Evolutionary Ecology of Health in Asian Elephants

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Abstract of Thesis

The definition of health is complex, involving the maintenance of a delicate balance between different physiological processes within an organism. The understanding of life-history traits and ecological factors affecting health in wild animal populations is still limited, especially in long-lived mammals. Here I investigated life-history traits and ecological factors affecting health in a semi-captive population of Asian elephants (*Elephas maximus*) from Myanmar. My study system is comprised of working timber elephants, employed by a government-owned company, the Myanma Timber Enterprise (MTE). These elephants are classified as semi-captive: they are managed during the day but free to roam in their natural habitat during the night without human supervision. I collected health measures from the elephants for a period of three years that encompass longitudinal quantification of several body systems and functions. Life-history data for these animals were obtained from detailed logbooks that are maintained for each individual from birth to death.

My study first established reference intervals for several health parameters, describing for the first time in Asian elephants both body condition score (BCS) outside of zoos and blood pressure, and reinforced the importance of evaluating health separately for males and females (Chapter 2, Franco dos Santos *et al*., *in press*). In chapter 3 I observed a decline in health with age, both at the individual health parameter and global health levels, providing an example of senescence in this long-lived mammal. Chapter 4 shows how elephant health is affected by ecological variation across different seasons of the year, driven mainly by rainfall and food quality/quantity fluctuations (Chapter 4). Finally, Chapter 5 quantified the risk parasites pose for their host health, especially in heavily parasitized animals, driven mainly by immune system activation (Chapter 5). These results improve our knowledge of health variation in wild populations, and have implications for the management and conservation strategies of this endangered species.

To Mother Nature,

For allowing me to contemplate its wonders

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To Him for never letting me of His hand. He was the hand that always raised me after falling.

Statement of intellectual contribution

The data chapters presented in this thesis (chapters 2 to 5) have benefited from advice and help from a number of collaborators, as mentioned below. All other work is my own, including, but not limited to, conceiving the ideas, the conceptual and methodological design, data collection and writing of the chapters. The research chapters are presented in the style of scientific papers. Virpi Lummaa supervised the research contributing to all chapters.

Chapter 2

Win Htut, U Kyaw Nyein and Htoo Htoo Aung facilitated the data collection in the field. I carried out the data analysis, with statistical guidance from John Jackson. All co-authors contributed critically to the drafts and gave final approval for submission.

Chapter 3

Win Htut facilitated the data collection in the field. Sophie Reichert and Mirkka Lahdenperä contributed to the conceptual design of the paper and Vérane Berger gave advice in the data analysis. All co-authors contributed to the manuscript.

Chapter 4

Win Htut, U Kyaw Nyein and Htoo Htoo Aung facilitated the data collection in the field. Vérane Berger and I carried out the data analysis on the multivariate models and LDA. All co-authors contributed critically to the drafts.

Chapter 5

Win Htut, U Kyaw Nyein and Htoo Htoo Aung facilitated the data collection in the field. Vérane Berger, Robin Cristofari and I carried out the data analysis on the multivariate models and LDA. Robin Cristofari provided statistical guidance on the analysis. All co-authors contributed critically to the drafts.

Contents

Chapter 1

Thesis introduction

Asian Elephant as Model for Health

Introduction

The publication of Charles Darwin's *On The Origin of Species* (1859) 160 years ago opened a broad field of studies focusing on how species evolve to adjust to prevailing environmental conditions. The field of evolutionary ecology is mainly concerned with survival (e.g. Catchpole, Morgan, Coulson, Freeman, & Albon, 2000; Martín et al., 2007), growth (e.g. Freed & Cann, 2009; Lester, Shuter, & Abrams, 2004) and reproductive success (e.g. Rebke, Coulson, Becker, & Vaupel, 2010; Robinson, Pilkington, Clutton-Brock, Pemberton, & Kruuk, 2006) differences between individuals and populations exposed to different environmental factors. However, less attention has been paid to what happens mechanistically to individuals encountering different environmental conditions, leading to the observed fitness differences. In contrast to the broad population view of evolutionary ecology, veterinary science focuses on the individual level, especially encompassing the diagnosis, treatment and prevention of disease in animals. Such an approach enables a better understanding of the physiological mechanisms within individuals, but may prevent a broader ecological understanding at the population level. Ecophysiology tries to bridge this gap, by focusing on the physiological boundaries within which an individual functions (Le Maho, 2002). Specifically, ecophysiologists aim to understand how the physiology of an organism responds to biotic and abiotic stressors (Ainsworth, Bernacchi, & Dohleman, 2016). However, within ecophysiology, the focus is often on rather narrow questions, looking at one system or body function at a time (Cheynel et al., 2017a; Garnier, Bento, et al., 2017; Jégo et al., 2014). Yet, in the real world, most body systems and functions are tightly interconnected. Thus, there is a need to understand the broader context of how the mechanisms for fitness differences, or *overall health*, responds to environmental challenges in nature.

The concept of health differs between the human and veterinary sciences (Table 1.1). For the World Health Organization, health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity (WHO, 2014). But while this definition can be applied to studies on human health, it does not apply well to studies on animal health. The health definition in veterinary science is not dogmatic, and is still debated. In its most simple form, health in veterinary science can be considered dichotomously as normality or the absence of disease (Baker & Greer, 1980). Health can also be defined as correctly functioning physiology in which the body functions perform properly structurally and functionally (Slauson & Cooper, 1990). However, such definitions still compartmentalize each body function too much. A more holistic approach defines animal health as the maintenance of a delicate balance within the organism or within a process in the organism (Gunnarsson, 2006), stressing the idea of interconnection between body systems and functions.

Table 1.1 - Different definitions of health by the World Health Organization (WHO) and in Veterinary Sciences

WHO	**Veterinary Science**		
State of complete physical, mental and social well-being (WHO, 2014)	Dichotomously as normality or the absence of disease (Baker & Greer, 1980)	Body functions perform properly structurally and functionally (Slauson & Cooper, 1990)	Maintenance of a delicate balance within the organism or within the processes in the organism (Gunnarsson, 2006)

This last definition of health highlights the importance of simultaneously studying not only the individual components of health but also the interconnection between the components, and the delicate balance within an organism. Our knowledge of how health is affected by life history and environmental factors in natural populations is, however,

limited by the challenges that such studies present. For example, it is important to get repeated measures for the same animal and record their sex and age, something that is challenging in natural study systems due to animal movements. In humans, the common practice of blood testing (Jang et al., 2013) is one of the easiest ways to assess individual health. For example, for the National Health Service in the United Kingdom, several blood tests are used as common diagnostic tools for health screening. For a full blood count, the Association for Clinical Biochemistry & Laboratory Medicine recommends tests for understanding blood components such as white cells, red cells and platelets ("Lab Tests Online UK," 2018). Blood tests are also valuable in veterinary medicine for the diagnosis and screening of individuals (Russell & Roussel, 2007) (Table 1.2).

However, these studies are difficult to perform in wild animals due to constraints including the need to capture the animals, restrain/anaesthetize them and estimate age (May-Júnior et al., 2009; Moen, Rasmussen, Burdett, & Pelican, 2010; Villegas, Sánchez, Costillo, & Corbacho, 2002). Such studies are valuable, but often have low sample sizes without repeated measures from the same animal. This creates the need for studies that monitor the same animals longitudinally with known age and sex in different environmental settings. In natural systems it is difficult to get repeats from the same animal and guarantee large sample sizes that are representative of life-history variation in the population and seasonal changes in the environment. Furthermore, it is important to note that the capture and restraint of wild animals can alter some physiological parameters (Gilot-Fromont et al., 2012).

Table 1.2. - Suggested basic elephant haematological and chemistry panel in zoos (Fowler & Mikota, 2006)

Haematocrit	Alkaline Phosphatase (ALKP)
Red Blood Cell Count	Gamma-Glutamyl Transferase (GGT)
Erythrocyte Sedimentation Rate	Bile Acids
Haemoglobin	Total Billirubin
Platelets	Creatinine Kinase (CK)
Total Protein (TP)	Lactate Dehydrogenase (LDH)
Albumin	Sodium
Blood Urea Nitrogen (BUN)	Chloride
Creatinine	Potassium
Aspartate Aminotransferase (AST)	Calcium
Fibrinogen	Phosphorus

Yet, a better understanding of “ecological medicine” in nature is timely and important, because in natural systems life-history trade-offs between investment in somatic maintenance and survival, growth, or reproduction are driven by the resource acquisition of individuals exposed to different biotic and abiotic constraints (Cheynel et al., 2017a). First, health patterns, and consequently survival and reproductive ability, can vary with animal life-stage or age. In roe deer (*Capreolus capreolus*), immune system function was found to decline at older ages, but such declines differed between populations depending on resource availability (Cheynel et al., 2017a). In the same populations, other health parameters also declined with age, including haematocrit (percentage of red blood cells in the blood), albumin (structural protein) and creatinine (a by-product of muscle metabolism) (Jégo et al., 2014), but some of these patterns differed for males and females. In wild Soay sheep (*Ovies aries*) senescence patterns differed between and within sexes for several phenotypic traits, suggesting that there is asynchrony in senescence and highlighting the need to understand the mechanisms underpinning senescence in wild populations (Hayward et al., 2015). Unfortunately, however, similar studies on other natural populations are rare, limiting our understanding

of which health functions underlie the senescence patterns in fitness observed in nature, and few previous attempts to quantify health holistically exist.

Second, health parameters can also be good indicators of climatic and nutritional variation (Garnier, Bento, et al., 2017; Garnier, Cheung, et al., 2017). In a natural environment, Soay sheep (*Ovies aries*) juveniles that prioritize immunity levels had increased overwinter survival rates, while older individuals survived better when they maintained a high-protein plane (Garnier, Cheung, et al., 2017), with an increase in antibodies levels and albumin/ total protein levels (Garnier, Bento, et al., 2017). Indeed, animals that live in natural systems are influenced by the circannual rhythm, allowing them to interpret seasonal cues and adapt to seasonal variation imposed by the climate (Bradshaw & Holzapfel, 2007; Paul, Zucker, & Schwartz, 2008). Accordingly, seasonal changes in health parameters have been documented in different mammal species such as grey wolves (*Canis lupus*), white-tailed deer (*Odocoileus virginianus*), Asiatic black bears (*Ursus thibetanus*) and European brown bears (*Ursus arctos arctos*), although the associations and trends in specific outcome variables differ between species (Butler, Ballard, & Whitlaw, 2006; DelGiudice, Mech, Kunkel, Gese, & Seal, 1992; Hissa et al., 1994; Yang, Jeong, & Lim, 2018). However, to my knowledge, the majority of such studies have been conducted in continental climates with strong differences between summer and winter, and less attention has been paid to animals in tropical climates or with different seasonal profiles.

Finally, an important mediator influencing animal responses to prevailing environmental conditions is infection with parasites that could tip the delicate balance between body systems and functions. Parasites are responsible for important fitness costs for the host (Dazak, Cunningham, & Hyatt, 2000; Wegner, Reusch, & Kalbe, 2003).

These costs can vary from short-term reactions such as decreased immunity, immunophatology or alteration of physiological parameters (Dimitrijević et al., 2016; Monteiro, Dietz, & Jansen, 2010) to long-term costs with increased susceptibility to other infections, disease complications or even death (Beldomenico et al., 2008). Nevertheless, most studies focus on how parasites modify one or two aspects of host physiology and lack repeated measures on the same individuals (Dimitrijević et al., 2016; Marcus, Higgins, & Gray, 2015; Rouatbi et al., 2016). Additionally, the rare studies that investigate several aspects of health simultaneously do not consider the interdependency between body functions preventing from uncovering general parasite-related health patterns.

Timber elephants in Myanmar: a unique population

In this thesis, I use Asian elephants (*Elephas maximus*) employed in timber camps in Myanmar as a model to study how the life-history stage (age), seasonal variation in the environment and parasitic infections associate with global measures of health and a wide range of underlying health parameters. This model system and the populations management system present a wide range of benefits for addressing these aims. Asian elephants are considered as an endangered species by the IUCN, with a declining population trend (Choudhury et al., 2008). Their habitat is highly fragmented across all of southeast Asia with a geographic range of about ~49 000km^2 (Figure 1.1), which has decreased from approximately 9 million km^2 in the past century (Blake & Hedges, 2004). The full population of wild Asian elephants is estimated to be between 40 000-50 000 individuals, with a further 14 000-15 000 held in captivity (Sukumar, 2006). India has the largest wild population with an estimate of 24 000-33 000 individuals followed by Myanmar with a rough estimate of 4 000 to 5 000 wild individuals (Mar, 2007; Sukumar, 2006), but potentially as low as 2 000 (Leimgruber et al., 2011). Myanmar is

also the home of the largest captive population, housing around 5 000 elephants, with ~2 700 individuals held by the state-owned Myanma Timber Enterprise (MTE) that are used as draught animals for timber logging (Mar, 2007). It is this population that is the focus of this thesis, because their husbandry system provided me with a unique opportunity to collect longitudinal blood samples and other health measures for a large number of individually recognized animals of known age and history, that forage, mate and interact naturally in the forests outside working hours.

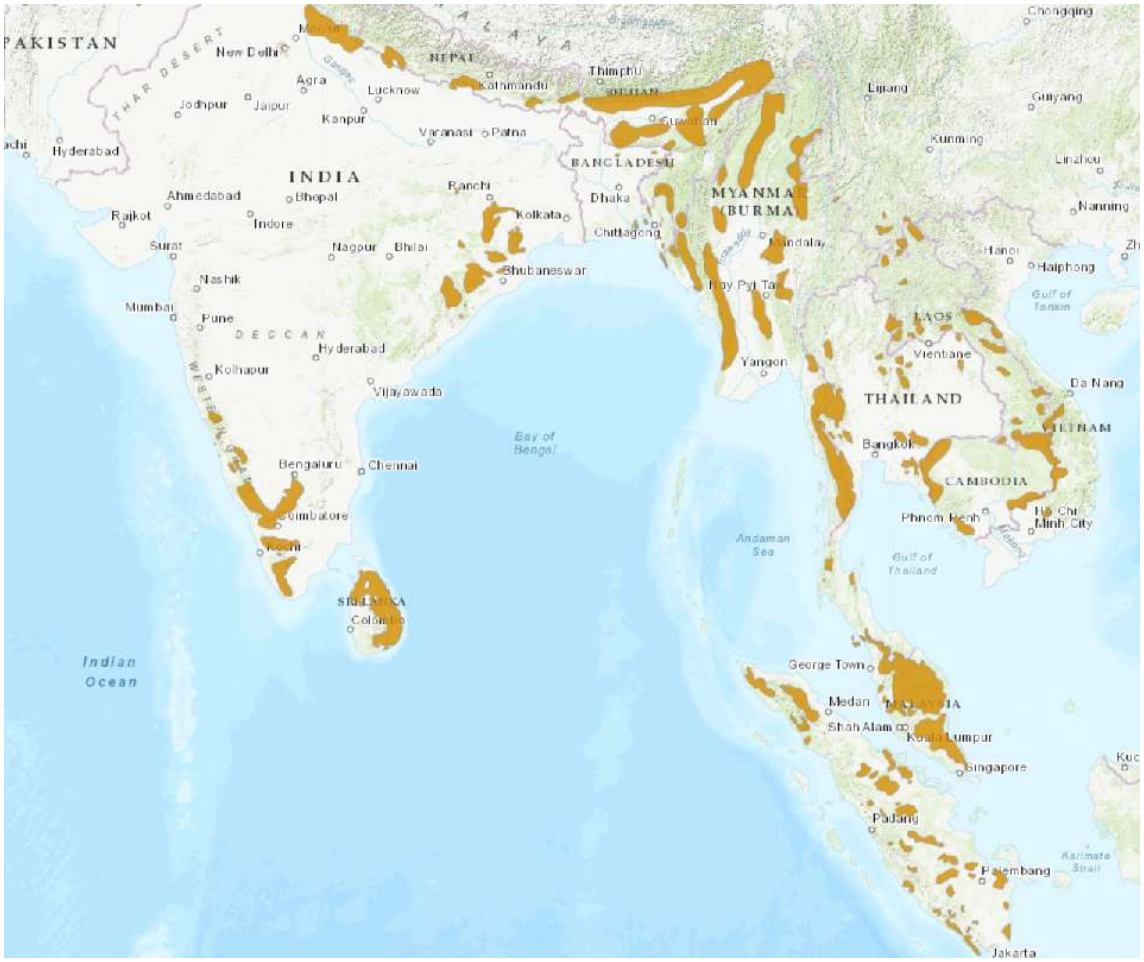


Figure 1.1 – Current distribution of Asian elephant in Asia (IUCN, 2018)

The current MTE population is composed of animals born in captivity and animals caught from the wild, although capture was banned in 1994 (Jackson, Childs, Mar, Htut, & Lummaa, 2019). Each animal has a caretaker that also rides the elephant, called a mahout (or *oozie*) (Crawley et al., 2019). The animals are grouped in working units, composed of 5/6 elephants of the same age group and managed by a head of mahouts (*sing-gaung*). All the camps from a specific area (MTE agency) are managed by the responsible of all the head of mahouts and camps, called the *sin-wun*. Each agency has also veterinarians responsible for managing diagnosis, treatment and disease prevention in the elephant population. They are responsible for providing wound and abscess treatment, basic gastrointestinal diagnosis/treatment and other general care. Veterinarians are also responsible for deworming the elephants twice a year in April and August, according to MTE regulations (Win Htut, personal communication). They also vaccinate for two infectious diseases, namely, anthrax in April and hemorrhagic septicemia twice a year, in June and December (Win Htut, personal communication). Due to their handling by mahouts and care by the veterinarians, timber elephants generally accept blood sampling and other regular health screening approaches.

The timber elephants are divided into four groups, in accordance with their age and work life. Elephants under 5 years old are kept with their mothers and allomothers (an individual female other than the biological mother of an offspring that performs the functions of a mother) and are then tamed to accept human handling after weaning, at age 4-5. From 5 to 17 years old they are trained and used in light work, and then enrolled into timber logging as full working elephants, until retirement by age 55 (Mar, Lahdenperä, & Lummaa, 2012). After this age, elephants are kept without working until death (Mar, 2007). Maximum lifespan in the MTE population is 69 for captive-born and 76 for wild caught (Chapman, Jackson, Htut, Lummaa, & Lahdenperä, 2019). In 2018, females that reproduced at 17 (this age represents the beginning of adulthood in this

population) were expected to survive 25 more years, while females that reproduced at 55 (age that represents the end of fecundity in this population) were expected to survive 5 more years (Chapman et al., 2019). It is also known that males have a shorter lifespan than females (Lahdenperä, Mar, Courtiol, & Lummaa, 2018). Pregnant females do light work until mid-pregnancy and are then given rest until the calf reaches one year of age (Gale, 1974). The workloads and rest periods are tightly regulated by the central government. These regulations establish the number of working hours per week, working hours per year and the tonnage allowed per elephant (Mar et al., 2012). The work season begins in mid-June and is highly dependent on the beginning of the monsoon season, and ends in mid-February when the hot season starts. MTE elephants live largely under natural conditions: they are released into the surrounding forest after working hours and are free to roam, forage naturally, interact and mate with wild and captive conspecifics (Lahdenperä et al., 2018). Reproduction and social interactions are not supervised or managed by humans (Hayward, Mar, Lahdenperä, & Lummaa, 2014). Thus, the semi-captive nature of the husbandry system provided me with an unusual opportunity to investigate the holistic health patterns of elephants in an ecological context that closely resembles conditions experienced in the wild (e.g. climate, nutrition, disease exposure, social life, reproduction).

Importantly for the current study, after taming at age 4-5 each elephant is designated a unique four-digit identification (ID) number, which is permanently marked (tattooed) on their haunches. At the same time, each elephant is assigned a log-book where all the information related to that elephant is recorded regularly (usually twice per month) by the vet in charge of the elephant. The logbook contains the exact date of birth (estimated for wild-caught individuals, see Lahdeperä et al., 2018), maternal lineages, sex, morphometric measures (when they are possible to collect), basic life-history notes, health and veterinary care, date of death and its cause (Mar, 2007). The ID numbers and

log-book maintenance systems provided my study with access to easily-recognizable animals with known age and health history, which were important for interpreting how physiological measures of health vary with age and across different environmental conditions longitudinally, and would not usually be possible in a truly wild population – at least for a similarly long-lived species.

Logging practices (and thus elephant workload), as well as nutrition, parasite exposure and presumably physiology, are strongly influenced by Myanmar's climate. Myanmar is characterized by a tropical monsoon climate (Beck et al., 2018), divided into three marked seasons. From March to June is the hot season, which is characterized by high temperatures and low rainfall, water shortages, low food quality and low parasite burden (Lynsdale, 2017). The monsoon season then extends from July to October and is characterized by high rainfall levels and high temperatures, good water supply and good quality food. The cold season is from November to February and has low temperatures and low rainfall, decreasing water supply, food quality and high parasite burden. Elephants have a small surface-to-volume ratio, a high energetic cost of body cooling and a lack of sweat glands and panting to dissipate heat (Weissenböck, Arnold, & Ruf, 2011), making it difficult to thermoregulate in the hot season. Consequently, the elephants are given rest from work during the hot season. The seasonal environment experienced by the timber elephants, combined with the ability to sample known animals longitudinally across seasons and years, provided my study with an opportunity to investigate how environmental variation affects holistic health and its different underlying parameters.

Finally, detailed longitudinal records of parasite infection in the population (Lynsdale, 2017) offer opportunities to investigate how parasite infection influences the

overall health of the host, as well as health parameter variation between the sexes. Parasites are known to have affected Asian elephants in Myanmar for over a century (Evans, 1910), and in the last century parasite associated mortality in this population was increased for males, juveniles and elderly adults (Lynsdale, Mumby, Hayward, Mar, & Lummaa, 2017). But while most of the knowledge about parasites in Asian elephants is descriptive and taxonomy related (Fowler & Mikota, 2006), no work to my knowledge has tried to understand the association they have with physiological health markers in the host and using ecophysiological approach. Such studies are needed because infection levels are known modulate the trade-off between the allocation of finite resources into immune responses and to other biological functions like reproduction and growth (Sheldon, Verhulst, & Sheldon, 1996; Zuk & Stoehr, 2002). Parasites also deteriorate different physiological functions, so leading to ill health (Dimitrijević et al., 2016; Marcus et al., 2015; Nnabuchi, Nwani, Ochang, & Somdare, 2015; Rouatbi et al., 2016). Although these studies suggest an interaction between health and parasite burden, few use repeated measures on individuals with known life-histories, and fewer still take the interdependency between body functions into account, which are important limitations for understanding how health responds to different parasite loads.

Thesis Chapters

This thesis aims to establish a quantitative assessment of holistic health and its underlying parameters in a long-lived, endangered model system; use these to investigate how age, ecology and infection modify health in nature; and determine the implications the results may bring to conservation and ecophysiology of semi-captive Asian elephants in Myanmar.

Thus, each of my chapters addresses the interconnection between health and elephant life-history traits and ecology. A better understanding of the interaction that health has with life-history traits and ecology will ultimately improve management decisions and conservation in this endangered species.

Chapter 2: Reference Intervals of Asian elephants in Myanmar

This chapter establishes reference values for several health parameters in the Myanmar timber elephant population and assess sex differences in these parameters.

In this chapter, I describe the reference values for several health parameters covering different systems and functions in the body. Given the physiological differences between the sexes and the accumulating evidence from human medicine that optimal health and drug dosage for men and women may differ (Anderson, 2008; Regitz-Zagrosek, 2012), I also investigate differences in health parameters between the sexes.

This chapter is under review in *Journal of Zoo and Wildlife Medicine* as *Reference Intervals and Sex Differences for Blood Pressure, Body Condition, Hematology and Serum Chemistry in Semi-Captive Asian Elephants (Elephas maximus) from Myanmar*.

Chapter 3: Age-related variation of molecular and physiological health markers in a long-lived mammal

After assessing “normal” health variation in this population, I investigate the influence that age has on health both individually and as a whole. In addition, I show evidence of a molecular and physiological health decrease with age in a free-living long-lived mammal. For this study, I used health data to understand how ageing influences

health parameters, focusing on the four life-stages that characterize the reproductive and working life of timber elephants.

Chapter 4: Hot, cold or rainy? Health seasonality in Asian elephants

In this chapter, I explore how seasons influence elephant health by shaping the environment around it. Again, we analyse the physiological parameters both individually and the interconnection between them. The elephant ecosystem faces strong seasonal changes, especially in water and food availability/quality, parasite abundance and temperature/rainfall, which have the potential to influence health.

Chapter 5: Health cost of heavy parasite burden in Asian elephants

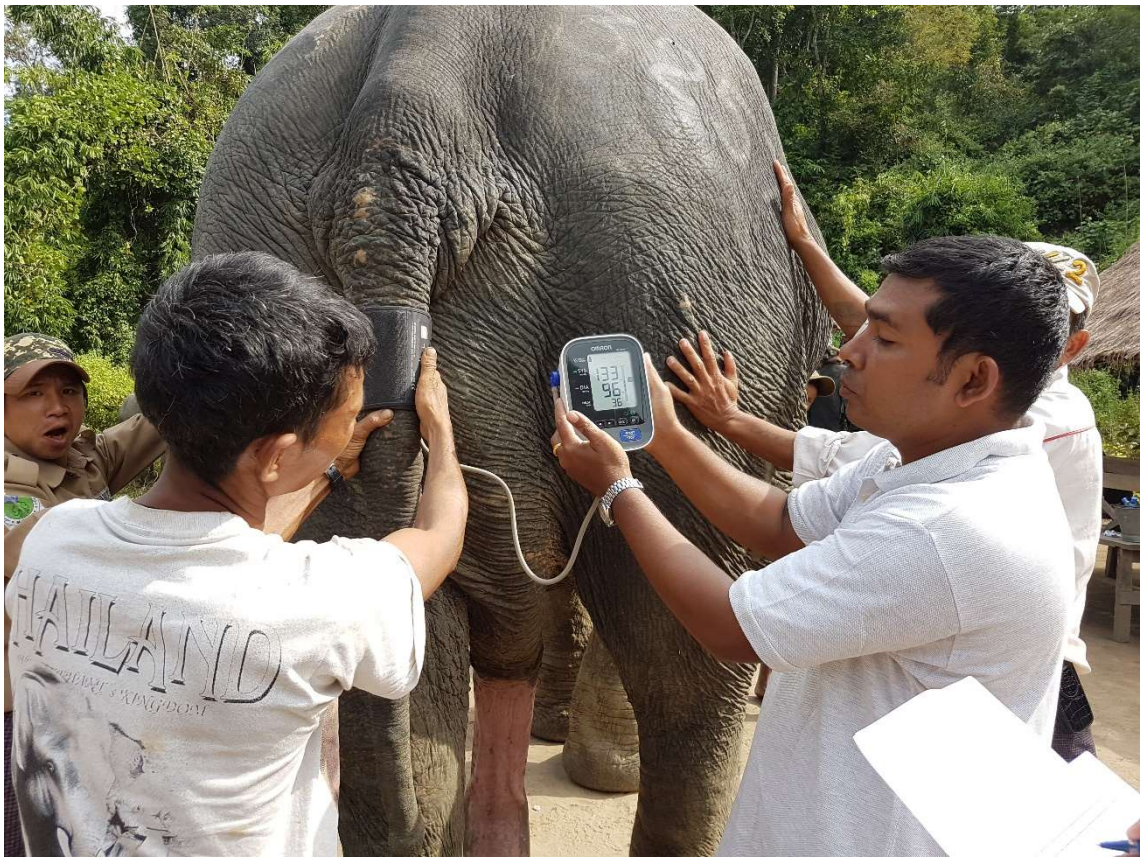
Parasites are known to have an effect on elephant life-history. In this chapter we study how parasite burden influences health both individually and as a whole to establish reasonable management and conservation measures. In this way, we intend to understand how the parasite dynamics influence the host health.

Chapter 6: Thesis discussion – Life history traits as health commanders and conservation issues

In the general discussion, I expand on the themes discussed in previous chapters, exploring how they offer new insights to the field and their implications for the conservation management of the Asian elephant in Myanmar. I also discuss the strengths and limitations of the current study.

Chapter 2

Reference Intervals of Asian elephants in Myanmar



This chapter is in press in *Journal of Zoo and Wildlife Medicine* as *Sex Differences in the reference Intervals of Health Parameters in Semi-Captive Asian Elephants (Elephas maximus) from Myanmar*. It is shown here in its submitted form with additional formatting changes.

Abstract

The reference intervals of health parameters are a valuable tool for veterinarians and conservationists to monitor the health status and viability of endangered species. Natural variation in the health of the long-lived Asian elephant (*Elephas maximus*) is poorly understood, particularly in relation to differences between males and females. Longitudinal health data was collected from clinical examination, hematology and serum chemistry analyses over three years from 227 healthy individually-marked Asian elephants varying in age and sex. The study population was semi-captive and used in Myanmar's timber industry, but maintained natural feeding and breeding behaviour. Body condition score (BCS) and blood pressure were investigated in clinical examinations. Hematological parameters included hematocrit, hemoglobin, total white blood cell count (TWBC), and differential blood cell counts. Serum chemistry parameters included blood urea nitrogen (BUN), creatinine, total protein, albumin, globulins, aspartate aminotransferase (AST), alkaline phosphatase (ALKP), triglycerides, creatinine kinase (CK), glucose, calcium, potassium, sodium and chloride. To the knowledge of the authors, this is the first description of BCS in an elephant population outside of zoos, and of blood pressure in this species using a novel adaptation of the Wrap Cuff pressure monitor. Several differences between the sexes were observed, with females generally having higher BCS and triglycerides, and males displaying higher ALKP and glucose levels. This study provides important clinical tools that can be used to assess the health status and improve management in this endangered species.

Introduction

In veterinary and human medicine, the reference intervals (RIs) of hematological and serum health parameters are commonly used tools in disease diagnostics, which can determine sub-optimal health status and improve our understanding of the physiological changes associated with disease (Catenacci et al., 2017; Friedrichs et al., 2012; Schmidt, Paulillo, Santin, Dittrich, & Gonçalves De Oliveira, 2007). Health surveys of animal populations conducted by veterinarians also aim to (1) establish baseline health values for a species or population, (2) monitor population health and its changes and (3) ensure population viability (Catenacci et al., 2017; Deem et al., 2001; López et al., 2017). As such, they are useful tools not only for veterinary medicine, but also for the *in situ* and *ex situ* conservation of endangered species.

Most health survey studies to date have focused on humans and domestic species such as mice, rabbits and dogs (Fukuda, Kawashima, Iida, Aoki, & Tokita, 1989; Fukuda, Tsuchikura, & Iida, 2004; Jeklova, Leva, Knotigova, & Faldyna, 2009; Touitou, Touitou, & Bogdan, 1986). In contrast, less data are available on natural populations, and particularly for endangered species. This is problematic, because health survey values for domestic populations may not be representative of wild animals that are exposed to different pathogens, levels of exercise and nutrition (López et al., 2017). Consequently, Deem et al., (2001) proposed that reference values should also be determined for wild populations, focussing on hematology, serum chemistry, vitamin and mineral levels, and surveys of infectious disease and chemical contaminants. However, the methodological challenges of conducting surveys in the wild has meant that currently only a few studies exist for animals living in their natural habitat.

Importantly, due to inherent physiological differences between the sexes, several studies have suggested that reference health values for males and females may also

differ (Bush, Smith, & Custer, 1981; Etim, Williams, Akpabio, & Offiong, 2014; Fairbrother, Craig, Alker, & Loughlin, 1990; Schmidt et al., 2007). Health parameters can also change with age, and in particular differences have been found during development , adulthood, and during senescence (Horning & Trillmich, 1997; Schmidt et al., 2007; Touitou et al., 1986; Videan, Fritz, & Murphy, 2008). Thus, distinguishing animals based on their age and sex is crucial when establishing reference values for health parameters.

This study uses a large population of semi-captive Asian elephants living in their natural habitat and maintaining their natural behaviour to: (1) establish reference values for clinical examinations, hematology and serum chemistry health parameters, and (2) establish if there are sex differences in the health parameters of this long-lived mega-herbivore, in which males and females exhibit different life-histories. The majority of previous studies on the health parameters of African elephants have focused on wild individuals, but in Asian elephants most studies have been conducted in captivity using only a small number of animals (Allen, Jacobson, Harvey, & Boyce, 1985; Gromadzka-Ostrowska, Jakubow, Zalewska, & Krzywicki, 1988; Niemuller, Gentry, & Liptrap, 1990; Salakij et al., 2005; Silva & Kuruwita, 1993a; White & Brown, 1978). Myanmar is unique because it employs over 5000 Asian elephants in the timber industry; the largest global captive population of this endangered species. The animals work for a maximum of 8h/day extracting logs but spend the rest of their time in comparable freedom, foraging in the forest and interacting unsupervised, and the population is thus considered semi-captive (Crawley et al., 2017). These timber elephants offer an opportunity to establish reference values for a large number of Asian elephants with known age, sex and life history. Another key advantage is the possibility to collect repeated blood samples from each individual because they are tamed and trained. The current study therefore offers an interesting comparison to existing studies on domestic species and zoo populations, and provides a rare insight into what the reference values for clinical evaluation, hematology, and serum chemistry may be in wild Asian elephants.

Materials and Methods

Study population

The study population is owned by the government-run Myanma Timber Enterprise (MTE), which has kept records of elephant births, deaths, wild-captures, and other events across the country for over 100 years.(Jackson et al., 2019; Seltmann, Helle, Adams, Mar, & Lahdenperä, 2018) These records provide information on each elephant's age, reproductive history, and recent health. Each animal has a unique ID number, which is recorded in a logbook and permanently marked on its back. For captive-born individuals (82% of the present sample), exact ages are known from recorded birth dates, and for wild-caught animals age is estimated by experienced veterinarians using body size, temporal/buccal depressions, ear folds, pigmentation and tusk size.(Arivazhagan & Sukumar, 2008) Working elephants are organized into small units with 6-7 animals of different sexes. After the working day, they are released into the forest to forage and mate without human intervention.

Elephants were sampled in 2016, 2017, and 2018 from three logging agencies in the Sagaing Division, namely, Kawlin (23°46' N, 95°40' E), East Katha and West Katha (40°26' N, 79°58' W). Overall, this study includes 227 elephants, of which 85 were male and 142 were female. Their ages ranged from 4 to 72 years old; blood collection is not possible for calves younger than 5 years because they have not been trained to be handled by humans. Repeated individual samples were collected three times a year, corresponding to the three seasons in Myanmar, namely, the hot and dry season (January-May), the monsoon season (June-September) and the cold season (October-December). However, not all of the study elephants were available in every sampling trip; 58 elephants were only sampled in one of the field trips, 50 in two, 25 in three, 17 in four, 19 in five, 22 in six, 17 in seven, 13 in eight, and five in all nine field trips over the three-year period.

Sample collection and analysis

To investigate hematological and serum chemistry levels, blood was collected from an ear vein using a Vacuette® (Greiner Bio-One, Kremsmünster, Austria, 4550) system into EDTA, heparin and serum separator tubes. The blood tubes were refrigerated until analysis in the laboratory (maximum of 24 hours post-collection). For serum chemistry, the samples were centrifuged, and sera were collected and frozen until analysis in a laboratory in Yangon using the IDEXX VetTest® (IDEXX, Westbrook, USA, 04092). The blood samples collected in EDTA were used to perform a manual count of leucocytes using Turk's solution. Differential leucocyte counts were performed manually using a blood smear stained with Romanowsky stain. Glucose levels were obtained using an ACCU-Chek® (Hoffmann–La Roche, Basel, Switzerland, 124) Aviva glucometer. Serum glucose was not evaluated in the field at the time of collection, and therefore results should account for glycolysis causing an artificially decreased serum glucose.(Fobker, 2014) Using samples stored in heparin, hematocrit, hemoglobin, sodium, potassium and chloride levels were obtained using a VetScan i-Stat® 1 (Abaxis, Union City, USA, 94587) with an E3+ cartridge. Blood pressure was collected using the Omron M6 Comfort IT (Omron, Kyoto, Japan, 617-0002) blood pressure monitor with an Intelli wrap cuff. The cuff was placed on the tail under the anal skin flap in an area where the diameter of the tail was more regular. The elephants were trained to accept tail handling and to keep the tail still.

To determine body condition, the elephants were weighed to the nearest kg using Eziweigh 3000 scales.(Crawley et al., 2017) When the scales were not available, body weight was estimated from the chest circumference and shoulder height using formulae for females and males from Chapman et al., (2016), which explain variation in the real body weight with an R^2 value of 0.87 for females and 0.94 for males in the current study population. In addition, the body condition score (BCS) of all animals was assessed using

a table developed by Morfeld et al., 2016 (Morfeld, Meehan, Hogan, & Brown, 2016) that scores each elephant between 1 (very thin) and 5 (obese), using the covering of fat on the ribs, pelvic bone and backbone as references.

Data analysis

RIs were established using the guidelines provided by the American Society for Veterinary Clinical Pathology (ASVCP) (Friedrichs et al., 2012). All statistical analyses were carried out in the R statistical package (R Core Team, 2017). The RIs were first calculated for the whole population, and subsequently by sex to test whether the reference intervals for each health parameter differed between males and females.

RIs and confidence intervals were calculated using the *singleRefLimit* function from the *referenceIntervals* package (Finnegan, 2014). Only animals that appeared healthy during a clinical evaluation and that had no record of recent illness were included in these calculations. The sample size used in each analysis varied because not all parameters were recorded in each season and year or for each individual, and because outliers were removed using Horn's method, (Friedrichs et al., 2012) which determines outliers in a Box-Cox transformed dataset using Tukey interquartile fences. The normality of each parameter was assessed using Shapiro-Wilk tests. Reference intervals were then calculated following the recommendations from the ASVCP guidelines: for health parameters with over 120 observations, non-parametric methods were used, and for sample sizes less than 120 (i.e. when separating sexes), robust methods were used, namely, bootstrapping to estimate the location and spread of the data (Friedrichs et al., 2012). Reference intervals (RI) were calculated with 90% confidence intervals in all tests.

To test for differences between the sexes, both linear mixed-effects models (LMMs) and generalized linear mixed-effects models (GLMMs) were used depending on the distribution of the health parameter, implemented with the lmer and glmer functions

from the *lme4* package (Bates, Mächler, Bolker, & Walker, 2015). In each model, the health parameter was the response variable and the link function used depended on the parameter's distribution. The majority of the health parameters displayed a Gaussian distribution and were analysed with LMMs. However, eosinophils, ALKP, CK, AST and triglycerides were positively skewed, and were analysed using GLMMs, with a poisson family and a log link function (eosinophils, ALKP and CK), a negative binomial family for AST, and a gamma family with a log link function for triglycerides. Sex was included as the main fixed factor of interest, and the age of each animal was controlled for by including age category (4 levels) as a fixed effect. The age categories were based on elephant life-history and working schedule and included: (1) young tamed animals in the early stages of training (4-10 years), which would still be dependent on their mothers and other herd members in the wild; (2) adolescent animals (10-20 years) that are finishing training, beginning to work and reaching sexual maturity; (3) full working-age animals (20-50 years) that are at peak reproductive ages; and (4) retired elephants (> 50 years) with declining fertility and survival rates (Lahdenperä, Mar, & Lummaa, 2014). The models also accounted for variation due to collection location (three levels), season (three levels) and year (three levels) as fixed factors, and elephant ID number (to account for repeated samples from individuals across years and seasons) as an intercept-only random effect. Statistical significance was determined at the 95% confidence level.

Results

Overall population

The results presented here establish reference health status values for Asian elephants managed in semi-captivity, in an effort to provide a valuable resource for veterinarians and conservationists. The frequency distributions of health parameters for the overall population are shown in Figure 2.1.

Regarding parameters obtained through clinical examination (Table 2.1), the BCS RI was 2-4 with a mean±SD (standard deviation used for all parameters) of 3.2±0.5; the first values obtained in a semi-captive elephant population. This study is the first to describe blood pressure in *Elephas maximus*, with an RI for systolic pressure ranging between 99-166 mmHg with a mean of 134±16 mmHg and an RI for diastolic pressure ranging between 67-127 mmHg with a mean of 96±15 mmHg.

For hematological health parameters (Table 2), the most notable findings were for the monocytes (RI of 21-55% and mean of 38±9%), which were the most abundant white blood cell, followed by the lymphocytes (RI of 16-46% with mean of 30±8%). RIs were also obtained for hematocrit, hemoglobin, and total white blood cell count (Table 2.2).

Serum chemistry parameters are rarely measured in elephants, and the RIs provided in Table 2.3 provide a basis for the clinical evaluation of health in this species, particularly for triglycerides (RI of 0-58 mg/dL and mean of 15±16 mg/dL) and CK (RI of 31-385 U/L and mean of 147±89 U/L). RIs were also obtained for blood urea nitrogen (BUN), creatinine, total protein, albumin, globulins, aspartate aminotransferase (AST), alkaline phosphatase (ALKP), glucose, calcium, potassium, sodium and chloride.

Table 2.1. Body condition score (BCS) and blood pressure values with Reference Intervals (RI) for semi-captive Asian elephants in Myanmar. A lowercase letter a indicates a statistically significant difference between males and females.

						95% Confidence Interval		
Parameter	**Sex**	**N**	**Mean±SD**	**Median**	**Range**	**RI**	**Lower**	**Upper**
BCS	All	748	3.4±0.6	3.0	2.0-5.0	2-4	2-2	4-4
	Male	281	3.2±0.5	3.0	2.0-4.0	2-4[a]	2-3	4-4
	Female	467	3.4±0.6	3.0	2.0-5.0	3-4[a]	2-2	4-4
Systolic Pressure (mmHg)	All	242	134±16	133	93-172	99-166	93-106	162-170
	Male	105	134±17	133	93-172	100-168	95-104	163-173
	Female	137	134±15	133	93-170	101-165	93-109	160-170
Diastolic Pressure (mmHg)	All	242	96±15	96	53-137	67-127	63-71	123-135
	Male	109	97±18	96	53-137	62-132	57-66	127-137
	Female	136	96±13	96	63-130	68-121	63-74	117-130

Table 2.2. Hematology values with Reference Intervals (RI) for semi-captive Asian elephants in Myanmar. A lowercase letter *a* indicates a statistically significant difference between males and females.

						95% Confidence Interval		
Parameter	**Sex**	**N**	**Mean±SD**	**Median**	**Range**	**RI**	**Lower**	**Upper**
Hematocrit (%)	All	765	34±3	34	28-44	29-42	29-29	41-42
	Male	278	34±3	33	28-43	28-41	28-29	41-42
	Female	487	35±3	35	28-44	29-42	29-30	41-43
Hemoglobin (g/dl)	All	603	11.7±1.2	11.6	8.5-17.0	9.5-14.3	9.5-9.9	13.9-14.6
	Male	218	11.5±1.2	11.6	9.2-15.9	9.3-14.3	9.2-9.5	13.9-14.6
	Female	385	11.8±1.1	11.9	9.2-16.0	9.9-14.3	9.9-9.9	13.9-14.6
WBC (10^9/L)	All	749	15.8±3.8	15.4	7.7-29.7	9.5-24.6	9.2-9.9	23.3-26.0
	Male	282	15.8±3.7	15.3	8.0-29.6	9.4-24.5	8.8-10.4	22.7-27.9
	Female	467	15.8±3.9	16.0	7.7-29.7	9.6-25.1	9.0-10.1	23.3-27.1
Lymphocytes (%)	All	662	30±8	30	11-52	16-46	14-16	44-47
	Male	249	30±8	30	11-52	17-46	12-18	44-48
	Female	413	30±8	30	11-50	15-46	14-16	44-47
Monocytes (%)	All	667	38±9	38	15-61	21-55	19-23	54-56
	Male	250	38±8	38	18-56	23-55	21-55	52-56
	Female	418	38±10	39	15-61	20-55	17-22	54-56
Heterophils (%)	All	660	27±8	27	11-52	14-44	13-15	43-45
	Male	247	28±8	28	12-52	14-44	12-16	42-51
	Female	413	27±8	27	11-51	14-44	12-16	43-47
Eosinophils (%)	All	667	4±3	4	0-14	0-11	0-0	10-13
	Male	250	4±3	3	0-14	0-11	0-0	9-11
	Female	416	4±3	4	0-14	0-12	0-0	10-13

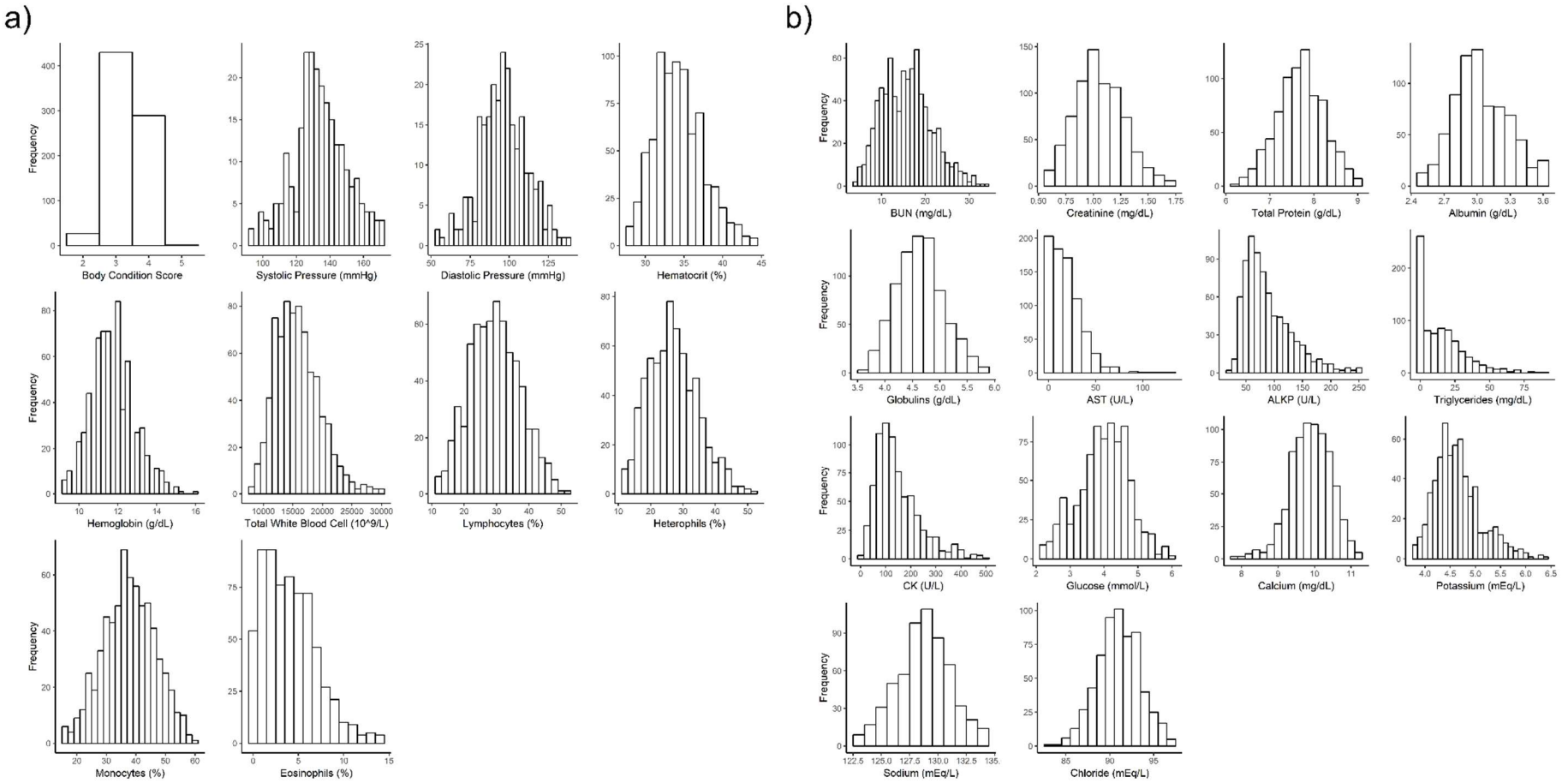


Figure 2.1. Frequency distributions for health parameters, including a) clinical examination/ hematology parameters, b) serum chemistry parameters, in a population of semi-captive Asian elephants in Myanmar.

Sex differences in health parameters

Clinical examination

The frequency distributions of health parameters for males and females are presented in Figure 2.2. Asian elephant males are considerably larger than females, with the body weight of males in this study sample ranging between 864 kg (4 years old) and 3601 kg (60 years old), compared to 894 kg - 3198 kg (5-39 years old) for females. The sexes also differed significantly regarding their Body Score Index (Table 1), with males displaying an overall BCS 6% lower than females (t = -2.640; P = 0.009). Systolic pressure was not significantly different between the sexes (t = 0.322; P = 0.748). Similarly, no significant difference was observed in diastolic pressure (t = 0.636; P = 0.527).

Hematology

Males and females did not differ significantly in their hematological health parameters (Table 2). The hematocrit levels (t = -1.281; P = 0.202), hemoglobin levels (t = -1.410; P = 0.160), TWBC (t =-1.033; P = 0.303), lymphocytes (t = -0.714; P = 0.476), monocytes (t = -0.304; P = 0.761), heterophil levels (t = 1.407; P = 0.161), and eosinophils (t = -0.901; P = 0.368) were not significantly different between males and females.

Serum chemistry

Significant sex differences were found for some of the serum chemistry health parameters (Table 2.3). Females had, on average, 5% lower glucose levels (mean = 4.0±0.7 mmol/L, n=491, and mean = 4.2±0.8 mmol/L, n=287 for females and males respectively; t=3.111; P=0.002), 19% lower ALKP levels (mean = 83±37 U/L, n=490, and mean = 102±48 U/L, n=289 for females and males, respectively; z=3.956; P<0.001), 30% higher triglyceride levels (mean = 17±17 mg/dl, n=498, and mean =12±13 mg/dl,

n=292 for females and males, respectively; t=-3.376; P<0.001) and 9% lower creatinine levels (mean =1.0±0.2 mg/dL, n=477, and mean = 1.1±0.2 mg/dL, n=284 for females and males, respectively; t=2.249; P=0.026). In contrast, sex differences in BUN (mean = 16±5 mg/dL, n=494, and mean =16±6 mg/dL, n = 290 for females and males, respectively; t=1.949; P=0.053), and total protein levels (mean = 7.8±0.5 g/dl, n=485, and mean = 7.6±0.6 g/dl, n=287 for females and males, respectively; t=-1.736; P=0.084) were only marginal. The levels of albumin (t =-1.365; P=0.174), globulins (t=-0.872; P=0.384), calcium (t=-1.330; P=0.186), potassium (t=-1.257; P=0.209), sodium (t=-1.121; P=0.264), chloride (t=-1.066; P=0.288), CK (z=1.619; P=0.105) and AST (z=-1.130; P=0.258), were not significantly different for males and females.

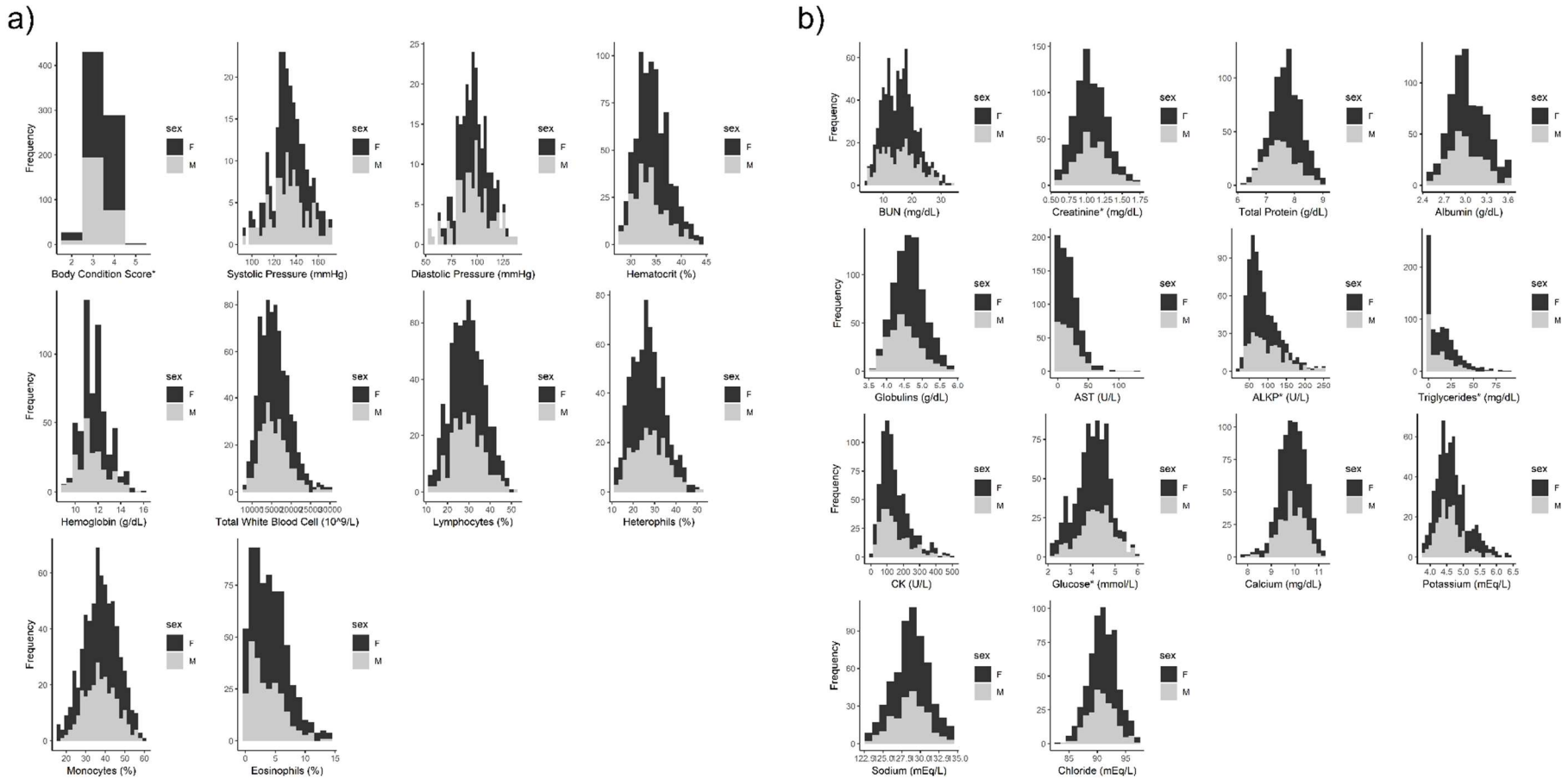


Figure 2.2 – Frequency distributions for health parameters, including a) clinical examination/hematology parameters, b) serum chemistry parameters, for male and female timber elephants. *health parameters significantly different between males and females

Table 2.3. Serum chemistry values with Reference Intervals (RI) for semi-captive Asian elephants in Myanmar. A lowercase letter *a* indicates a statistically significant difference between males and females.

						95% Confidence Interval		
Parameter	**Sex**	**N**	**Mean±SD**	**Median**	**Range**	**RI**	**Lower**	**Upper**
BUN (mg/dL)	All	784	16±6	16	4-34	6-28	6-7	27-29
	Male	290	16±6	16	5-34	5-28	5-7	27-29
	Female	494	16±5	16	4-33	7-28	6-7	26-30
Creatinine (mg/dL)	All	761	1.1±0.2	1.1	0.6-1.7	0.7-1.5	0.6-0.7	1.5-1.6
	Male	284	1.1±0.2	1.1	0.6-1.7	0.7-1.6[a]	0.6-0.7	1.5-1.7
	Female	477	1.0±0.2	1.0	0.6-1.7	0.7-1.5[a]	0.6-0.7	1.4-1.6
Total Protein (g/dL)	All	772	7.7±0.5	7.8	6.2-9.0	6.7-8.8	6.6-6.8	8.7-8.9
	Male	287	7.6±0.6	7.5	6.2-9.0	6.6-8.7	6.4-6.7	8.6-8.9
	Female	485	7.8±0.5	7.8	6.2-9.0	6.8-8.9	6.6-6.9	8.7-8.9
Albumin (g/dL)	All	746	3.0±0.2	3.0	2.5-3.6	2.6-3.6	2.5-2.6	3.5-3.6
	Male	279	3.0±0.2	3.0	2.5-3.6	2.6-3.6	2.5-2.6	3.4-3.6
	Female	467	3.1±0.3	3.0	2.5-3.6	2.6-3.6	2.5-2.6	3.6-3.6
Globulins (g/dL)	All	771	4.7±0.4	4.7	3.6-5.8	3.9-5.6	3.9-4.0	5.5-5.6
	Male	289	4.6±0.4	4.5	3.6-5.8	3.8-5.6	3.8-3.9	5.4-5.7
	Female	482	4.8±0.4	4.8	3.7-5.8	4.0-5.6	3.9-4.1	5.5-5.6
AST (U/L)	All	782	19±17	16	0-128	0-58	0-0	53-69
	Male	289	18±18	16	0-117	0-55	0-0	50-105
	Female	493	19±17	16	0-128	0-62	0-0	55-71
ALKP (U/L)	All	779	90±42	78	20-249	37-199	35-39	187-212
	Male	289	102±48	92	27-249	38-229[a]	36-40	199-247
	Female	490	83±37	73	20-247	36-185[a]	31-39	171-194
Triglycerides (mg/dL)	All	790	15±16	11	0-88	0-58	0-0	52-62
	Male	292	12±13	8	0-70	0-44[a]	0-0	39-50
	Female	498	17±17	13	0-88	0-61[a]	0-0	57-68

Table 2.3 (continuation). Serum chemistry values with Reference Intervals (RI) for semi-captive Asian elephants in Myanmar. A lowercase letter *a* indicates a statistically significant difference between males and females.

						95% Confidence Interval		
Parameter	**Sex**	**N**	**Mean±SD**	**Median**	**Range**	**RI**	**Lower**	**Upper**
CK (U/L)	All	761	147±89	123	11-491	31-385	26-33	369-407
	Male	282	146±90	121	19-486	29-386	22-41	335-455
	Female	479	147±88	124	11-491	38-385	26-35	367-408
Glucose (mmol/L)	All	778	4.0±0.7	4.1	2.2-6.0	2.5-5.5	2.4-2.6	5.4-5.6
	Male	287	4.2±0.8	4.2	2.3-6.0	2.6-5.8[a]	2.5-2.8	5.5-5.9
	Female	491	4.0±0.7	4.1	2.2-6.0	2.4-5.3[a]	2.3-2.6	5.0-5.4
Calcium (mg/dL)	All	767	9.9±0.6	10.0	7.8-11.2	8.6-11.0	8.4-8.9	10.9-11.1
	Male	283	9.9±0.5	9.9	8.0-11.2	8.9-10.8	8.2-9.0	10.8-11.1
	Female	484	10.0±0.6	10.0	7.8-12.2	8.6-11.0	8.4-8.9	10.9-11.1
Potassium (mEq/L)	All	601	4.7±0.5	4.6	3.8-6.4	3.9-5.8	3.9-4.0	5.6-5.9
	Male	218	4.6±0.4	4.6	3.8-5.9	3.9-5.6	3.4-4.0	5.4-5.8
	Female	383	4.7±0.5	4.6	3.8-6.4	3.9-5.9	3.8-4.0	5.7-6.0
Sodium (mEq/L)	All	589	129±2	129	123-134	124-133	123-124	133-134
	Male	213	129±2	129	123-134	124-133	123-125	132-134
	Female	376	129±2	129	123-134	124-134	123-124	133-134
Chloride (mEq/L)	All	606	91±2	91	83-97	86-96	86-87	95-96
	Male	213	91±3	91	84-97	86-96	84-87	94-96
	Female	376	91±3	91	83-97	86-96	85-87	95-96

Discussion

This study is the first to provide evidence of health parameter variation in a large population of Asian elephants managed in their natural habitat. These results contribute to the small but much-needed group of studies focused on health variation in free-ranging systems. The observed health parameter variation is largely comparable with studies published on other species, and supports the need to differentiate animals according to their sex when considering typical health parameter values in a population (Dangolla, Malitha, & Silva, 2004; Gromadzka-Ostrowska et al., 1988; Mel & Weerakoon, 2014; Morfeld et al., 2016; Niemuller et al., 1990; Nirmalan, Nair, & Simon, 1967; Ratnasooriya, Gunasekera, & Goonesekere, 1999; Salakij et al., 2005; I. D. Silva & Kuruwita, 1993a; Sreekumar & Nirmalan, 1992; Tuntasuvan, Teeraphan, Phoengpong, Jitnupong, & Lungka, 2002; Van Sonsbeek, Van der Kolk, Van Leeuwen, & Schaftenaar, 2011). Some sex differences e.g. in body score index (higher BCS in females) and serum chemistry parameters (lower creatinine, ALKP, glucose and higher triglycerides in females) are in contrast to other findings in captively managed Asian elephant populations, which highlights interesting potential differences arising as a result of management. For example, between-population differences may be explained by differences in exercise opportunities, disease exposure and foraging patterns between captive and wild/semi-captive animals. Importantly however, variation in health parameters may also result from differences in location, sample size, the collection and analysis methods used, population demography (sex and age), study duration (including possible seasonal differences) and the life-history or state of the animal (working, pregnant, lactating/nursing females, sick, etc).

Very few studies have successfully quantified variation in health parameters for wild populations. To establish the reference values presented here, the recommendations of the American Society for Veterinary Clinical Pathology (ASVCP)

were used (Friedrichs et al., 2012). Unfortunately, adhering to these guidelines is often challenging in field conditions, especially for endangered species *in situ* with small population sizes and fragmented habitats. The current study on semi-captive animals provided a unique opportunity to obtain reference values that may be comparable to wild systems while adhering to ASVCP guidelines. First, the study population lives, feeds and mates within their natural environment with minimal human interference. Second, using trained elephants enabled the collection of repeated samples for a significant number of individuals, across several seasons, and without having to use anaesthesia. Third, the presence of accurate life-history information for each animal also allowed the study of health variation in relation to demographic variation. Finally, the detailed records for each individual enabled us to avoid animals that are a clinical concern to veterinarians overseeing the study elephants. Ultimately, establishing range values for health parameters in natural systems is crucial for veterinary and conservation management.

A key result from the analyses was the determination of range values for BCS, which inform us about the overall perceived nutritional condition of the population. This study is the first to the authors' knowledge to apply a BCS to elephants living and foraging independently in their natural environment without significant human provisioning. Consequently, the results presented differ from those obtained for zoo elephants in America, where most of the animals were very fat (4) or obese (5) (Morfeld et al., 2016). MTE elephants forage naturally in the forest with changes to food availability throughout the year, and their BCS was considerably lower than in American zoo elephants. Morfeld et al., (2016) also presented results for the triglyceride level associated with each BCS score in American zoo elephants, comparing BCS to a major component of body fat content. Only one other study quantified triglycerides in Asian elephants, but this time using wild individuals from Sri Lanka (Silva & Dangolla, 2002). Our results are comparable with the results obtained in the study on wild elephants, whereas the values obtained by Morfeld et al., (2016) on zoo elephants are considerably higher. These

results reinforce the utility of the timber elephant population as a model for wild elephants.

To the knowledge of the authors, systolic and diastolic blood pressure have not been described in elephants until now. The description of blood pressure in elephants was made possible using a novel adaptation of the Wrap Cuff pressure monitor, which was applied under the anal skin flap around the tail. High and low blood pressure reading are widely used as an indicator of health in the cardiovascular system, but also for the function of other organs such as the kidney, both in humans and non-human animals (Brown et al., 2007; Kannel, 1996). Although there are no other studies available in which to compare the range values for blood pressure in elephants, these results are consistent with range values described in humans, cats and dogs (Brown et al., 2007; Kannel, 1996; Pickering et al., 2005) and provide a useful benchmark for veterinarians studying Asian elephants in the future.

The biggest differences to previous studies on Asian elephant health parameter values were seen in the differential white blood cell counts, especially between lymphocytes, monocytes and neutrophils. One reason for this may be confusion due to bilobed and sometimes trilobed cells that have been wrongly identified as lymphocytes (Allen et al., 1985; Gromadzka-Ostrowska et al., 1988; Nirmalan et al., 1967; White & Brown, 1978). Silva & Kuruwita (1993b) analysed these cells and saw that their granules were peroxidase positive and identical to the granulocyte (neutrophil and monocyte), but did not stain in Leishman-stain blood smears, similar to the non-bilobed monocyte. With the Leishman-stain the cytoplasmic granules of neutrophils are coloured, and so these cells could only be monocytes and not lymphocytes. Similar findings were described by Salakij et al., (2005) who used Sudan Black B, α-naphthyl acetate esterase and β-glucuronidase to show that the bilobed and trilobed cells stain in a very similar way to non-bilobed monocytes. This classification was adopted to distinguish between

lymphocytes and monocytes. The results from this study are in accordance with those reported by Salakij et al.(2005) who recorded more monocytes than lymphocytes in captive Asian elephants in Thailand. However, in contrast, using the same distinction for lymphocytes and monocytes for Sri Lankan elephants, Silva & Kuruwita, (1993b, 1993a) consistently found more lymphocytes than monocytes.

Reference intervals were established for a range of serum chemistry indices, which are used for disease diagnostics, to quantify the general level of health, and to understand the physiology of this endangered species. Different range values were obtained here compared to other studies, stressing the importance of having different RIs for different populations. For example, BUN levels indicate urea nitrogen in the blood, a waste product of protein digestion in the liver, and higher BUN levels were found here than in all previous studies available in Asian elephants (Silva & Kuruwita, 1993b, 1993a; Tuntasuvan et al., 2002). However, at present without kidney or liver pathology tests, it is assumed that this value represents the normal range for this population. In contrast, creatinine levels were lower here than in all other available studies, but as with BUN, since they are comparable or under the levels observed in other species such as the horse, they were considered normal (Aoki & Ishii, 2012; Lumsden, Rowe, & Mullen, 1980; Silva & Kuruwita, 1993b, 1993a; Van Sonsbeek et al., 2011). Total protein levels in the blood are used to understand the composition of the structural and defensive proteins in the blood and were found to be in accordance with previous studies (Silva & Kuruwita, 1993b; Tuntasuvan et al., 2002). However, differences were found in the composition of these proteins (Niemuller et al., 1990; Silva & Kuruwita, 1993a). A higher albumin:globulin ratio was observed than previously reported, and although the reasons for such differences are not clear, they could reflect between-population variation. CK was also investigated, which is indicative of muscle stress and damage. CK levels have been reported in one other study using a sample of captive elephants from the forestry department in India (including babies and non-working adults) and were lower than in

the current study (Sreekumar & Nirmalan, 1992). The higher values seen in the MTE population may be due to the fact that these individuals are working animals that experience higher levels of both stress and muscular exertion (Fowler & Mikota, 2006).

One interesting finding is the consistent difference between males and females in several health parameters. First, females generally displayed higher BCSs than males, despite individuals of both sexes being considered healthy. Higher BCSs in females were also observed in North American zoo elephants, where 27.1% of females had a BCS of 4 (fat) and 48.2% of females had a BCS of 5 (obese), compared to 47.8% of males with a BCS of 4 and 17.4% with a BCS of 5 (Morfeld et al., 2016). Males in our study also exhibited lower overall levels of triglycerides than females, which is also indicative of poorer body condition. This result is in line with those of Morfeld et al., (2016) who found a correlation between body condition and serum triglycerides, except between BCS 2 and 3 due to small sample sizes. Similarly, in wild and captive Asian elephants in Sri Lanka, females had higher levels of triglycerides than males (Silva & Dangolla, 2002). Some of the observed sex differences could be explained by the differences in life-history between males and females in the study population. In MTE elephants, females stop working when pregnancy is detected, and are given rest for 2 years after birth, whilst males only stop working when they are sick and need treatment. Additionally, males are under the influence of testosterone that is responsible for an anabolic metabolism of fat, decreasing fat storage (De Maddalena, Vodo, Petroni, & Aloisi, 2012; Kaufman & Vermeulen, 2005). In older women, the diminished influence of oestrogen due to menopause increases the risk of fat storage (Brown & Clegg, 2010) These findings are consistent with the results obtained in this study.

Regarding hematology, in other studies males have tended to have a higher red blood cell count, packed cell volume, and hemoglobin level than females due to the positive effect of testosterone in erythropoiesis (Howell et al., 2003; Kelani & Durotoye,

2002; Riviello & Wirz, 2001). Surprisingly, no significant differences between males and females were found in hematocrit or hemoglobin level in our study. Similarly, no significant sex differences in white blood cells, either in terms of total number or composition were observed, contrary to some descriptions in other species (Bush, Custer, & Whitla, 1980; Bush, Custer, Whitla, & Montali, 1983).

Concerning sex differences in serum chemistry levels, previous studies on other species (wild and lab populations) have reported mixed findings (Bush et al., 1980; Howell et al., 2003; Riviello & Wirz, 2001; Zhou & Hansson, 2004). In the current study, no differences between the two sexes were observed in either total protein, or any of its components. CK levels are predicted to be higher in males than in females due to an increase in muscular activity and muscle mass in male elephants, but no differences were found in the timber elephants (Sreekumar & Nirmalan, 1992). In contrast, females displayed lower levels of ALKP and glucose, which is in line with other studies, mainly in humans, but the physiological mechanisms and the reasons for this difference remain unknown (Rahmioglu et al., 2009; Schwartz, 2007). In the case of creatinine, females have lower values than males. As creatinine is a metabolite produced by muscle, males may have higher levels of creatinine due to increased muscle mass. Adding to this, given that the excretion rate of creatine is constant, it is expected that males with a higher body mass should have higher creatinine levels (Braun, Lefebvre, & Watson, 2003).

Overall, the sex differences observed in health parameters mirror the differences in body size, behaviour and life-history between male and female Asian elephants. Male elephants do not reach peak reproduction until their 30s or 40s (Lincoln & Ratnasooriya, 1996) and experience a significantly higher mortality risk across all ages (Lahdenperä et al., 2018). These sex differences may also have important implications for the clinical examination of this species. Specifically, normal health measure values in females may actually represent a clinical signal in males, and vice versa. Thus, it is crucial to

acknowledge sex differences when considering physiological status in conservation and population management.

In conclusion, the current study establishes baseline reference intervals for physical examinations, and analyses of serum chemistry and hematology in a large semi-captive population of Asian elephants managed in their natural habitat. The reference values obtained in this study provide a useful tool not only for MTE veterinarians, but for use in the *in situ* and *ex situ* management of this endangered species. The findings in this study also highlight the need for caution when comparing results not only between species, but also between populations of the same species and between the sexes. Understanding health parameter variation in free ranging populations can be used as a clinical tool to assess the health status of animals, working towards providing better management of species in the wild and in captivity.

Chapter 3

Age-related Variation of Molecular and Physiological Health Markers in a Long-lived Mammal

Abstract

Most organisms experience senescence, accumulating damage with ageing that can lead to loss of function or death. While this phenomenon has been widely studied in humans and model animals, less is known in wild populations that face other environmental challenges. In this study, we investigate ageing effects on the health of Asian elephants (*Elephas maximus*). We used global and individual trait-by-trait analyses of ageing effects on health by utilising data from a large number of animals ranging in age from 4 to 72 years, with repeated measures for several health parameters. Overall, age was associated with significant variation in a global measure of health that incorporated a range of parameters related to haematology, blood chemistry, immune, and liver function. When investigating the different traits individually we found age-related changes in immune defence (*eosinophils and lymphocytes*), liver function (*ALKP*), kidney function (*creatinine*), fat storage (*triglycerides*), and protein levels (*albumin*). We provide the first evidence of the underlying molecular and physiological health functions decreasing with age in a long-lived mammal living in natural conditions.

Introduction

The world's population comprises an ever-higher proportion of elderly individuals. With age being the primary risk factor for a number of pathologies that significantly affect late-life health and well-being (Niccoli & Partridge, 2012), understanding how and why we age has become a key challenge in science. Most organisms experience senescence, a process by which individuals accumulate damage with increasing age, leading to loss of function and eventually death (Monaghan, Charmantier, Nussey, & Ricklefs, 2008; Nussey, Froy, Lemaitre, Gaillard, & Austad, 2013). A large number of studies in humans, in model organisms, but also in wild vertebrates have now documented reproductive and actuarial senescence (Hawkes, 2003; Nussey et al.,

2013). However, our knowledge on the molecular and physiological mechanisms underlying such senescence patterns comes largely from studies on model organisms, which are usually relatively short-lived, genetically homogenous, and maintained under standard conditions. In contrast, very little is known about the molecular and physiological parameters associated with increasing age in animals living in natural conditions. Limiting ageing research to model organisms might cause us to miss important factors that help explain key ageing processes in long-lived species like ourselves, and leaves the wider evolutionary significance of the detected age-related changes uncertain. Moreover, although a couple of studies have explored immune and haematological parameter variation with age in wild mammals (Cheynel et al., 2017a; Jégo et al., 2014; Nussey, Watt, Pilkington, Zamoyska, & McNeilly, 2012), none have investigated a wide range of health parameters simultaneously, and considered multiple functions of health in populations living under natural conditions. However, such studies are needed since all biological functions might not decline at the same pace with age (Hayward et al., 2015).

Here, we present the first evidence of age-related variation in health markers in a mammal as long-lived as the Asian elephant (*Elephas maximus*). We take advantage of a unique health dataset of semi-captive Asian elephants (age range 4-72 years) for which physiological health measures and exact age are known accurately, to investigate age-related differences in muscle, hepatic and immune functions in a long-lived mammal population living under natural conditions. The study population is described as “semi-captive” because it is comprised of state-owned, individually-marked Asian elephants in Myanmar that are used daily as draft and transport animals in the timber industry by the Myanma Timber Enterprise (MTE), but released at other times into the forest to forage, mate and socialize independently under natural conditions (Lahdenperä et al., 2018; Robinson, Mar, & Lummaa, 2012), leading to mortality and reproductive patterns matching those of wild elephants (Clubb et al., 2008). Importantly, the semi-captivity

enables close monitoring of physiological health markers of animals with known birth dates, not possible in any wild population of a species this long-lived. Many features of the present study system parallel those of humans (the extreme longevity, reproductive patterns, as well as the working and retirement schedules), thus offering a unique comparison with human life-history trajectories.

Between March 2016 and April 2018, we collected blood samples from 205 females and males, ranging from 4 to 72 years old to examine age related variation in blood haematology and chemistry (haematocrit, haemoglobin, globulin, albumin, and total proteins), immune (total white blood cells counts), liver (aspartate aminotransferase-AST, alkaline phosphatase-ALKP), and muscle (creatinine kinase) and kidney (blood urea nitrogen-BUN, creatinine) functions, and fat storage (triglycerides); these are known to change with age in humans or laboratory model systems. Age was categorized in four groups corresponding to the different life/working stages experienced by the elephant population: young elephants undergoing a procedure aimed at habituating calves to humans, and for the calf to accept a rider (4-10 years old - calves); training elephants used for light work tasks only: 11-20 (juveniles); working elephants used in timber logging: 21-50 (adults), retired elephants not subject to physical work: 51-72 (seniors). In this species, and our population especially, the appropriate age threshold to separate juveniles from adults would be around 20 years old, which corresponds both to the age at which growth on average stops and reproduction begins (Lahdenperä et al., 2014; Mumby et al., 2015), and also the age when fertility peaks (Hayward et al., 2014).

To test the influence of life stages on health, we first assessed the overall age-related variation in health using a novel multivariate mixed model framework that considers that some health parameters co-vary with each other. Second, to determine the most important molecular and physiological parameters underlying the age-related declines in

overall health, we analysed variation in each trait separately, also testing for any sex differences in health deterioration with age, given that like in humans, male elephants experience several years' shorter lifespan compared to females (Lahdenperä et al., 2018).

Materials and Methods

Study population

Myanmar has the largest captive population of elephants worldwide, of around 5,000 elephants (Sukumar, 2006), 2,700 of which are government-owned through the Myanma Timber Enterprise for sustainable logging (Leimgruber et al., 2011). Government-owned Myanmar Timber elephants inhabit forest camps, distributed across Myanmar. Elephants work as transport and draught animals during the day, they are released at night for up to fourteen hours, and in rest periods to forage naturally, interact with conspecifics and mate unsupervised. Breeding rates are natural, with no reproductive management of the population, and timber elephants are never culled, numbers are not restricted or managed. As there is no additional supplementation, or selective breeding, they are classed as a semi-captive population.

Calves born in captivity are raised by both biological mothers and allomothers. Reproductive females are given rest from mid-pregnancy (11 months into gestation) until the calves reach their first birthday. Mothers are then used for lighter work duties until the calf reaches age four and is capable of foraging independently. Calves are separated from their mother and tamed/trained at around the age of four to five (Min Oo, 2010a), at which point they are assigned a rider, name, logbook and registration number. After the training period, elephants are used for light work duties until the age of around 20, at which point they enter the full workforce until retirement at around 50. The Myanma

Timber Enterprise (MTE) imposes regulations on the daily and annual workload of elephants, which cannot be exceeded and are consistent for all individuals in the study population (Khin Zaw, 1997). The work season lasts from mid-June to mid-February, with a rest period during October. This working season correspond to the monsoon (July-October) and cool (November-February) seasons, so that no work is done during the dry season (March-June) when temperature-related mortality is highest (Mumby, Courtiol, Mar, & Lummaa, 2013b). There are strict limits for the annual maximum tonnage of logs each elephant can move and also strict limits of weekly days and hours of work (in 2010; limits were set to a daily maximum of eight hours, with a break at noon, and five days of work in a week) (Hayward et al., 2014).

Each elephant is marked with a unique identification (ID) number and has important life-history information recorded in logbooks. Logbooks include crucial individually-based information, such as the identification number and name of each animal, their birth origin (captive-born or wild-caught), date of birth, latest location, mother's identification number and name (if known), year and place of capture (if wild-captured), year or age of taming, date of death or last known date alive, and cause of death. Moreover, the elephants receive monthly checks by trained veterinarians throughout their lifetimes, and these vets closely monitor and collect in logbooks changes in elephant body condition, health, and specific illnesses. The MTE maintains the logbooks until death or departure of the individual.

Elephant health's parameters

We measured a set of 17 health parameters in order to investigate age effects on several physiological responses from 2016 to 2018 over 3 seasons (monsoon/hot/cold 2016, monsoon/hot/cold 2017, hot 2018). In total, we had 586 repeated measurements of health parameters from 205 individuals for the analyses. Age ranged from 4 to 72 years of age. All elephants were measured and sampled in mornings on non-workdays.

To investigate hematological and serum chemistry levels, blood was collected from an ear vein in three different tubes, namely EDTA, heparin and serum separator tubes. The collected blood tubes were refrigerated for a maximum of 24 hours until analysis in the laboratory. For serum chemistry, the samples were centrifuged, and sera were collected and frozen until analysis in a laboratory in Yangon. The blood samples collected in EDTA were used to perform a manual count of leucocytes using Turk's solution (Franco dos Santos et al., 2020). Differential leucocyte counts were performed manually using a blood smear stained with Romanowsky stain (Franco dos Santos et al., *submitted*). Using VetScan i-Stat 1 hematocrit, and hemoglobin levels were obtained.

Blood cells and hematology

We measured age-related variation in immune responses by counting total white blood cells (TWBC; N=179) and each group of white blood cells (lymphocytes, monocytes, heterophils, eosinophils and basophils). The number of lymphocytes measured the adaptive immunity, the number of monocytes and heterophils measured the innate response, and the eosinophils and basophils measured the immunity against internal and macro-parasites, and the inflammatory response (Cheynel et al., 2017b; Karasuyama, Mukai, Obata, Tsujimura, & Wada, 2011). We measured the percentage of red blood cells using hematocrit (N=178), and the oxygen carrying capacity using hemoglobin (N=161) (Fowler & Mikota, 2006).

Proteins and triglyceride levels

To investigate age effects on homeostasis, we first measured the albumin, globulins levels, and the sum of albumins and globulins, which represents the total proteins (N=180). Albumin maintains the osmotic pressure and transport of several hormones, vitamins and hemoglobin. Globulin intervenes in the immune and inflammatory responses (Fowler & Mikota, 2006). We also quantified age effects on the

state of lipid storage using triglyceride levels (N=181) which are expected to decline as a result of senescence of body condition (Nussey et al., 2011).

Enzyme activity, and kidney, liver and muscle functions

Second, we investigated age effects on kidney function by measuring blood urea nitrogen (BUN; N=181) and creatinine (N=178). As end products of protein and muscle metabolism, they are good indicators of age-related variation in kidney function (Fowler & Mikota, 2006). Third, we measured the age-related variation in enzyme activity in the liver by assessing aspartate transaminase, important in amino acid metabolism (AST; N=181), and alkaline phosphatase working on energy metabolism (ALKP; N=180) (Fowler & Mikota, 2006). To measure age effects on enzyme activity in muscle, we measured creatinine kinase (CK; N=177).

Statistical analysis

To investigate the age-related variation in health parameters, we used three complementary methods: a multivariate health parameter analysis, a multivariate blood cells analysis, and a single-trait analysis. To prevent our analyses being driven by outlier points, we removed them using Horn's method. This method determines outliers in a Box-Cox transformed dataset using Tukey's interquartile (IQR) fences. A point was considered as an outlier when it lied outside 1.5 * IQR from the 1st or 3rd quartile point. For all methods, age was included as a 4-level variable (calves: 0-10 years old; juveniles: 11-20; adults: 21-50, seniors: 51-72).

Overall health: multivariate mixed model framework

As health parameters will co-vary with each other, we analysed the overall age-related variation in 13 health parameters using a multivariate mixed-model framework. First, we used a Gaussian multivariate mixed model (MCMCglmm), which allowed us to

include multiple response variables and we extracted residuals. We included hematocrit, the absolute white blood cell count (lymphocyte, monocyte, heterophil, and eosinophil), protein levels (albumin, globulin), triglycerides, kidney function (BUN, creatinine), and enzyme activity in the liver (ALKP, AST) and in the muscle (CK) as multi-responses. Because of a large number of missing values, we removed hemoglobin and basophils from the multivariate mixed model. We included the elephant's camp, season of sampling and wild or captive origin as fixed factors. We fitted the covariance between different data points from the same individuals by including individual as a random effect. We also checked whether our results were dominated by the priors (visual evaluations of the posterior distributions). MCMCglmm uses inverse-Wishart distributed priors for variances. We here specified proper priors with parameter "V" for the variances in **R** and in **ID** set at the repeatability for each trait. The parameter "nu" (degree of belief) was equal to the number of health parameters to be estimated in **R** and **ID**. Second, we performed a first Linear Discriminant Analysis (LDA) in which we included the residuals of the multivariate mixed model and 4-level age category, and a second LDA including the residuals of the multivariate model and the interaction between 4-level age category and sex. We tested for the significance of the discriminant values (Eigen values) using a multivariate analysis of variance (MANOVA) with a Pillai test.

Single-trait analysis

We analysed the age-related variation in immune function (*TWBC*), in hematology (*hematocrit, hemoglobin*), blood chemistry (*globulin, albumin, and total proteins*), fat storage (*triglycerides*), and kidney (*BUN, creatinine*), liver (*AST, ALKP*), and muscle (*creatinine kinase*) functions. We first determined the probability distributions for each health parameter. To do so, we fitted univariate distributions to each parameter. Using linear mixed models, we fitted the total white blood cells count, hematocrit, hemoglobin, total protein, globulins, albumin, and creatinine, as dependent variables using a Gaussian distribution. Using generalized mixed models, we fitted AST, ALKP

and CK as dependent variables using a Poisson error distribution, and triglycerides and BUN as dependent variables using a Gamma distribution with a log link. To test the influence of age on each health parameters, we included age as a 4-level factor and we used Wald tests with adjusted p-values for multiple testing to measure the contribution of the 4-level age factor. We included sex, captive or wild-caught origin of the elephant, season of sampling and the elephant camp as fixed factors, and individual identity, year of birth and year of sampling as random factors. Because of singularity issues, we removed the year of birth as a random factor for TWBC, globulin and creatinine, and we removed the random factors for triglycerides. Moreover, as numerous studies evidenced sex-specific senescence patterns (e.g. Clutton-Brock & Isvaran, 2007), we also tested the interactive effect between age and sex using Wald tests.

White blood cells: Multivariate generalized linear model framework

As the blood cell count was performed using a manual differential approach (see Franco dos Santos et al., 2020), we analysed specifically the age-related variation in white blood cells (*lymphocytes, monocytes, heterophils, basophils and eosinophils*) using a multivariate generalized linear model framework with a Dirichlet distribution, which allowed us to include multiple response variables. As the Dirichlet distribution is provided in GLM model, we were not able to include random effects for this analysis.

All analyses were conducted using R version 3.5.1 (R Development Core Team, 2018) and using the functions *fitdistr* from the package “fitdistrplus” (Delignette-Muller & Dutang, 2015), *horn.outliers* from the package “referenceIntervals” (Finnegan, 2014), *wald.test* from the package “aod” (Lesnoff & Lancelot, 2012); the given p-value are adjusted for multiple testing using a Benjamini & Hochberg correction, *lmer* and *glmer* from the package “lme4” (Bates et al., 2015), *DirichReg* from “DirichletReg” package (Maier, 2015), *MCMCglmm* from “MCMCglmm” package (Hadfield, 2010). The LDA was performed with “ade4” and the function *discrimen* (Dray & Dufour, 2007).

Results

Overall analysis

Our multivariate health parameter analysis and the Linear Discriminant Analysis (LDA) provide strong support that, over the extraordinary long lifespan of elephants, overall health declined with age (df_{health}=413, df_{age}=3, Pillai=0.16, F=1.72, p<0.01). Despite the higher age-specific mortality risk in males compared to females, the overall health declines were consistent for both males and females (sex x age interaction: df_{health}=409, $df_{age*sex}$=7, Pillai=0.27, F=1.08, p=0.06). LDA projects the 13 health parameters onto a lower-dimensional space with age class separability. LDA provides, then, a discriminant function that is a combination of variables (health parameters) with the associated discriminant coefficients (Table 1). The linear discriminants are axes that maximize the separation between age groups. We show that the 13 health parameters were strongly discriminated according to the age group of the individuals (figure 3.1). The linear discriminant 1 explained 68% of the variance of health parameters and maximized the separation of elephant age groups chronologically based on their health (Appendix – Figure 3.1). The linear discriminant 2 explained 25% of the variance in health parameters and clustered senior elephants from adults, which could represent an axis of work hardship. Overall, the age term influenced mainly five health functions: immune defence (*eosinophils and lymphocytes*), liver (*ALKP*), kidney (*creatinine*) fat storage (*triglyceride*s), and protein level (*albumin*), which showed the highest standardized discriminant coefficients (Table 1). In particular, liver function had the highest coefficients along linear discriminant 1 and the triglycerides, lymphocytes and albumin had the highest coefficients along linear discriminant 2. We show that changes in fat storage and immunity are associated with senior elephants, changes in liver function is associated with the youngest elephants, and changes in protein level and kidney activity are associated with adult elephants.

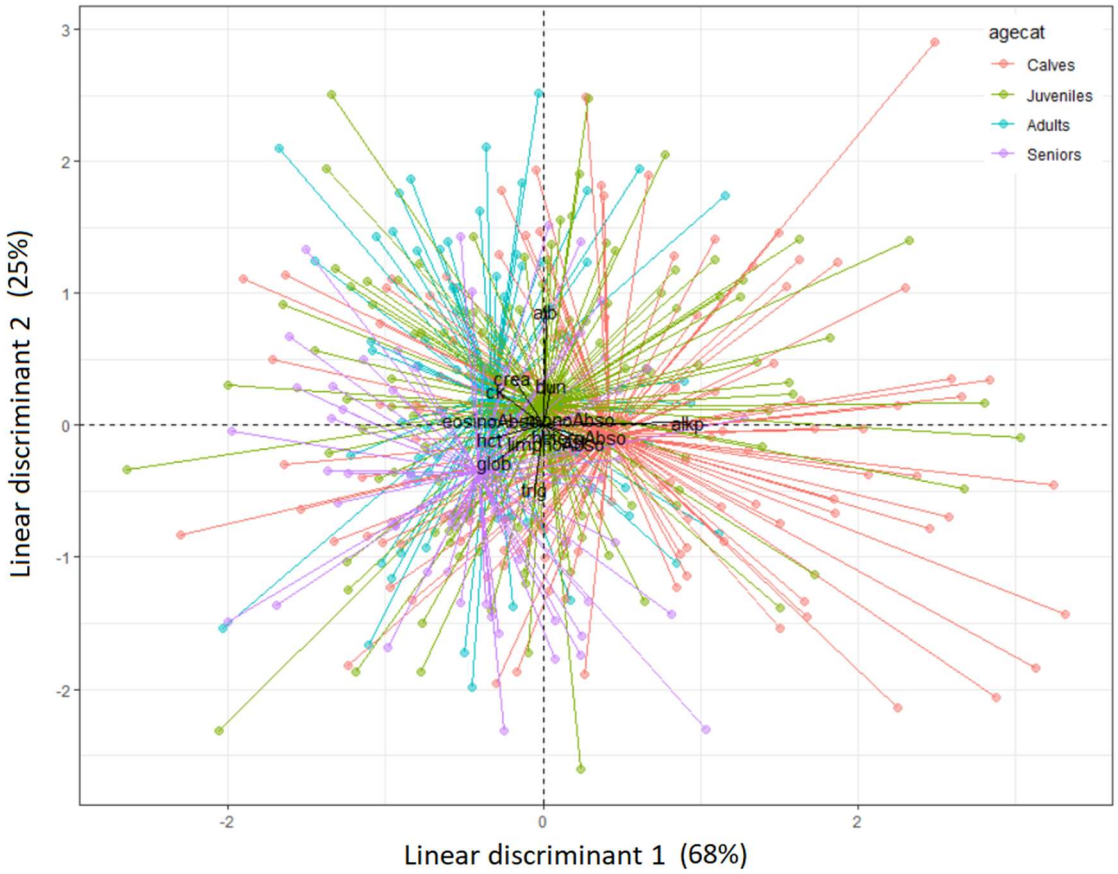


Figure 3.1. Biplot from the LDA showing clustering of “calves”, “juveniles”, “adults”, and “seniors” elephants across 13 health parameters. The figure combines the standardised coefficients of health in black (i.e. canonical weights of the linear discriminant functions on the two axes of the linear discriminant analysis, axis1: linear discriminant 1, axis 2: linear discriminant 2), and the projection of the health samples with gravity centres of each life stage (“calves” in red, “juveniles” in green, “adults” in blue, and “seniors” in purple).

Table 1. Standardized loadings (standardized coefficients) for the canonical variables (health parameters) from the Linear Discriminant Analysis. Health parameters with the highest loadings are highlighted in grey.

	Axis 1	Axis 2
Hematocrit	-0.340	-0.108
Monocytes	0.170	0.052
Eosinophils	-0.329	0.046
Lymphocytes	0.087	-0.132
Heterocytes	0.232	-0.085
Albumin	0.018	0.867
Globulin	-0.304	-0.282
Triglycerides	-0.054	-0.478
BUN	0.052	0.301
Creatinine	-0.189	0.367
AST	0.195	-0.112
ALKP	0.922	0.027
CK	-0.295	0.261

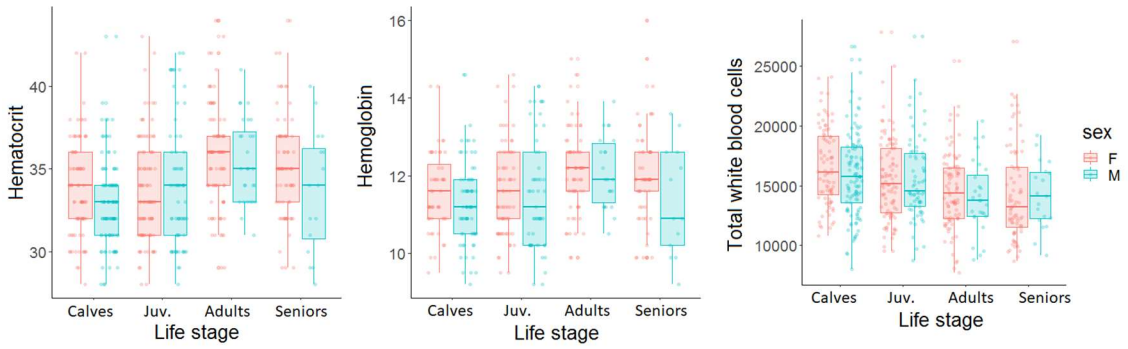
Single trait analysis

Hematology

Elephant's age influenced red blood cell levels (i.e hematocrit: χ^2= 11.2, df= 3, p=0.01, p-adjusted=0.02; hemoglobin: χ^2= 7.8, df= 3, p=0.05, p-adjusted=0.06). Hematocrit varied between 28 and 44% with an average of 34.25%. We detected an increased hematocrit between the calf and the adult life stages (β= 1.58 ± 0.59, t = 2.68) whereas haematocrit remained constant across adulthood (β= -0.08 ± 0.61, t = -0.13). We did not detect a significant effect of sex (β= -0.39 ± 0.35, t = -1.11) or effect of the interaction between age and sex (χ^2= 4.9, df= 3, p=0.18, p-adjusted=0.30), indicating that haematocrit varied similarly with age in both males and females. Hemoglobin varied between 9.20 g/dL and 16 g/dL with an average of 11.71 g/dL. Similarly to haematocrit, hemoglobin increased between calf and adult life stages (β= 0.38 ± 0.23, t = 1.63) and

remained across adulthood (β= 0.12 ± 0.24, t = 0.53). We did not detect any effect of sex (β= -0.21 ± 0.14, t = -1.48) or the interaction between sex and age (χ^2= 2.7, df= 3, p=0.44, p-adjusted=0.53).

Elephant's age also substantially influenced total white blood cells (TWBC; χ^2= 17.3, df= 3, p<0.01, p-adjusted<0.01). On average, our sampled elephants had 16,738 x10^9 white blood cells/L but this ranged widely between and within animals sampled multiple times from 7,722 x10^9 to 27,856 x10^9 white blood cells/L. We showed that TWBC strongly decreased over the life stages (Appendix - Table 3.1). We did not detect any effect of an interaction between age and sex (χ^2= 0.9, df= 3, p=0.83, p-adjusted=0.83),



confirming that this decline was consistent in both males and females.

Figure 2. Age-related changes in hematocrit, hemoglobin and total white blood cells in both sexes. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and lowest values of the results

Protein levels

On average, elephants displayed 7.67 ± 0.53 g/dL of total proteins including 2.99 ± 0.22 g/dL of albumin and 4.66 ± 0.42 g/dL of globulin. Age strongly influenced the variation in the three measures of protein levels (*Total proteins*: χ^2= 61.7, df= 3, p<0.01, p-adjusted<0.01; *Albumin*: χ^2= 17, df= 3, p<0.01, p-adjusted<0.01; *Globulin*: χ^2= 94.1,

df= 3, p<0.01, p-adjusted<0.01). Elephants displayed a similar age-related patterns with an increase until working life stage and then a slight decline during retirement for total proteins (β= -0.07 ± 0.08, t = -0.79), a significant decline for albumin (β= -0.12 ± 0.04, t = -3.00), and no difference for globulin (β= 0.05 ± 0.07, t = 0.66) (Appendix – Table 3.2). Therefore, the decline of protein levels during retirement was mostly driven by the decline of albumin in senior elephants. We did not detect any sex differences in any of the proteins as we did not observe this effect on total proteins or globulins (*Total proteins*: χ^2= 4.7, df= 3, p=0.19, p-adjusted=0.30; *Globulin*: χ^2= 3.5, df= 3, p=0.32, p-adjusted=0.43). Overall, we observed stronger old age effects on albumin than globulin.

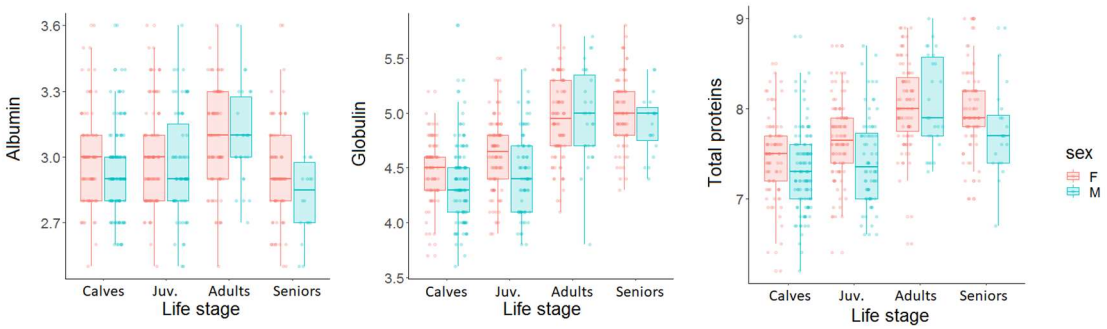


Figure 3. Age-related changes in total proteins, albumin, and globulin in both sexes. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and lowest values of the results.

Triglyceride levels

We measured fat storage using triglyceride levels that averaged 15.17 mg/dL across the samples but ranged from 0.10 to 88.00 (χ^2= 9.1, df= 3, p=0.03, p-adjusted=0.04). Triglyceride level remained constant for calves, juveniles and adults and declined for seniors and especially for males (β= -1.06 ± 0.32) but this effect remained non-significant after performing the Benjamini-Hochberg FDR-controlling method for

multiple hypothesis testing (χ^2= 9.6, df= 3, p=0.02, p-adjusted=0.12) (Appendix – Table 3.3).

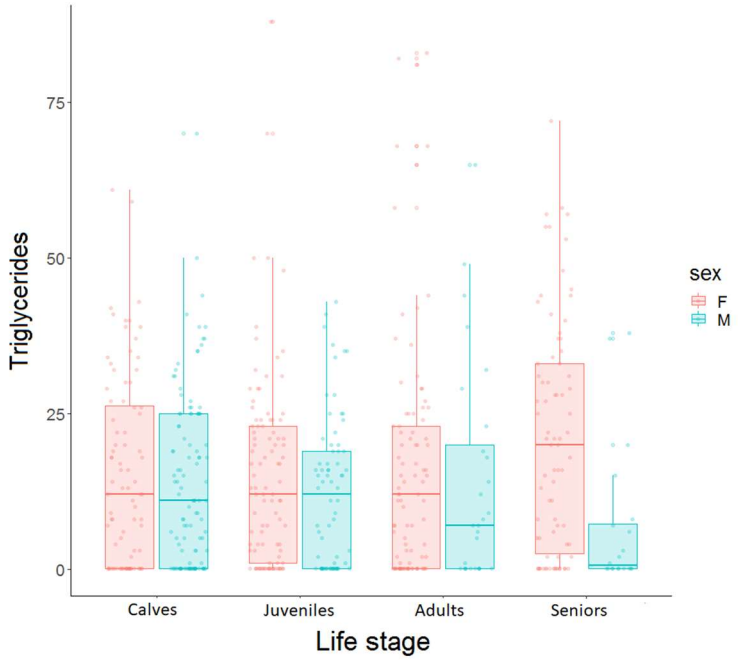


Figure 4. Age-related changes in triglycerides in both sexes. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and lowest values of the results

Kidney activity

We measured kidney activity using two parameters BUN (14.77 ± 5.21 mg/dL, on average) and creatinine (1.07 ± 0.23 mg/dL, on average). While we showed an absence of age effect on BUN (χ^2= 5.1, df= 3, p=0.17, p-adjusted=0.18; fig. 5), we evidenced a significant interactive effect between age and sex on BUN (χ^2= 8.8, df= 3, p=0.03, p-adjusted=0.12; Appendix - Table 3.4) where working males showed the highest level of BUN. By contrast, we did not show any effect of age (χ^2= 3.3, df= 3, p=0.35, p-adjusted=0.35) or interaction between age and sex (χ^2= 6.4, df= 3, p=0.10, p-adjusted=0.24) on creatinine.

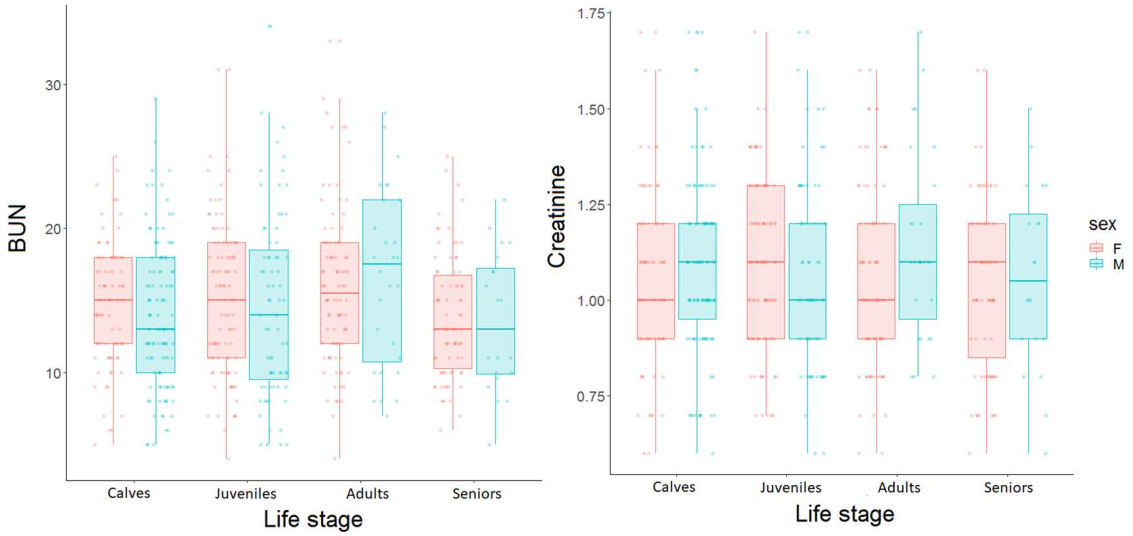


Figure 5. Age-related changes in BUN and creatinine in both sexes. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and lowest values of the results.

Liver and muscular activities

We measured three enzymes namely, AST (18.48 ± 17.37 U/L, on average), ALKP (89.82 ± 42.90 U/L, on average), CK (147.45 ± 90.95 U/L, on average) which were associated with the liver (AST, ALKP) and muscular (CK) activity, and expected to decrease with age. Age strongly influenced the liver activity (*AST*: χ^2= 10.00, df= 3, p=0.02, p-adjusted=0.03; *ALKP*: χ^2= 171.4, df= 3, p<0.01, p-adjusted<0.01) but not the muscular activity (*CK*: χ^2= 5.5, df= 3, p=0.14, p-adjusted=0.17). We observed the same pattern of decline with age for both liver enzymes (Appendix - Table 3.5). We detected a small effect of the interaction between age and sex only for AST (χ^2= 9.7, df= 3, p=0.02, p-adjusted=0.12) and no effect on ALKP (p-adjusted=0.30) and CK (p-adjusted=0.18).

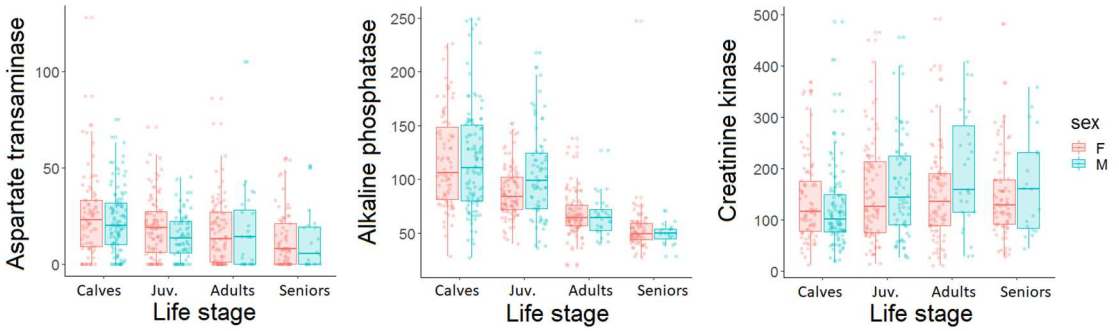


Figure 6. Age-related changes in AST, ALKP, and CK in both sexes. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentiles, and the whiskers indicate the highest and lowest values of the results.

Multivariate-blood cells analysis

Although we showed a significant effect of age on white blood cells (*lymphocytes, heterophils, monocytes, eosinophils, and basophils*), we did not detect a significant effect of the interaction between age and sex (χ^2= 18.3, df= 15, p=0.25; fig. 7). We showed that the differential count of heterophils and eosinophils increased with age (fig. 7; Appendix - Table 3.6). As in the single-trait analysis, TWBC decreased with age, and our results showed that the decrease in immunity was mainly driven by a decline of lymphocytes, monocytes, and basophils.

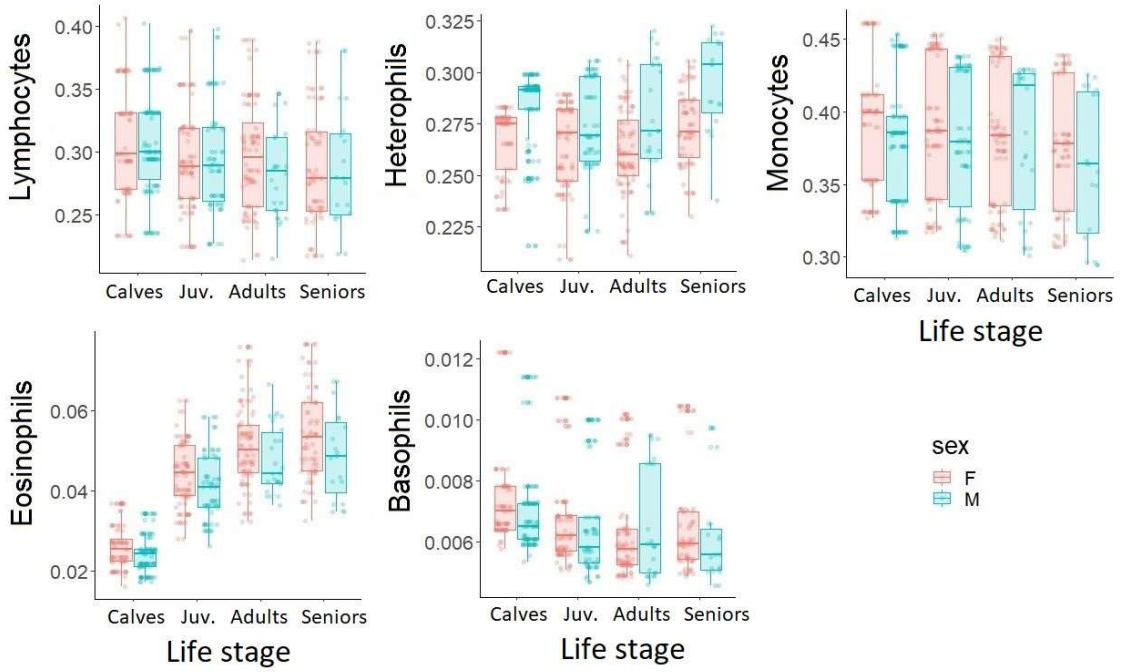


Figure 7. Age-related variation in white blood cells measured in the multivariate generalized model (with a Dirichlet distribution). The horizontal line within the box indicates the median, boundaries of the box indicate the 25th and 75th percentile, and the whiskers indicate the highest and lowest values of the results.

Discussion

Our data complement the accumulating evidence that declines in survival and reproduction with age are observable in natural populations, by providing the first evidence of the underlying molecular and physiological health functions decreasing with age in a long-lived mammal, to date only documented in humans and laboratory mammals. Studies testing evolutionary predictions in natural populations could therefore provide key insights into the causes of inter-individual variation in ageing patterns and into the mechanisms underlying such variation. Our relatively “short-term”, cross-sectional sample relative to the extraordinarily long elephant lifespan precluded us from detecting causal links between the physiological parameters we measured and individual survival and/or reproductive senescence. However, the age categories in which we

observed the most decline in the traits we measured correspond to the ones in which we also observed severe declines in reproduction and survival (Lahdenperä et al., 2014). By providing the first evidence for age-related differences in a broad range of physiological markers in a long-lived mammal living under natural conditions, our data suggest a certain ubiquity in the patterns previously observed in laboratory models and in humans. Our results also suggest that such age-dependent differences in health markers might be a target for natural selection in wild populations and emphasise the need for more longitudinal research in wild populations to shed further light on the underlying mechanisms, the evolutionary causes and the consequences of their variation in ageing.

While the elephants' ages influenced overall health, we did not detect many differences between males and females in the way they aged. Age was a strong discriminating factor on the axis 1 of the LDA based on 13 health parameters. The axis 2, which discriminated seniors from adults based on health, could represent an axis of work hardship. Overall, age influenced mainly four health functions: immune function (*eosinophils and lymphocytes*), liver function (*alkp*), fat storage (*triglyceride*s), and proteins level (*albumin*) as they showed the highest coefficients (called hereafter *loadings*; Table 1). In particular, the liver function (*alkp*) had the highest loading on the axis 1 and the triglycerides, creatinine and albumin had the highest loadings on the axis 2. This discrimination of elephant's age according to health parameters allowed us to show that fat storage and immunity were associated with senior elephants, liver function was associated to the youngest elephants, and proteins level were associated with adult elephants.

We then determined the most important molecular and physiological parameters underlying the age-related declines in overall health by investigating each trait in a separate analysis, and found several parameters to co-vary strongly with the elephant's

age, with a significant effect of age on the variation in haematology, blood chemistry, immune, and liver function (Figure 3.2). Interestingly, for the kidney and muscle functions, we did not detect any effect of age, as creatinine, BUN and creatinine kinase levels stayed constant across all age groups. By contrast, as demonstrated in humans (Helman & Rubenstein, 1975), we showed that haematocrit and hemoglobin levels increased with age; and the serum chemistry (albumin), the lipid metabolite levels (triglycerides), the immune function (TWBC) and liver (ALKP) function decreased sharply between the working and retired life stage. Although white blood cell counts overall declined with age, a more detailed analysis on the different white blood cells revealed that the pattern slightly varied depending on the cell type. While lymphocytes, monocytes, and basophils decreased with age, heterophils and eosinophils increased. Nonetheless, both the decrease in total white blood cells and the increase in globulins with age are characteristic of a decrease in immune function with age, as previously shown in humans and few vertebrates in the wild (Franceschi et al., 2006; Nussey et al., 2012). This increase of globulin might suggest that a progressive dysregulation of the inflammatory response occurs at old ages, leading to an increase in the production of related inflammatory products, which could be responsible for chronic inflammation causing tissue degeneration. The declines in both the innate and the adaptive systems and the increase of globulin levels with age indicate the existence of immunosenescence in Asian elephants. In parallel, we also observe a decline in lipid metabolite levels, which often reflects an alteration of body condition with age (Jenni-Eiermann & Jenni, 1994; Toth & Tchernof, 2000). In addition, the decrease in AST and ALKP levels with age implies liver damage and/or a decline in liver function, which are symptomatic of an ageing hepatic function (Zeeh & Platt, 2002). Moreover, using a Benjamini-Hochberg False Discovering Rate (FDR) controlling method, we did not detect any sex differences in age-related variation in health parameters, except a small (but non-significant) effect of age-related variation in AST.

In conclusion, our results demonstrate the first evidence of senescence in a wild mammal as long-lived as humans. Ageing effects on health were observed when evaluating health globally but also in the majority of the individual traits, especially in immunity, fat storage and liver function. These results match with the observed declines in survival and reproduction, and call for more studies that could create the link between those life-history traits and physiological mechanisms.

Chapter 4

Hot, cold or rainy? Health Seasonality in Asian Elephants

Abstract

Seasonal variation can have unknown effects on animal physiological processes. Theoretically, long-lived species are predicted to be buffered against climate variation: high longevity means low annual mortality and reproduction rates, and annual variability in climate may therefore have a smaller impact on population growth rates of long-lived species as compared to short-lived ones. However, little is known of the physiological mechanisms for such patterns in long-lived species. In this study, we investigated seasonal variation in the health of Asian elephants from a seasonal monsoon climate. We used global and individual trait-by-trait analyses of seasonal effects on health by utilising data from a large number of animals with repeated measures for several health parameters. In the global analysis, we highlighted the biggest differences in health between the hot and monsoon seasons. More generally, the results suggest that even long-lived, large mammals may experience physiological changes in response to seasonal variation in their environment, that can in extreme circumstances pose a significant health risk.

Introduction

In an effort to understand the widespread effects of climate change on natural systems, research has been increasingly directed towards understanding how climate influences life-history traits (Loarie et al., 2009; Parmesan & Yohe, 2003). Accumulating evidence now documents how climatic variability in a range of taxa affects key traits such as body mass (Perret & Aujard, 2001), reproduction (Perret & Aujard, 2001; Rosa & Bryant, 2003) and survival (Mumby et al., 2013b; Schaub & Vaterlaus-Schlegel, 2001), and even how these traits evolve in response to environmental change, at least in relatively short-lived species (Charmantier et al., 2008) – however, far less is known of

the underlying physiological mechanisms between environmental variation and vital rates. Within each year climate variation not only alters temperature, rainfall and other weather patterns, but is also responsible for changes in food availability (Post & Forchhammer, 2008), pathogen exposure (Dukes et al., 2009; Lafferty, 2009) and seasonal timings of biological events (Farrell et al., 2008; Pörtner & Farrell, 2008). Understanding how these multiple interacting factors affect physiological markers of health, which ultimately lead to reduced (or increased) growth, reproduction and survival of individuals, is essential to the development of better intervention strategies. Unfortunately, compared to the rapid pace of climate change, the data needed to explore this are slow to accumulate, particularly for long-lived species.

One approach to study the effects of environmental variation on physiological markers of health in long-lived species is to monitor species inhabiting seasonal environments, and observe longitudinally how their different health parameters respond to weather fluctuations and the accompanying environmental changes. Seasonal variation is known to affect health in many species across different taxa living in a variety of environments, including mammals (gray wolves: Butler, Ballard, & Whitlaw, 2006; harbour seals: Trumble, Castellini, Mau, & Castellini, 2006; beluga whales: Norman et al., 2012), birds (Eurasian skylarks: Hegemann, Matson, Both, & Tieleman, 2012) and reptiles (yellow-marginated box turtle: Yu, Wu, & Chi, 2014). Most studies have, however, focused on animals that live at high latitudes with significant differences in temperature and rainfall, as well as day length and subsequent differences in photoperiodicity between summer and winter, or with hibernating mammals such as bears (Butler et al., 2006; DelGiudice et al., 1992; Hissa et al., 1994; Yang et al., 2018). Yet, even contemporary humans living in economically developed regions such as the US experience differences in physical and mental health between summer and winter (Jia & Lubetkin, 2009). By contrast, little research exists on long-lived animals exposed

to moderate climates and stable photoperiodicity, but still with seasonal fluctuations in weather. Moreover, most previous studies have focused on one or two aspects of physiology rather than a global quantification of health, and they rarely monitor the same individuals longitudinally across different seasons, which limits our ability to understand different responses of the same individuals to ecological challenges.

Asian elephants (*Elephas maximus*) are large, terrestrial mammals with very long lifespans (more than 80 years in some cases), that range in tropical regions with pronounced seasonal variation (Mumby et al., 2013b). In their core distribution area, it is possible to distinguish three different seasons with marked differences in weather and ecology (Romatschke & Houze, 2011). The hot season from March to June is characterized by high temperatures and no rainfall, the monsoon season from July to October also displays high temperatures but with very high rainfall levels, and the cool season from November to February is characterized by low temperatures and a reduced rainfall, but extreme weather patterns during the hot and monsoon season are on the rise (Wassmann et al., 2009). For studying the link between environmental variation and health, Asian elephants are a particularly interesting study species as they are not seasonal breeders, but instead females can conceive any time of the year during their 16-week oestrus cycle. Nevertheless, their birth rate varies between months, being highest in the cold season and lowest in the monsoon (Mumby, Courtiol, Mar, & Lummaa, 2013a). Similarly, mortality varies seasonally, with the lowest mortality rates occurring during the monsoon season compared to the relatively higher rates of the cold and hot seasons (Mumby et al., 2015). Asian elephant populations both in captivity and in the wild are becoming increasingly threatened and are classified by IUCN as endangered (Choudhury et al., 2008), so understanding how seasonal, and more generally, climatic variation affects the physiological measures of health which underlie observed increases in mortality and reductions in fertility, is important for the conservation of existing

populations. Yet, to date, little is known of the within individual global health patterns that respond to seasonal variation across all species, particularly those found in tropical regions. High variation in rainfall and temperature are predicted to have both direct and indirect effects on health, by changing food availability and quality, and even the presence and transmission of certain parasites and diseases (Mumby et al., 2013b). For example, it is known that the lack of water and excessive temperatures can lead to dehydration and heat stroke, which may be detectable in commonly monitored health parameters as increases in red blood cell count, because of the dehydration and of increases in blood urea nitrogen (BUN) and creatinine secretion by the kidneys in response to the lack of systemic fluids (Fowler & Mikota, 2006). However, because studies into such “ecological medicine” in natural populations are exceedingly rare, we currently know very little concerning how different individuals cope with ecological challenges over time and in a range of conditions.

In this study, we investigate seasonal variation in a large range of health parameters in Asian elephants of different ages and sexes in a tropical monsoon climate (Beck et al., 2018). We focus on 23 different health parameters that cover a wide range of physiological functions, such as haematological, immunological, muscular, kidney and liver functions, as well as protein balance and electrolytes to gain a global understanding of health variation in different ecological settings. Our study design allows longitudinal monitoring of 225 individually marked elephants with known age, reproductive and health history, with repeated measures per individual taken across seasons over a 26-month study period. This study population is part of the world’s largest population of captive Asian elephants (~2,700), consisting of government-owned working elephants in timber camps, employed and centrally managed by the Myanma Timber Enterprise (MTE) (Jackson et al., 2019). Although these elephants are managed as draught and transport animals by the MTE, they are considered “semi-captive” and live largely under natural

conditions: they are released in the surrounding forests outside working hours and at night to forage and to socialise with wild and other captive conspecifics, and breeding rates are not managed by humans, with mating and birth occurring independently in forests at night. MTE elephants benefit from twice a month veterinary checks, but usually only traditional medicines were available during the study period. Hence, the population offers a unique opportunity to collect longitudinal measures of the health of individually marked animals with known age, breeding history and veterinary records, but that exhibit foraging, lifespan and breeding patterns comparable to wild elephants (Lahdenperä et al., 2018). Given the seasonally varying mortality and fertility rates in the population (Mumby et al., 2013a, 2013b), we expect that season will have a strong effect on the underlying health parameters, and that our study will contribute towards creating a better health management system for this endangered species.

Materials and methods

Study population

Our study population of government-owned MTE elephants inhabits forests and logging camps distributed across Myanmar. Captive-born timber elephants that comprise most of the current population (Jackson et al., 2019; Lahdenperä et al., 2018) are raised by their mothers and allomothers. Pregnant females are given rest from work mid-pregnancy (11 months) until their calf reaches one year of age, and after that, mothers continue light work until their calf is four and capable of foraging independently. At age four or five, calves are separated from mothers and are tamed (Min Oo, 2010b). At the end of this process they are assigned a rider (mahout), a name, a logbook and a registration number that is marked on their haunches. From age five to 17, the elephants are used in light work only, such as transportation, and from 18, they are then employed to drag logs until their retirement at age 55. The regulations from MTE impose a

maximum daily and annual workload for each elephant that was consistent in the study population (Tun Aung & Thoung Nyunt, 2001), and during retirement, the elephants are held in nursing camps and cared for until their death.

The logbooks of each elephant are also maintained until death, providing our study with vital information on demography and veterinarian care for every MTE elephant, such as sex, date of birth, and date of last medical treatment. They include individual-specific information including identification number and name, their birth origin (captive-born or wild-caught), date of birth, most recent location, mother's identification number and name (if known), year and place of capture (if wild-captured), year or age of taming, date of death or last known date alive, and cause of death. Moreover, the elephants receive twice a month checks by trained veterinarians throughout their lifetime, who closely monitor and register in logbooks any changes in elephant body condition, health, and specific illnesses.

The working season of MTE elephants lasts from mid-June until mid-February, i.e. during the monsoon (July-October) and cool (November-February) seasons. The resting period corresponds to the hot season (March-June), when elephant thermoregulation is compromised due to their small surface-to-volume ratio and the high energetic costs of cooling (Weissenböck et al., 2011) – when temperature related mortality is the highest (Mumby et al., 2013b, 2013a).

Study sample and data selection

We collected seasonal health parameters longitudinally from March 2016 to November 2018 for 225 elephants (females: 139, males: 86) ranging in age from four to 72 years old. We sampled elephants from three logging camps in the Sagaing Division

in Myanmar, namely, Kawlin (524 samples), East Katha (176 samples) and West Katha (98 samples). Samples were collected in three different seasons: the hot season in March-April (283 samples), the monsoon season in July (244 samples) and the cold season in November (271 samples). Despite efforts to sample the same elephants every season, this was sometimes impossible due to translocation or death, but overall we sampled: five individuals across nine seasons, 13 individuals across eight seasons, 17 individuals across seven seasons, 22 individuals across six seasons, 19 individuals across five seasons, 17 individuals across four seasons, 24 individuals across three seasons, 52 individuals across two seasons and finally, 56 individuals across one season only.

Health measurement

We measured a set of 23 health parameters in order to understand the physiological responses to environmental cues in different seasons. All elephants were measured and sampled during the morning of non-workdays (for details, see Franco dos Santos et al., submitted).

Analysing haematological and serum chemistry parameters (see below for details) required the collection of blood from an ear vein using a Vacuette® system (Greiner Bio-One, Kremsmünster, Austria, 4550) with three different tubes, namely, EDTA (8 mL), heparin (2 mL) and serum separator tubes (9 mL). The blood tubes were refrigerated for a maximum of 24 hours until analysed in the laboratory. For serum chemistry, the samples were centrifuged, and sera were collected and frozen until analysis in a laboratory in Yangon using the IDEXX VetTest® (IDEXX, Westbrook, USA, 04092). Serum chemistry included analysis on kidney function (blood urea nitrogen (BUN) and creatinine), protein activity (total proteins and their two biggest constituents,

albumin and globulins), liver function (aspartate aminotransferase (AST) and alkaline phosphatase (ALKP)), lipid storage (triglycerides), muscle function (creatine kinase (CK)) and calcium levels. Of these complementary serum chemistry health parameters, BUN is the end product of protein metabolism and creatinine is the end product of creatine metabolism in the muscles, and they are both excreted from the body by the kidney (Fowler & Mikota, 2006). They can be changed by the nutritional status (BUN) or muscle mass (creatinine) that varies according to the season (Butler et al., 2006; DelGiudice et al., 1992), or can increase due to dehydration during the hotter seasons (Fowler & Mikota, 2006). Proteins are responsible for several biological processes (such as molecular transport or even immunity; Macrae, 1993), and are expected to increase in the hot season due to dehydration, but could also increase due to infectious agents that increase globulins (Fowler & Mikota, 2006). AST is present in both liver and muscle, with ALKP being liver-specific and CK muscle-specific. Consequently, an increase in AST with CK would suggest muscle damage, especially in seasons entailing loss of muscle mass (Fowler & Mikota, 2006). Since the sampled elephants are working, we also expected to observe an increase in the two enzymes during the working season, which takes place during the monsoon and cold seasons (Lahdenperä et al., 2018). Finally, triglycerides indicate fat storage levels and are expected to increase in the season(s) when nutritional status is better (Fowler & Mikota, 2006).

The blood samples collected in EDTA were used to perform a manual count of white blood cells using Turk's solution (Franco dos Santos et al., submitted). Differential leucocyte counts were performed manually using a blood smear stained with Romanowsky solutions (Franco dos Santos et al., submitted), which allowed us to understand the immune behaviour of each individual by observing the presence of five leucocytes that have different functions in the immune system: lymphocytes, monocytes, heterophils, eosinophils and basophils. Lymphocytes take part in adaptive immunity

while monocytes and neutrophils, being phagocytic cells, are involved in the innate response (Cheynel et al., 2017a). Eosinophils induce immunity against internal parasites and inflammatory response (Cheynel et al., 2017a), while basophils act as a defence against macroparasites (Karasuyama et al., 2011).

We also monitored haematocrit, haemoglobin, sodium, potassium and chloride levels. Haematocrit and haemoglobin are measurements of red blood cell function, and both can be affected by seasonal variation in animal nutritional status, food intake or even dehydration (DelGiudice et al., 1992; Hellgren, Rogers, & Seal, 1993). Electrolytes are responsible for normal cell function, electrochemical impulses and acid-base balance (Fowler & Mikota, 2006), which may become elevated during the hot seasons due to water deprivation (Fowler & Mikota, 2006). These data were obtained using a VetScan i-Stat® 1 (Abaxis, Union City, USA, 94587) with an E3+ cartridge.

Finally, blood pressure is known to vary seasonally in some humans due to temperature variation, with temperature making the main vessels of the body constrict or dilate (Rosenthal, 2004). Blood pressure was measured using the Omron M6 Comfort IT (Omron, Kyoto, Japan, 617-0002) blood pressure monitor with an Intelli wrap cuff (Franco dos Santos et al., submitted), which was applied under the anal skin lap in an area where the diameter of the tail was constant. The elephants were trained by their mahouts to accept tail handling and to keep their tail still.

Statistical analysis

To investigate the influence of the three seasons (hot, monsoon and cold) on health parameters, we used two complementary methods: a multivariate joint analysis of health parameters, and a univariate parameter-by-parameter analysis. The multivariate

analysis allowed us to capture an overall association of seasons with health by taking into account the covariation of health parameters. In the single-trait analysis, we analysed each health parameter separately in order to describe specific, potentially parasite-related changes in physiology.

Outliers in the health parameters were detected and removed using Horn's method, where outliers were determined from a Box-Cox transformed dataset using Tukey's interquartile (IQR) fences. A datapoint was considered an outlier when it lay 1.5 times the IQR outside the 1st or 3rd quartile point. After removing outliers, the sample sizes varied between health parameters (see Appendices – Table 4.1).

Association between the three seasons and overall health: Multivariate mixed procedure

We proceeded in two steps to investigate whether seasons were associated with variations in health, taking into account the expected correlation between health measures. First, we used a Bayesian multivariate mixed model (MCMCglmm: Hadfield, 2010) implemented in a framework using Markov chain Monte Carlo (MCMC) sampling, with a simple Gaussian error structure and identity link. MCMCglmm uses inverse-Wishart distributed priors for variances. Here we specified proper priors with parameter "V" for the variances in **R** (matrix containing the residual covariances) and in **ID** (matrix denoting the between individual covariance) set at the repeatability for each trait (Brommer, Karell, Ahola, & Karstinen, 2014). The parameter "nu" (degree of belief) was equal to the number of health parameters to be estimated in **R** and **ID** (Brommer et al., 2014).

This model allowed us to include multiple response variables: haematology (TWBC, haematocrit, the absolute count of lymphocytes, monocytes, heterophils,

eosinophils measured as [white blood cell/100] * TWBC), serum chemistry (albumin, globulin, ALKP, CK, triglycerides, AST, creatinine, BUN) and electrolytes (calcium). Because of a large number of missing values, we removed five health responses from the multivariate mixed model (number of missing values out of 798 individual measures: haemoglobin: 195, basophils: 129, k: 187, cl: 188, na: 187). We included age, sex, region (Kawlin, East and West Katha), and birth origin (wild or captive) as predictors, and fitted covariance between individuals by including individual as a random effect. Results were checked to see whether they were dominated by the priors (visual evaluations of the posterior distributions).

We extracted the residuals of the multivariate Gaussian model, which can be interpreted as estimators of each health parameter, linearly corrected for the confounding effect of the covariates that are not the direct focus of this study (age, sex, region, birth origin). We used a Linear Discriminant Analysis (LDA) in order to assess overall variations in health between individuals with the three seasons: discriminant functions are constructed as linear combinations of corrected health estimators, to maximise the variance between the seasons. The importance of the contribution of each health parameter to these discriminant functions can therefore be interpreted as a measure of how much this parameter varies between seasons, while accounting for the high correlation between health parameters. We tested for the significance of the discriminant values (eigenvalues) using a multivariate analysis of variance (MANOVA) with a Pillai test. The LDA was performed with the “ade4” R package and the function *discrimin* (Dray & Dufour, 2007).

Association between seasons and differential blood cell count: Multivariate procedure

As the differential blood cell count was performed using a manual approach where a fixed total of 100 cells was counted, errors are necessarily correlated between

the different cell types. We analysed the parasite-related variation in white blood cells (*lymphocytes, monocytes, heterophils, basophils and eosinophils*) using a multivariate generalized linear model framework with a Dirichlet distribution which allowed the inclusion of multiple response variables (*MGLMreg* from the package "MGLM"). Because of implementation limitations, we were not able to include random intercepts in this model, so pseudo-replication may be a possible confounding factor in this case. Season of sampling, age, sex, origin (captive or wild-caught) and the region were included as explanatory variables.

Season-related patterns of health parameters: Single-trait analysis

Next, we investigated how seasons were associated with each of the 23 health parameters, independently of the variation in other parameters – a less realistic approach, but one which has the advantage of being robust to possible measurement errors in individual parameters. Using linear mixed models (*lmer*, package "lme4": Bates, Mächler, Bolker, & Walker, 2015), we fitted haematocrit, haemoglobin, total protein, globulin, albumin, BUN, CREA, chloride, sodium, systolic pressure and diastolic pressure as dependent variables with a Gaussian distribution. Using generalized mixed models (*glmer*, package "lme4"), we fitted AST, ALKP and CK as dependent variables using a Poisson error distribution and triglycerides, total white blood cell count, calcium and potassium as dependent variables using a Gamma distribution with a log link. Our main predictor variable of interest was the three-level season fitted in each model as a fixed factor. Our models also controlled for any differences in the health parameters due to age, sex, origin, and location. We included individual identity as a random intercept to avoid pseudo-replication issues.

We performed AIC-based model selection. For each health parameter, we compared (1) a first model including the confounding variables, (2) a second model including the confounding variables, and the season of sampling, and (3) a third model

including the confounding variables, and an interaction between the season of sampling and the sex of the elephant (to test whether the health of males and females responds differently to seasonal variation in climate and environment). The most likely models were selected using the Akaike information criterion (AIC), considering each random effect as one parameter (Pinheiro and Bates 2002), and selecting the model with the lowest AIC as the best model. Where the difference in AIC between competing models was less than two, we retained the simplest model (Burnham and Anderson 2002). Akaike weight (AICw) was also calculated for each model to provide the relative likelihood that it was the best among the candidate models. Further details about model selection are presented in Appendices -Table 4.2. The figures presented represent count data and do not take into account model covariates

Results

Global Analysis

LDA suggests that season is associated with the overall health (df_{health}=527, df_{season}=2, Pillai=0.772, F=21.53, $p<0.001$). According to the LDA, linear discriminant 1 (68.6%) clustered the three seasons from hot, cold to monsoon based on measures of 15 health parameters, which can be interpreted as an axis of precipitation/humidity. Linear discriminant 2 (31.4%) clustered the cold season (red in Figure 1; Figure 2) from the hot and monsoon seasons (blue and green in Figure 1), which can be interpreted as an axis of temperature. Particularly, the discrimination between seasons was higher for immunological health parameters (the total white blood cells, lymphocytes, monocytes and heterophils) and two nutritional driven parameters, BUN and triglycerides that showed the highest loadings (Table 4.1). These results suggest that the immune function, kidney activity and body condition are mainly influenced by seasonal variations. These results are not confounded by sampling variation between seasons according to

host sex, age, origin or location of sample collection, or the fact that we sampled the same individual several times, as all of these were controlled for in the analysis. The use of a multivariate method to establish the difference between hot and monsoon seasons offered the advantage of detecting and accounting for the interdependency between health parameters, which is not possible when analysing individual traits.

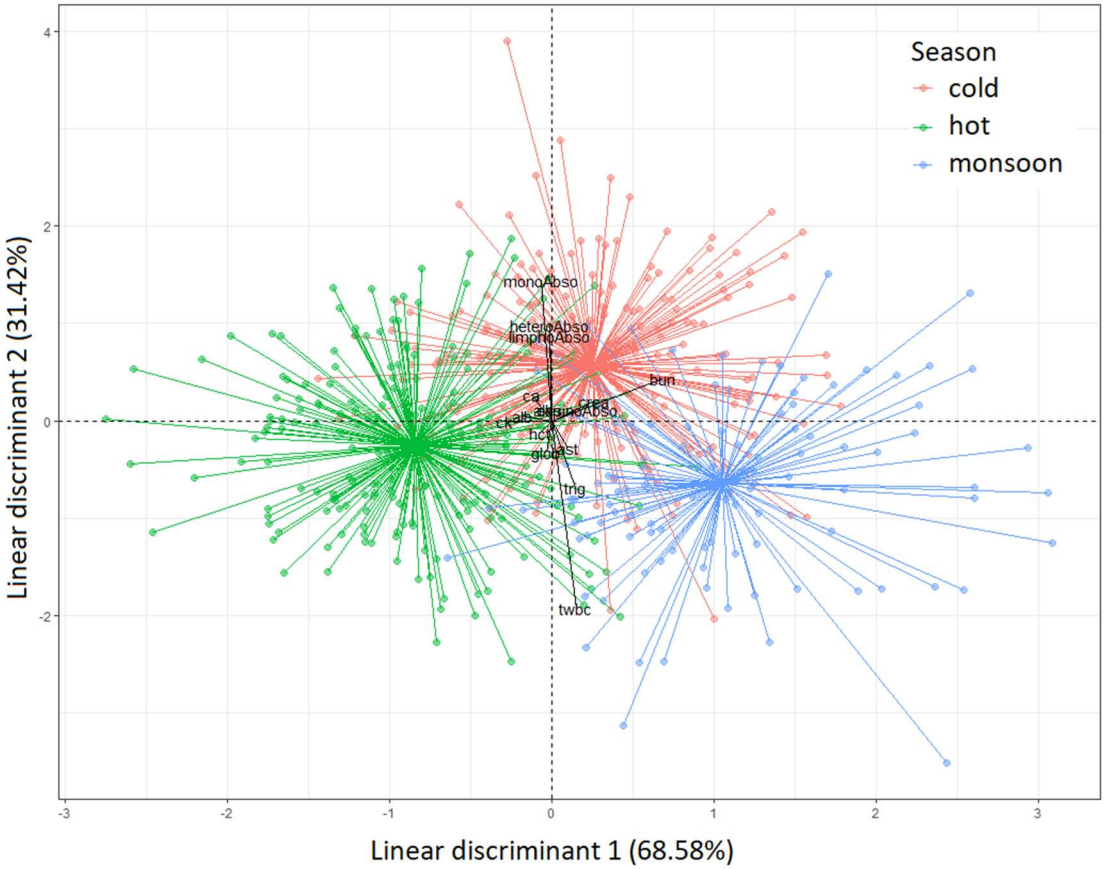


Figure 1. Biplot from the LDA showing clustering of “hot”, “cold” and “monsoon” season across 15 health parameters. The figure combines the standardised coefficients of health in black (i.e. canonical weights of the linear discriminant functions on the two axis of the linear discriminant analysis), and the projection of the health samples with gravity centres of each season (“cold” in red, “hot” in green, and “monsoon” in blue).

Table 4.1. Standardized coefficients for the health parameters of linear discriminant 1 (LD1) and linear discriminant 2 (LD2). Health parameters with the highest loadings are highlighted in grey.

Health Parameter	Axis 1	Axis 2
Haematocrit	-0.072	0.124
Lymphocytes	-0.015	-0.874
Monocytes	-0.066	-1.446
Heterophils	-0.011	-0.985
Calcium	-0.122	-0.272
Albumin	-0.176	-0.052
Globulins	-0.028	0.322
BUN	0.688	-0.435
Creatinine	0.262	-0.208
CK	-0.290	0.004
ALKP	-0.026	-0.112
AST	0.097	0.275
TWBC	0.155	1.927
Triglycerides	0.146	0.683
Eosinophils	0.160	-0.114

Specific Analysis of Health Parameters

Overall, our results strongly support the hypothesis that seasonal variation is associated with variation in health parameter values in Asian elephants, ranging from haematology to blood pressure, liver, muscular and kidney function, electrolytes and fat storage.

Haematology

On average, elephants had 16,506 $x10^9$ white blood cells/L (TWBC), ranging between 15,759 and 17,289 cells/L across seasons and individuals. Both males and females showed, on average, lower TWBC in the hot season than in the cold (β = -0.030 ± 0.015, t=-2.003), the latter being when food availability is better but the conditions for infectious agents are also better. This result was supported by the AIC-model selection that showed that the model including season as a 3-level factor was the best fit to the

data (AIC = 14,079.37, wAIC = 0.623; see Appendices – Table 4.3). For the differential white blood cell count which used a multivariate generalised model, the results varied depending on the cell type considered: basophil numbers were not associated with season, lymphocytes were higher in monsoon than in cold season (β = 0.173 ± 0.085, z=2.048), heterophils (β = -0.203 ± 0.074, z=-2.726) and monocytes (β = -0.254 ± 0.074, z=-3.431) decreased in the hot season, and eosinophils were influenced by season (AIC = -12,830.25, wAIC = 0.977; see table Appendices – table 4.4), being lower in the hot season (β = -0.172 ± 0.082, z=-2.086) and higher in the monsoon (β = 0.324 ± 0.092, z=3.542) compared to the cold season.

Red blood cells were similarly associated with seasonal variation. On average, elephants presented 34.8 ± 0.5 % haematocrit, with large variance among samples (28 – 44). In the monsoon season when elephants are recovering their nutritional status, they presented 2.3% less haematocrit (β = -0.829 ± 0.289, t=-2.872) than in the cold season. AIC-model selection confirmed that the model with interaction between season as a 3-level factor and sex as a 2-level factor was the best at explaining variation in haematocrit (AIC = 3,806.503, wAIC = 0.736; Appendices – Table 4.5). Although males had lower haematocrit than females in all seasons, this sex difference was largest during the cold season (β = 1.121 ± 0.475, t=2.363). For haemoglobin levels, on average, elephants presented 11.8 ± 0.2 g/dL, ranging from 9.2 to 16. Compared to cold season, haemoglobin levels increased 3.4% in the hot season (β = 0.414 ± 0.101, t=4.092), when water is less available and temperatures are much higher, and a decrease of 2.5% in the monsoon season (β = -0.282 ± 0.094, t=-3.003). This result was supported by the AIC-model selection with the best model including season as a 3-level factor (AIC = 1,810.522, wAIC = 0.778; Appendices – Table 4.5).

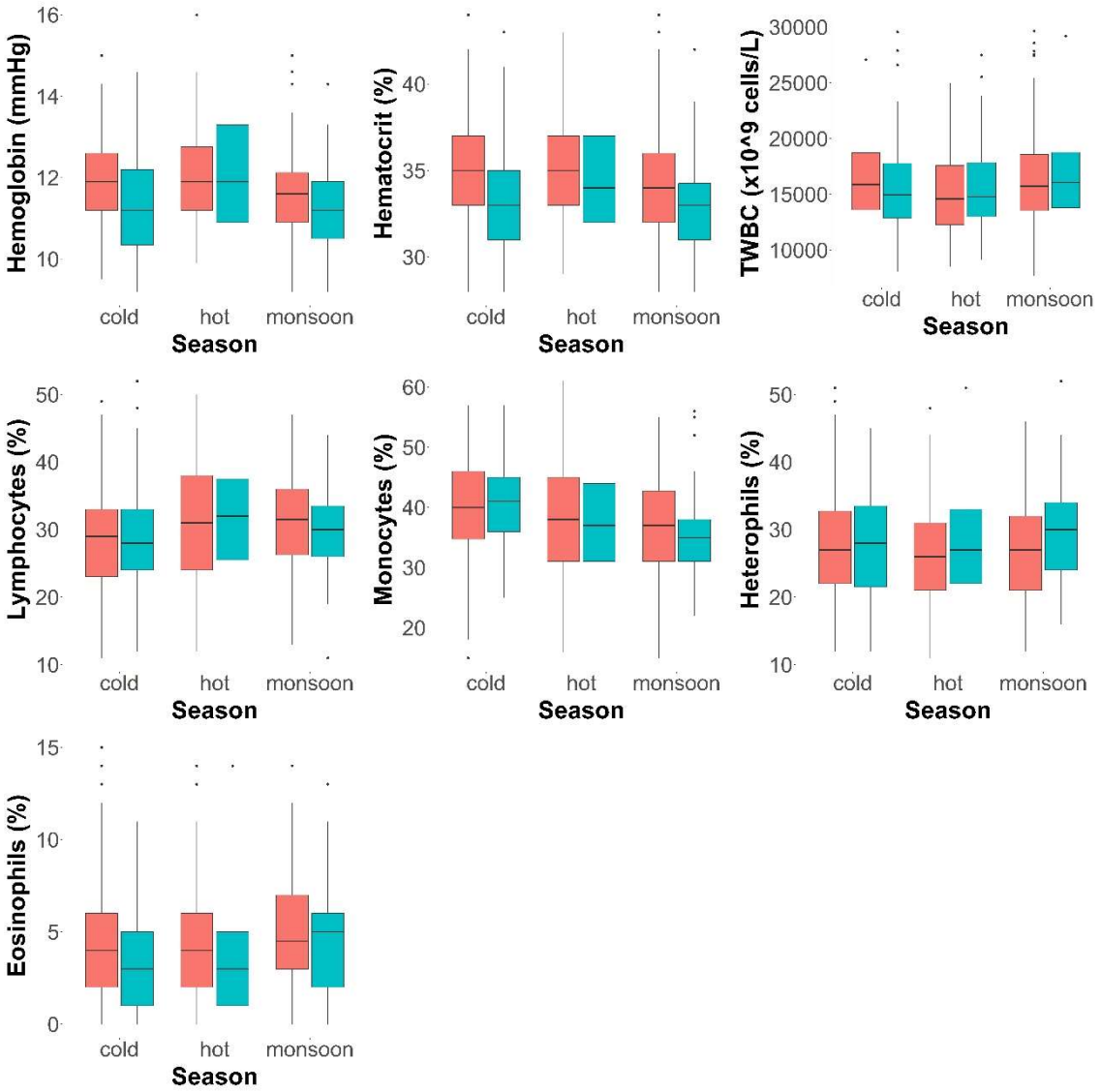


Figure 4.1. Seasonal changes in haematological parameters by sex. Females are represented in salmon and males in blue. The horizontal line within the boxes indicates the median, boundaries of the boxes indicate the 25th- and 75th-percentile, and the whiskers indicate the highest and lowest values of the results.

Blood Pressure

On average elephants had a systolic pressure of 137 ± 4 mmHg, but there was large variation within and between individuals (93 – 172). In both the hot (β = -4.184 ± 3.001, t=-1.394) and monsoon seasons (β = -5.395 ± 3.609, t=-1.495), which both experience high temperatures (dry in the former and with heavy rainfall in the latter),

elephants had 2.9% and 3.6% lower systolic pressure than in the cold season, respectively. Systolic pressure was influenced by the interaction between season as a 3-level factor and sex as a 2-level factor (AIC = 2,002.262, wAIC = 0.952; Appendices – Table 4.6). Females exhibited higher systolic pressure than males in the cold and monsoon seasons, but lower systolic pressure in the hot season. On average, elephants had 95 ± 3 mmHg of diastolic pressure, with large variation among samples (62 – 140). Similar to systolic pressure, diastolic pressure was influenced by an interaction between season and sex (AIC = 1,975.204, wAIC = 0.980; Appendices – Table 4.6).

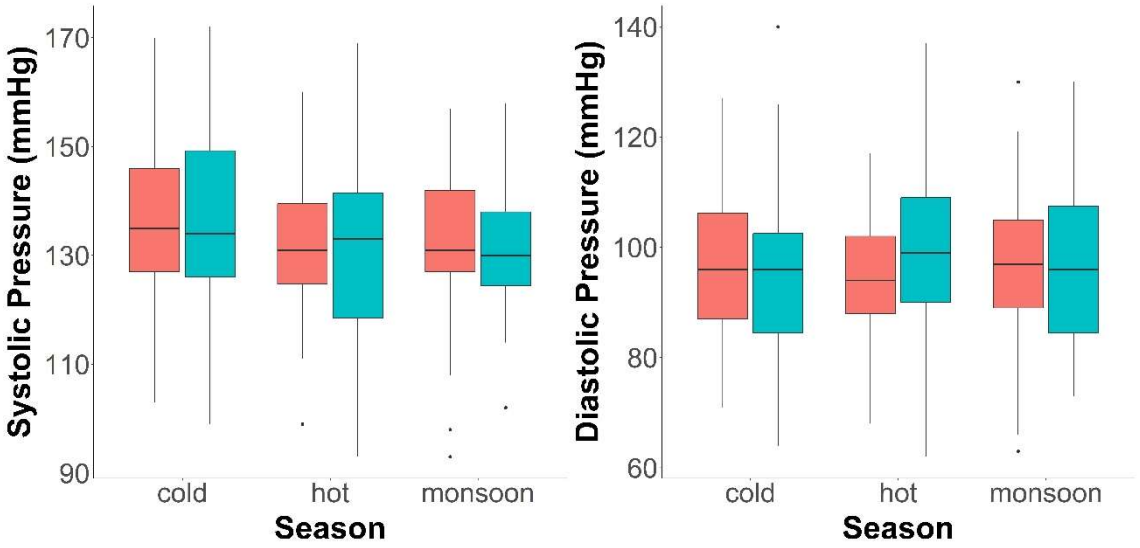


Figure 4.2. Seasonal changes in blood pressure parameters by sex. Females are represented in salmon and males in blue. The horizontal line within the boxes indicates the median, the boundaries of the boxes indicate the 25th- and 75th-percentile, and the whiskers indicate the highest and lowest values of the results.

Protein activity

On average, elephants had 7.62 ± 0.07 g/dL of total protein, ranging from 6.2 to 9.0. Elephants had on average 3.15 ± 0.03 g/dL albumin (ranging from 2.5 to 3.6) and 4.42 ± 0.05 g/dL globulins (ranging from 3.6 to 5.8). We did not detect any association

between protein activity and season, as the base model was selected as the best model for total proteins, albumin and globulin (AIC = 1,009.757, wAIC = 0.998; $AIC_{albumin}$ = 26.544, $wAIC_{albumin}$ = 1; $AIC_{globulins}$ = 479.6142, $wAIC_{globulins}$ = 0.999 see Appendices – Table 4.7).

Kidney function

Elephants displayed 18.7 ± 0.6 mg/dL blood urea nitrogen (BUN) on average (ranging from 4 to 34), which decreased by 22.5% in the hot season, when food and water availability is lower, compared to the cold season (β = -4.230 ± 0.374, t=-11.301). We observed the opposite trend in monsoon (with heavy rainfall and good food quality) when BUN increased by 18.2% compared to the cold season (β = 3.356 ± 0.377, t=8.911). The interaction between season and sex also influenced BUN (AIC = 4,316.746, wAIC = 0.987; Appendices – Table 4.8). Males in the hot (β = -2.036 ± 0.615, t=-3.313) and monsoon seasons (β = -1.316 ± 0.629, t=-2.094) showed less BUN than females in the cold season but in cold season, males had higher BUN levels than females (β = 1.877±0.558, t=3.365). Creatinine levels in elephants were of 1.14 ± 0.03 mg/dL on average (sample variance of 0.6 – 1.7). No seasonal effect was observed and the result was supported by AIC-model selection with the best model without season (AIC = -132.862, wAIC = 0.342; Appendices – Table 4.8).

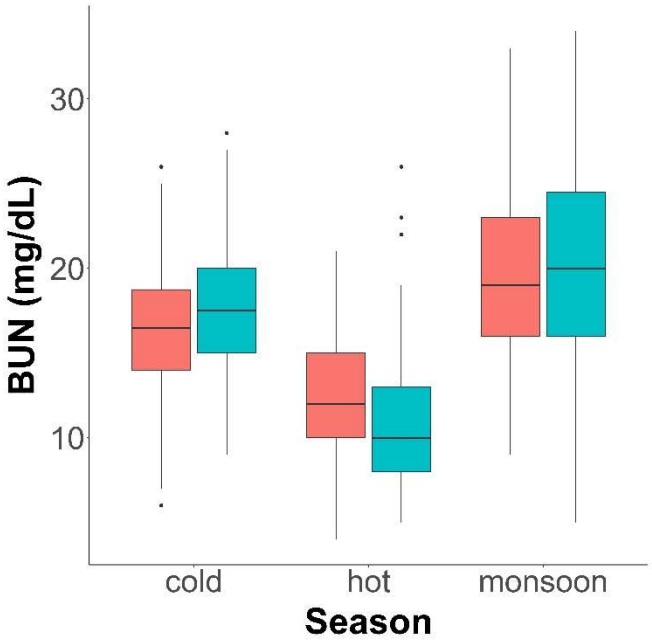


Figure 4.3. Seasonal changes in kidney activity parameters by sex. Females are represented in salmon and males in blue. The horizontal line within the boxes indicates the median, the boundaries of the boxes indicate the 25th- and 75th-percentile, and the whiskers indicate the highest and lowest values of the results.

Enzymes

We characterised liver function using two enzymes, AST and ALKP, and for the muscular function, CK. The level of AST displayed by elephants was 28.6 ± 1.2 U/L on average, with large variation between individuals and samples (0 – 128). The best AST model selected had an interaction between season and sex (AIC = 11,933.80, wAIC = 0.965; Appendices – Table 4.9). We showed that AST was 9.3% higher in the monsoon than the cold season (β = 0.311 ± 0.026, z=11.776), with males showing lower AST levels than females in the cold season (β =-0.211±0.101, z=-2.099). Elephants displayed an average of 114 ± 1 U/L ALKP (ranging from 20 to 249), and similarly to AST, we observed that seasonal variation increased ALKP levels in the hot season (β = 0.038 ± 0.013, z=2.953) but decreased levels in the monsoon season (β = -0.058 ± 0.013, z=-4.588), compared to the cold season. The model with an interaction between sex and

season was retained as the best model (AIC = 10,142.99, wAIC = 1; Appendices – Table 4.9). Males in the hot season showed higher ALKP levels than females in the cold season (β = 0.100 ± 0.020, z=5.102), and males had consistently higher ALKP compared to females in the cold (β = 0.082±0.035, z = 2.332) and hot seasons (β = 0.182±0.035, z = 5.194), but no difference in the monsoon season. With an average of 136 ± 1 U/L ranging from 11 to 491, seasonal trends in CK were similar to ALKP, and had an increase of 6% in the hot season when temperatures were high and the elephants were resting (β = 0.296 ± 0.010, z=29.775), and a decrease of 2% in the monsoon season (β = -0.102 ± 0.010, z = -9.868) compared to the cold season. As for ALKP and AST, the best model retained had an interaction between sex and season (AIC = 25,767.59, wAIC = 1; Appendices – Table 4.9). CK levels were higher in males compared to females in the cold (β = 0.090 ± 0.044, z = 2.060) and hot seasons (β = 0.132±0.044, z = 3.018) but not in the monsoon season.

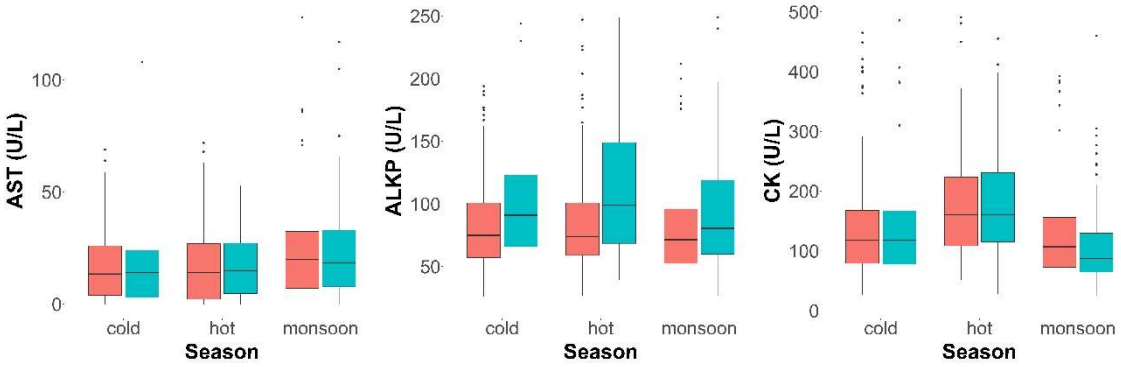


Figure 4.4. Seasonal changes in kidney activity parameters by sex. Females are represented in salmon and males in blue. The horizontal line within the boxes indicates the median, boundaries of the boxes indicate the 25th- and 75th-percentile, and the whiskers indicate the highest and lowest values of the results.

Fat storage

Fat storage was measured using triglyceride level, and elephants displayed 11.4 ± 1.1 mg/dL of triglycerides on average, with large variation between and within individuals (0.5 to 88). Season played a key role in regulating triglycerides levels, with an increase of 10% and 30% in the hot (rest) and monsoon (better quality food due to heavy rain) seasons, respectively, compared to the cold season (β_{hot} = 0.252 ± 0.088, t=2.867; $\beta_{monsoon}$ = 0.735 ± 0.091, t=8.041). Males had lower triglyceride levels than females (β = -0.248 ± 0.079, t=-3.127). This result was supported by AIC-model selection with the best model including season as a 3-level factor (AIC = 5,780.397, wAIC = 0.514; Appendices – Table 4.10).

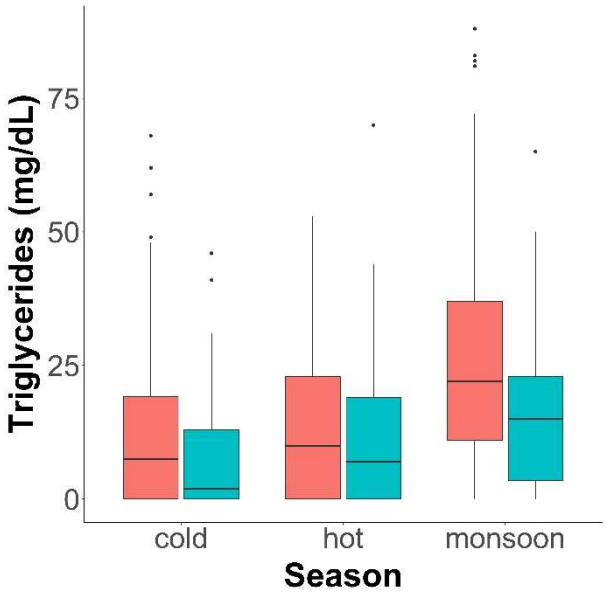


Figure 4.5. Seasonal changes in fat storage by sex. Females are represented in salmon and males in blue. The horizontal line within the boxes indicates the median, boundaries of the boxes indicate the 25th- and 75th-percentile, and the whiskers indicate the highest and lowest values of the results.

Electrolytes

To test whether season could influence electrolyte levels, we measured potassium (average 4.92 ± 1.01 mEq/L, ranging from 3.8 to 6.4), sodium (average 129.68 ± 0.34 mEq/L, ranging from 123 to 134), chloride (average 91.8 ± 0.3 mEq/L, ranging from 83 to 97) and calcium (average 10.0 ± 1.0 mg/dL, ranging from 7.8 to 11.2). We observed an effect of season on sodium levels, with elephants in the monsoon season showing lower levels of electrolytes compared to the cold season (β = -1.025 ± 0.217, t=-4.727). We also observed a lower calcium level in the monsoon season (β = -0.009 ± 0.004, t=-2.078) and a higher calcium level in the hot season, a season characterized by high temperatures and low humidity (β = 0.010 ± 0.004, t=2.285), compared to the cold season which has milder temperatures and little rainfall. We found lower potassium level in the monsoon season (β = -0.079 ± 0.008, t=-9.834), but contrary to calcium, this low level was also observed in the hot season compared to the cold season (β = -0.077 ± 0.009, t=-8.871). These results were supported by the AIC-model selections of sodium, calcium and potassium, which retained the model including season as a 3-level factor as the best model (AIC_{sodium} = 2,667.559, $wAIC_{sodium}$ = 0.834; $AIC_{calcium}$ = 1,208.970, $wAIC_{calcium}$ = 0.774; $AIC_{potassium}$ = 596.1262, $wAIC_{potassium}$ = 0.591; Appendices – Table 4.11). No seasonal differences were observed for chloride (AIC = 2,771.599, wAIC = 0.810; see Appendices – Table 4.11).

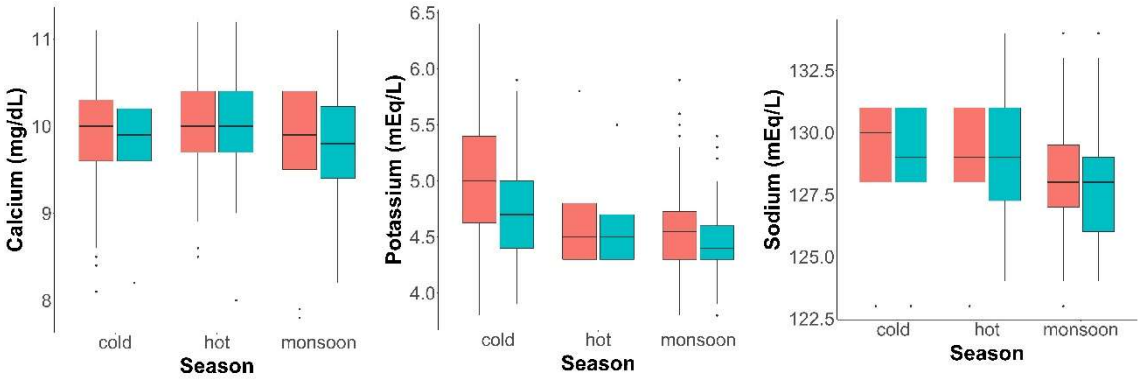


Figure 4.6. Seasonal changes in electrolytes by sex. The horizontal line within the boxes indicates the median, boundaries of the boxes indicate the 25th- and 75th-percentile, and the whiskers indicate the highest and lowest values of the results.

Discussion

Several seasonally varying environmental factors can have both direct and indirect effects on animal physiological processes, such as temperature (Pörtner & Farrell, 2008), food abundance (Hanya et al., 2006) or rainfall (Strong & Sherry, 2000). Climate change has created a bigger need to understand such seasonal variation in physiology, given that the accompanying changes in weather patterns, food availability (Post & Forchhammer, 2008) or even the exposure to diseases (Dukes et al., 2009) can increase mortality risk and lead to lower reproductive output. Theoretically, long-lived species are often predicted to be buffered against climate variation: longevity means low annual mortality and reproduction rates, and annual variability in climate may therefore have a smaller impact on the population growth rates of long-lived species as compared to short-lived ones (Morris et al., 2008). Nevertheless, a longitudinal study of wild African elephants in Amboseli, Kenya, found that drought years were associated with higher calf mortality (Foley, Pettorelli, & Foley, 2008; Moss, 2001). Similarly, warm, wet and windy winter conditions were associated with reduced juvenile survival but increased adult fecundity and survival in Soay sheep (Forchhammer, Clutton-Brock, Lindström, & Albon, 2001), but little is known of the physiological mechanisms for such patterns. In this study,

we investigated seasonal variation in the physiology of Asian elephants occupying tropical forests with a strong seasonal monsoon climate (Beck et al., 2018). As we sampled a large number of animals with repeated measures for several health parameters, we could utilise both a global and individual trait-by-trait analysis of seasonal effects on health. The global analysis highlighted the biggest differences in health between hot and monsoon seasons. Previous studies have found Asian elephants to display higher survival in months with intermediate temperatures and more rainfall (Mumby et al., 2013b), and our results may offer some mediating mechanisms for such seasonally increased mortality risk. More generally, the results suggest that even long-lived, large mammals that are generally thought to be well equipped against climate variation, may experience physiological changes in response to regular seasonal variation in their environment that can, in extreme circumstances, pose a risk.

We measured altogether 23 health monitoring parameters related to haematology, blood pressure, liver, muscular and kidney function, electrolytes and fat storage. While each parameter was informative of the underlying ecophysiology in their own right, we also developed an innovative approach to take into account the interdependency of the overlapping physiological functions (Trayhurn, 2005). Such a global approach revealed that season was a major driver of health, especially during periods characterized by the extremes of rainfall, namely, the hot season with no rainfall and the monsoon season with heavy rainfall. Seasonality has a strong effect on the growth and production of vegetation, with rainfall being one of the most important factors in predicting vegetation mass (Arndal et al., 2009; Austin, 2002). The health factors that had the biggest contribution to overall health variation across seasons were the immune system parameters and two parameters related to nutrition. Since most infectious diseases have pronounced seasonal patterns due to variation in transmission and reproduction rate (Altizer et al., 2006; Pascual & Dobson, 2005), one leading contributor

to seasonal variation in health could therefore be disease. For example, in this population, gastrointestinal nematode burden increases during the monsoon and cold seasons (Lynsdale, 2017), potentially leading to a more active immune response. Alongside the immune system markers, however, BUN levels, which indicate nutritional status, contributed significantly to the seasonal variation in overall health, with the lowest fat storage levels apparent in the hot season and the highest levels during the monsoon season. This reflects food quality and quantity fluctuations across the year: during the hot and dry seasons Asian elephants are largely browsers, while in the monsoon season they are grazers and may eat fruit more (Sukumar, 2003). This influences their protein intake, since in the dry season elephants ingest low levels of protein (browse period) and in wet seasons they consume higher protein levels (grazing periods) (Brown & White, 1979; McCullagh, 1969). Such foraging differences in our study elephants were also detectable in their BUN levels, with BUN being the main nitrogenous end product of protein metabolism (Fowler & Mikota, 2006).

The hot season is characterized by high temperatures, no rainfall and consequently, low food quality and quantity as well as lower water availability. In the MTE elephant population, the hot season also corresponds to the resting period when elephants do not work. During this season, we observed a peak in haemoglobin, from the monsoon until hot season, possibly due to increases in nutritional condition and food intake, and haemoconcentration attributed to dehydration (DelGiudice et al., 1992; Hellgren et al., 1993). CK also reached the highest level in the hot season, however, as many of our sample elephants worked little or not at all during the study period, the CK variation we observed may reflect natural seasonally varying physiological processes rather than workload. Physical activity at high temperatures and an increase in respiration rate in hot temperatures can cause the CK to increase (Chulayo & Muchenje, 2013). We also found ALKP (liver specific protein) but not AST (which is also present in

the liver tissue) to increase in the hot season, but in contrast, this is when blood pressure, BUN displayed their lowest levels. Blood pressure is known in humans to be affected by season (Lewington et al., 2012; Rosenthal, 2004), and in our study, males had higher blood pressure than females in hot season. High temperatures during the hot season cause increased vasodilatation (widening of the arteries and veins) and loss of water and salt from sweating, leading to lower blood pressure (Rosenthal, 2004). Elephants have difficulty sweating and losing heat (Hiley, 1975), so this decline in blood pressure during hot months was likely produced by increased vasodilatation. BUN levels were lowest during the hot season, alongside lowest food availability, indicating that protein metabolism in each season may play a stronger role in influencing these parameters than the glomerular filtration rates from the kidneys (Butler et al., 2006; Crooks, Scott, Bowen, & Vuren, 2000; DelGiudice et al., 1992).

Unlike the hot season, the monsoon season is characterized by strong and prolonged precipitation associated with high temperatures, as well as increased food quality, food quantity and ease of access to water. We observed an increase in the total and differential white blood cell count during the monsoon season which, disregarding lymphocytes, were also maintained at high levels during the cold season. Such increases may be driven by infectious diseases given that infectious agents require specific climate conditions to spread and infect their hosts, and the hosts in turn may present different resistance to their infection during different seasons (Altizer et al., 2006; Hosseini, Dhondt, & Dobson, 2004). The host immune system can adapt to such a seasonality in infection and be more active in times when the conditions for the infectious agents are more prevalent (Hosseini et al., 2004). In line with this, the levels of the gastrointestinal parasite prevalent in our study population begins to rise in June from low levels during the hot season, until the peak levels recorded in December/January of the cold season (Lynsdale, 2017). Another health parameter to rise during the monsoon was AST, which

is present in several tissues, especially in the liver, cardiac muscle and skeletal muscle. The observed AST rise in the monsoon season here may be an indicator of increased liver activity, as there is often also a decrease in CK in this season, that is more sensitive to muscular damage than AST (Fowler & Mikota, 2006). The cause behind the rise in AST during the monsoon season is not clear to us at present as ALKP, another specific liver enzyme, peaks in the hot season, not in the monsoon. BUN also increased in the monsoon season, and as stated before, may be explained more by nutritional status than by glomerular filtration in the kidneys (Butler et al., 2006; Crooks et al., 2000; DelGiudice et al., 1992). We also observed that male BUN levels were higher than those of females in the monsoon and cold seasons. Conversely, haematocrit and haemoglobin had their lowest values during this season. Males normally have higher levels in haematocrit and haemoglobin due to the positive effect of testosterone in erythropoiesis (Howell et al., 2003; Riviello & Wirz, 2001), but for reasons still unknown to us, we found that haematocrit was consistently lower in males and that there was no difference in haemoglobin.

The cold season is characterized by low temperatures with a lower rate of rainfall and a better quality and quantity of food at the beginning of the season, decreasing in the run up to the hot season. In this season there is a peak in gastrointestinal parasite abundance (Lynsdale, 2017), and accordingly, the total and differential white blood cell counts remained high. Adding to this, the decrease in photoperiod has been shown to have a positive effect on the ability of the immune response to anticipate immunological challenges (Nelson, 2004). It is possible that the increase in white blood cells observed in the monsoon and cold seasons could be related to both the infectious agent's seasonality challenge and photoperiod (3 hours difference between the longest and the shortest daylight days). Blood pressure (specifically systolic pressure) also peaked during the cold season, with males displaying less or the same blood pressure as

females. The reason may similar to humans, where cooler temperatures increase the sympathetic nervous system tone, increasing this way the blood pressure (Rosenthal, 2004).

Studying seasonal variation of health in natural environments is difficult to conduct in wild populations. Utilising a semi-captive population such as the MTE timber elephants has numerous advantages. First, these animals preserve many natural behaviours such as roaming, feeding and mating, and may carry these out with wild conspecifics and without any human control (Lynsdale et al., 2017). Secondly, climate also determines the workload in the timber industry, with elephants resting in the hot and dry seasons (February until June) and working in the monsoon and the cold season (Mumby et al., 2013a) – which needs to be taken into account when investigating seasonal changes in health, especially in working-age elephants (20 to 50 years of age). Finally, elephants are monitored by trained veterinarians who check their health every month, ensuring the animals can be handled, identified and used for longitudinal health data collection. However, our study does have some limitations that need to be considered. Data collection once every season limits the possibility of observing trends in health from season to season, and investigating how quickly or slowly health changes diminish. Further, few studies exist from similar climate systems, preventing us from drawing comparisons as the majority of previous work has been conducted in either (i) continental climate systems (Beck et al., 2018), (ii) with hibernating species (Hissa et al., 1994; Huber, Kusak, Žvorc, & Rafaj, 1997; Yang et al., 2018), (iii) with predator species such as wolves (Butler et al., 2006; Seal & Mech, 1983) or (iv) with aquatic species (Norman et al., 2012; Trumble et al., 2006; Yu et al., 2014). This clear gap in our knowledge calls for more research on health variation to be conducted on a wider range of species occupying a wider range of climates.

In conclusion, our results demonstrate that season is a major driver in health parameters, with all except two of the 23 parameters differing between seasons. Climate change is predicted to increase extreme weather patterns in monsoon climates and could bring major changes in natural systems, especially for species already endangered, as is the case for Asian elephants. A third of the remaining Asian elephant population lives in captivity, mostly in countries exposed to seasonal monsoon climate, meaning our results also have practical and relevant implications for Asian elephant management, and highlight the importance of considering seasonal health variation when assessing veterinary care and workload decisions.

Chapter 5

Overall health cost of heavy parasite burden: A 3-year longitudinal study in Asian elephants

Abstract

Parasites affect hosts by imposing resource costs that lead to fitness reductions, but how parasitism alters physiological processes and more largely health remains poorly studied. The aim of this study is to understand the interaction between parasites and health in a long-lived mammal, the Asian elephant (*Elephas maximus*), evaluating several physiological markers, both individually and within the individual, analysed globally. We collected longitudinal measures of gastro-intestinal parasite load and health data, encompassing haematology, protein activity, kidney and liver systems, electrolytes and fat storage. Measurements were taken over 3 years from 208 individually-marked Asian elephants, from a semi-captive population used in Myanmar's timber industry, that maintain natural behaviours and feeding. Heavily parasitized individuals showed the poorest health, most strongly evident in our measures of the immune system. Individually, 19 out of 21 health parameters were affected by parasite load, and these changes were most visible at the higher end of the parasite load spectrum. Our results highlight the importance of investigating effects of parasites on host health globally to unravel the physiological link between parasites and immunity outcomes, and to better inform parasite control strategies.

Introduction

While parasitism is ubiquitous across the tree of life, its consequences for the hosts still remain debated (Dobson, Lafferty, Kuris, Hechinger, & Jetz, 2008). Parasites can influence many aspects of their host's life including their life-history, behaviour, and physiology (Albon et al., 2002; Murray, Keith, & Cary, 1998), which often lead to important fitness costs (Dazak et al., 2000; Wegner et al., 2003). Such fitness costs are thought to stem from the immune response that organisms develop in response to infection and from the damage done by the parasite. An immune response is costly to

produce (Martin, Scheuerlein, & Wikelski, 2003), and thus infection is mediated by resource-allocation trade-offs between investment in, e.g., growth and reproduction, and in immune responses which can sufficiently counter or mitigate infection (Sheldon et al., 1996; Zuk & Stoehr, 2002). Such consequences of infection on host survival and fecundity are well documented. For example, in wild populations of red grouse (*Lagopus lagopus scoticus*), nematode (*Trichostrongylus tenuis*) infection was associated with drastically lower host fertility, and population crashes (Hudson, Dobson, & Newborn, 1998). In Svalbard reindeers (*Rangifer tarandus*), higher gastrointestinal nematode loads were associated with decreased body mass, back fat depth and fertility in late winter (Albon et al., 2002; Stien et al., 2002).

A growing number of studies have recently shown parasite-related deterioration of different physiological parameters leading to ill health, with health being defined here as the ensemble of all biological processes that maintain an organism's homeostasis (Gunnarsson, 2006). This homeostasis can be crucially compromised by parasitism, which can vary from short-term reactions, such as an alteration of physiological parameters (Dimitrijević et al., 2016; Monteiro et al., 2010), to long-term costs including disease complications, susceptibility to secondary infections and mortality (Beldomenico et al., 2008). However, individual life-history responses to a given parasite load appear heterogeneous, and some studies have shown tolerance to parasites as measured through fitness outcomes (Stjernman, Råberg, & Nilsson, 2008; Svensson & Råberg, 2010). In the particular case of gastrointestinal parasites, a number of deleterious consequences of infection make them a threat to host health. Generally in vertebrates, nematodes cause damage to the gut wall, leading to decreased nutrient absorption and haemorrhages, causing anaemia and ultimately affecting host health (Coop & Kyriazakis, 2001; Cooper, Whyte-Alleng, Finzi-Smith, & Macdonald, 1992; Gilman, 1982). Several studies have focused on consequences of infection in specific taxa. In sheep (*Ovies*

aries), individuals with higher levels of gastrointestinal nematodes showed both decreased red blood cell counts and haematocrit, indicating blood loss by parasite consumption and/or lack of production by the host, as well as increased levels of neutrophils and eosinophils – white blood cells responsible for the immune response to infection (Dimitrijević et al., 2016). In a wild Australian sea lion population (*Neophoca cinerea*), nematode burden decreased red blood cell count and total proteins, the latter decreasing due to gastrointestinal mucosa destruction and protein loss by the intestine (Marcus et al., 2015). Similar deleterious effects of parasitism on haematology and serum chemistry were also observed in clariid catfishes (*Clariid gariepinus* and *C. anguilaris*) infected with protozoans, cestodes and nematodes. Infected individuals showed increased white blood cell counts, liver enzyme levels (i.e. aspartate amino transaminase (AST); alanine aminotransferase (ALT) and alkaline phosphatase (ALKP)), and decreased kidney function (measured by urea and creatinine levels) (Nnabuchi et al., 2015). In an experimental study, rams (*Ovies aries*) artificially infected with nematodes showed diminished haematological parameters including haematocrit, red blood cell counts, and haemoglobin, as well as lower albumin, total protein and glucose levels, compared to non-infected rams (Rouatbi et al., 2016).

Such findings provide some understanding of how parasites modify different physiological parameters, but to date the evidence for how parasites affect health and fitness outcomes remain mixed. For example, although gastrointestinal parasitism effects on health tend to converge in decreased red blood cell components, increased white blood cells, decreased protein levels and increased enzyme activity, opposite results are also widely evidenced (López-Olvera, Höfle, Vicente, Fernández-de-Mera, & Gortázar, 2006; Matanović et al., 2007; Monteiro et al., 2010; Nnabuchi et al., 2015; Rohlenová et al., 2011; Rouatbi et al., 2016; Shender, Botzler, & George, 2002). Yet, recent literature on animal models of human disease has shown that endoparasites, for

example nematodes, can in fact promote host health by aiding self-recognition and therefore suppressing inflammatory and autoimmune diseases (Navarro et al., 2016; Smallwood et al., 2017). This absence of clear patterns highlights the need to determine the proximate effects of parasitism on host physiological processes and more largely on health, which underlie the effects of infection on fitness outcomes, in a wider breadth of host taxa.

These mixed results might be explained by the fact that gastrointestinal parasitism is commonly measured by counting eggs in the faeces (Faecal egg count, FEC) (López-Olvera et al., 2006; Matanović et al., 2007; Nnabuchi et al., 2015; Rouatbi et al., 2016; Vanimisetti, Andrew, Zajac, & Notter, 2004) and measuring their influence as a linear effect (Barger & Dash, 1987; López-Olvera et al., 2006; Matanović et al., 2007; Monteiro et al., 2010; Rouatbi et al., 2016). Some headway has been made regarding this issue, with a number of studies focusing on relationships between a number of key markers of vertebrate health and measures of infection in domestic and wild host taxa, yet studies differ in their statistical approaches, making results harder to interpret more broadly across systems. For example, nematodes are aggregated within vertebrate host populations, and therefore observed nematode loads are highly variable between individuals, such that only a small proportion of hosts are heavily parasitized and the majority of hosts harbour low burdens (Poulin, 2013; Shaw, Grenfell, & Dobson, 1998). In line with this, some previous studies have investigated non-linear effects of parasites on different outcomes of health and showed that negative parasitism effects on host health were exacerbated in individuals with the highest infection levels (Dimitrijević et al., 2016), i.e. those with the highest burdens suffered the most severe consequences. Moreover, when parasite burden was included as a discrete factor, only heavily infected individuals were found to suffer from poor health, whereas the pattern is less clear for individuals with medium parasite burden (Dimitrijević et al., 2016). The

absence of a clear pattern of parasite-related health effects, even among gastrointestinal parasites, reinforces the need to investigate effects within different categories of infected individuals. Furthermore, several limitations in previous studies prevent us from outlining how overall health responds to a given parasite load. Previous studies often focused on one or two aspects of an individual's physiology, or did not include repeated measures from the same individuals, which is a key limitation if we are to understand the heterogeneity in individual responses to given parasite loads. Additionally, studies which investigated several aspects of health simultaneously did not consider the interdependency between such physiological functions. More work is therefore needed in order to understand a general parasite-related association with individual host health that can be achieved by having a longitudinal, global (within individuals) and integrated view of health.

In the present study, we aim to understand the physiological mechanisms underlying a response to gastrointestinal parasitic infection in a long-lived mammalian host, the Asian elephant (*Elephas maximus*). Importantly, we investigate how overall health affects host homeostasis, and we analyse specifically how health parameters (haematology, immunology, liver and kidney activity, fat storage) respond to gastrointestinal parasite infection levels. We sampled 208 elephants ranging in age from 4 to 72 years old that were longitudinally monitored over 3 years. We monitored gastro-intestinal (GI) parasite levels from faecal egg counts (FECs) longitudinally, in order to link within and between-individual variation in infection to 21 different health parameters, enabling us to outline both specific and global associations between health and infection. With 706 samples of 21 different physiological markers, we cover a wide range of physiological functions such as haematological, immunological, muscular, kidney and liver functions, protein balance and electrolytes. Some of these physiological markers are already known to change with heavy parasitism in both domestic/agricultural and wild

species, e.g. white blood cell count (and in particular specific types of cells such as eosinophils) is known to increase due to parasite presence; albumin is lost due to destruction of the intestine barrier by high worm numbers and globulins increase due to infection (Garside, Kennedy, Wakelin, & Lawrence, 2000; Sullivan, Lunn, Northrop-Clewes, & Farthing, 1992). However how other vital bodily functions are expected to be affected by parasites directly or indirectly in nature through their connections to the affected organs or functions remains largely unknown.

Our study population is part of the world's largest population of captive Asian elephants (>5000), consisting of government-owned, individually-recognised elephants working in timber camps, employed and centrally managed by the Myanma Timber Enterprise (MTE) (Lahdenperä et al., 2018). Although these elephants are managed as draught and transport animals by the MTE, they are considered as "semi-captive" and live largely under natural conditions. They are released into the forest outside working hours to forage naturally, and interact and socialise with wild and other semi-captive conspecifics, and breeding rates are not managed by humans with mating occurring independently in the forest at night. Elephants benefit from bi-monthly veterinary checks including deworming (Lynsdale et al., 2017). The population offers a unique opportunity to collect longitudinal measures of health and parasitism of individually marked animals with known age, breeding history and veterinary records, but exhibiting foraging, lifespan and breeding patterns comparable to wild elephants. Importantly, Lynsdale et al., (2015) already described important variation in gastrointestinal parasite burden, specifically nematodes, between individuals in our population, where a few hosts suffered from intense parasite load. Finally, all our study subjects were administered anthelminthic treatment at different stages of the study period to allow us to better quantify the physiological correlates of health in a range of infection level scenarios (Lynsdale, 2017). The population thus provides an opportunity to determine at a fine scale the

consequences of parasite burden (low, medium, high) on a multi-faceted picture of health in an endangered species (Lynsdale et al., 2017).

Materials and Methods

Study population

Myanmar has the largest captive Asian elephant population - around 5,000 individuals (Sukumar 2006), 2,700 of which are government-owned through the Myanma Timber Enterprise (MTE) for sustainable logging (Leimgruber et al., 2011). Government-owned MTE elephants inhabit forest camps, distributed across Myanmar. Elephants work as transport and draft animals during the day and they are released each night for up to fourteen hours, as well as in extensive rest periods (e.g. during the hot season), to forage naturally, interact with conspecifics and mate unsupervised. Breeding rates are natural with no reproductive management of the population, timber elephants are never culled, and numbers are not restricted or managed, so that this population is usually considered semi-captive. This population is composed of captive-born and wild caught animals, despite wild capture been forbidden since the 90's (Jackson et al., 2019).

We collected parasite and health parameter data longitudinally from March 2016 to November 2018 for 208 elephants (females: 129, males: 79). We sampled elephants in three logging camps in the Sagaing Division of Myanmar, namely, Kawlin (481 samples) East Katha (168 samples) and West Katha (88 samples). We collected samples in three different seasons annually; hot and dry in March-April (268 samples), monsoon in July (223 samples) and cold season in November (246 samples). However, not all of the study elephants were available in every sampling trip; 57 elephants were only sampled in one of the field trips, 38 in two, 24 in three, 18 in four, 17 in five, 24 in

six, 13 in seven, 12 in eight, and 5 in all nine field trips over the three-year period. The study elephants ranged in age from 4 to 72 years of age.

Parasitism and anthelminthic treatment

We collected faecal samples to obtain estimates of nematode load following a non-invasive sampling protocol, standardized for use in Asian elephants (Lynsdale et al., 2015). The faecal samples were stored at +4°C and analysed within a seven-day period after collection as recommended by Crawley, Chapman, Lummaa, & Lynsdale (2016). The samples were analysed to obtain an indirect measure of parasite burden through faecal egg counts (FECs). FECs have been widely used as a reliable tool to estimate nematode load in other host taxa (Denwood et al., 2012; Seivwright, Redpath, Mougeot, Watt, & Hudson, 2004). However, FECs only reflect the presence of reproductive adult parasites, and their rates of egg shedding: FECs do not directly encompass numbers of immature larvae, variation in female parasite fecundity and egg output, or heterogeneity in parasite breeding cycles. As such, FECs are considered as an estimation only of nematode burdens in any host system. To obtain the FEC, a modification of the McMaster faecal flotation method was used on a 100x magnification compound microscope (MAFF, 1986). We visually identified nematode eggs, recognizing indicative morphological characteristics, e.g. size, shape, internal structure and developmental stage (Bowman, 2014; Lynsdale et al., 2017; MAFF, 1986).

Since the MTE considers parasites as a threat to elephant health and survival (Lynsdale et al., 2017), anthelminthics are used routinely for deworming these animals. Two drug active ingredients have been used regularly since the 1990's, namely ivermectin and albendazole. Since 2017, an organophosphate insecticide (trichlorfon) has also been used. Deworming, according to the MTE regulations, is usually carried out twice a year with rotation of these drugs (Oo, 2012; personal correspondence with Dr

Win Htut). The timing of seasonal deworming events relative to our sampling events led to a strongly bimodal distribution of time between treatment and sampling. Consequently, we created a binary variable to reflect individual deworming status, and considered that elephants dewormed less than four months ago were “freshly dewormed”, and other elephants were “not freshly dewormed”. Another consequence of the deworming schedules was that the time elapsed since deworming and the sampling season, are not independent, and therefore temporal effects have to be handled carefully while interpreting the results

Characterization of elephant health

Field sampling protocol

All blood samples were collected in the morning, outside of elephant work periods. Blood was collected from the main auricular vein using three vacuum tubes (Vacuette©) containing EDTA, heparin, and silica particles (serum separator tube), respectively, with a total volume of ca. 30 mL. The collected blood tubes were refrigerated until analysis at +4°C for a maximum of 12 hours. For serum chemistry, the serum separator tubes were centrifuged, and the sera were collected and frozen at -20°C until analysis.

Blood cells and haematology

In vertebrates, haematological markers are commonly used to draw conclusions about animal health, and several white blood cell types play key roles in vertebrate immune function and are expected to vary in response to pathogenic threats (Fowler & Mikota, 2006). Lymphocyte abundance is known to measure variation in adaptive immunity, the number of monocytes and heterophils measure variation in innate immune

response, and eosinophils and basophils reflect specific immunity against internal and macro-parasites, and inflammatory response (Cheynel et al., 2017a).

To investigate the parasite-related variation in blood cells and haematology, we measured the total number of white blood cells (TWBC; 201 elephants) and differential counts of each group of white blood cells (lymphocytes, monocytes, heterophils, eosinophils and basophils). Total white blood cell counts were done manually using Turk's solution staining protocol, from blood stored in the presence of EDTA, with a Neubauer hematocytometer. After mixing the sample, 380 µL of Turk's solution was mixed with 20 µL of blood and allowed to rest for two minutes to allow for red blood cell lysis. The hematocytometer was loaded with the sample and the count performed using the 10x objective lens. All nine squares were counted in each side of the Neubauer chamber. Total white blood cell count was calculated by taking the mean of the two chamber counts, multiplying it by the depth of the chamber and the dilution factor and dividing it by the area counted.

The differential counts were performed manually using a blood smear stained with Romanowsky stain (QuickDiff© kit), from blood stored in EDTA. For each smear, we performed identification for a total of 100 randomly chosen cells, classified into one of five categories (lymphocytes, monocytes, heterophils, eosinophils and basophils). We measured the percentage of red blood cells using haematocrit (201 elephants), and the oxygen carrying capacity using haemoglobin (187 elephants) (Fowler & Mikota, 2006). Haematocrit and haemoglobin levels were obtained using Abaxis's VetScan I-Stat® 1 with an E3+ cartridge, using blood stored in the presence of heparin.

Proteins and enzymes, kidney, liver and muscle function

Proteins and enzymes contribute to crucial biological processes (e.g. immunity, molecule transport and storage) which can be altered by parasitism (Macrae, 1993). We measured the albumin and globulins levels, and the sum of albumins and globulins as an estimator of the total proteins. Albumin is involved in the maintenance of osmotic pressure and in the transport of several hormones, vitamins and haemoglobin (Fowler & Mikota, 2006). Globulins intervene in the immune and inflammatory responses (Fowler & Mikota, 2006). We assessed kidney function by measuring blood urea nitrogen (BUN) and creatinine. We measured the enzyme activity in the liver by measuring the aspartate transaminase (AST) and alkaline phosphatase (ALKP). Finally, in muscle, we measured the creatinine kinase (CK). These parameters were evaluated using an IDEXX VetTest® serum analyser.

Triglycerides and electrolytes

Fat and electrolyte levels have an impact on the homeostasis of organisms. We measured triglycerides in blood to assess the state of lipid storage (Ferguson & Leese, 2006; Fowler & Mikota, 2006). We also measured electrolytes by quantifying calcium, sodium, potassium and chloride levels in blood samples. Triglycerides and calcium were analysed using IDEXX VetTest®. The other electrolytes were quantified using Abaxis's VetScan I-Stat® 1 with an E3+ cartridge.

Statistical analysis

To investigate the influence of parasites on health parameters, we used two complementary methods: a multivariate joint analysis of health parameters, and a univariate parameter-by-parameter analysis. The multivariate analysis allowed us to capture an overall association of parasite load with health by taking into account the

covariation of health parameters. In the single-trait analysis, we analysed each health parameter separately in order to describe specific, potentially parasite-related changes in physiology. Parasite load was measured in faecal egg count (FEC) and its distribution was highly skewed to the left (only 9 cases showed an FEC > 100). In all our analyses, we considered parasite load as a 3-level categorical variable ("Negligible": 0-10 FEC, "Mild": 11-49, "Heavy": 50-208) (Döpfer, Kerssens, Meijer, Boersema, & Eysker, 2004; Nielsen, Baptiste, Tolliver, Collins, & Lyons, 2010). The relatively low repeatability of FECs within individuals (Lynsdale et al., 2015) justified the use of a categorical variable, rather than of the continuous point FEC measurements. The range covered by each category was based on the literature in horses (Döpfer et al., 2004; Nielsen et al., 2010). All analyses were conducted using R version 3.5.2 (R Development Core Team, 2018).

Measurement repeatability

We tested the repeatability of serological measures done on the IDEXX VetTest® analyzer by replicating measurements for a subset of individuals (N=28, technical replicates only). Repeatability was assessed by variance decomposition using the R package *rptR* (Stoffel, Nakagawa, & Schielzeth, 2017). For serology, one individual had abnormally low measures (outside viable range) for Ca, BUN, and CK in one replicate, hinting at an analyzer malfunction, so we did not include data for this individual.

Repeatability tests stressed the possibility of measuring artefactual extreme values due to analyser failures in serology tests. As a consequence, we removed extreme health parameter values using Horn's method (Finnegan, 2014) (see Appendices – Table 5.1). This method determines outliers in a Box-Cox transformed dataset using Tukey's interquartile (IQR) fences. A data point was considered as an

outlier when the point lay 1.5 * IQR outside of the 1st or 3rd quartile point. Results were qualitatively the same with and without the outliers.

Association between parasite load and overall health: Multivariate mixed procedure

We proceeded in two steps to investigate whether negligible, mild or heavy parasite loads were associated with variations in health taking into account the expected correlation between health measures. First, we used a Bayesian multivariate mixed model (package *MCMCglmm* by Hadfield, 2010) implemented in a framework using Markov chain Monte Carlo (MCMC) sampling. We used a Gaussian error structure and identity link. MCMCglmm uses inverse-Wishart distributed priors for variances. Here we specified proper priors with parameter "V" for the variances in **R** (matrix containing the residual covariances) and in **ID** (matrix denoting the between individual covariance) set at the repeatability for each trait (Brommer et al., 2014). The parameter "nu" (degree of belief) was equal to the number of health parameters to be estimated in **R** and **ID** (Brommer et al., 2014). This model allowed us to include multiple response variables.

We first assessed the potential effect of the high correlation between two covariates, the time since last deworming and the season of sampling. We included haematology (TWBC, haematocrit, the absolute count of lymphocytes, monocytes, heterophils, eosinophils measured as [white blood cell/100] * TWBC), serum chemistry (albumin, globulin, ALKP, CK, triglycerides, AST, CREA, BUN) and electrolytes (calcium) as multi-responses. Due to a large number of missing values, we removed five health responses from the multivariate mixed model (number of missing values out of 706 individual measures: haemoglobin: 180, basophils: 111, potassium: 180, chloride: 181, sodium: 180). We included age, sex, and birth origin (wild-caught or captive-born) as predictors. We fitted the covariance between individuals by including individual as a

random effect. Then, we compared three models using the Deviance Information Criterion (DIC): one model containing the season of sampling as a factor, a second model containing the deworming time period and a third model containing both the deworming time period and the season of sampling. According to the DIC, we retained the multivariate mixed model including both anti-helminthic treatment and the season of sampling. We checked the robustness of the selected model by using a cross-validation method (e.g. Geisser, 1975; Stone, 1974). We also checked whether our results were dominated by the priors through visual evaluations of the posterior distributions.

We extracted the residuals from the best multivariate Gaussian model out of the three tested. These residuals can be interpreted as estimators of each health parameter, linearly corrected for the confounding effect of the covariates that are not the direct focus of this study (age, sex, birth origin, season of sampling, or time since deworming). We used a Linear Discriminant Analysis (LDA) in order to assess overall variation in health between individuals with Negligible, Mild or Heavy parasite loads: discriminant functions are constructed as linear combinations of corrected health estimators, to maximise the variance between the three parasite load groups. The importance of the contribution of each health parameter to these discriminant functions can therefore be interpreted as a measure of how much this parameter varies between parasite load groups, while accounting for the high correlation between health parameters. We tested for the significance of the discriminant values (eigen values) using a multivariate analysis of variance (MANOVA) with a Pillai test. The LDA was performed with “ade4” and the function *discrimin* (Dray & Dufour, 2007).

Association between parasite load and differential blood cell count: Multivariate procedure

As the differential white blood cell count was performed using a manual approach where a fixed 100 cells were counted, errors are necessarily correlated between the different cell types. We analysed the parasite-related variation in white blood cells (*lymphocytes, monocytes, heterophils, basophils and eosinophils*) using a multivariate generalized linear model framework with a Dirichlet distribution which allowed us to include multiple response variables (*MGLMreg* from the package "MGLM" by Zhang, Zhou, Zhou, & Sun, 2017). Due to implementation limitations, we were not able to include random intercepts in this model, and therefore pseudo-replication may be a possible confounding factor in this case. Parasite count, age, sex, origin (captive-born or wild-caught), season of sampling (hot, dry, monsoon) and the location (Kawlin, East and West Katha) were included as explanatory variables.

Nematode-related patterns of health parameters: Single-trait analysis

Next, we investigated how Negligible, Mild or Heavy parasite load was associated with each of the 21 health parameters, independently of the variation in other parameters – a less realistic approach, but one which has the advantage of being robust to possible measurement errors in individual parameters. Using linear mixed models (*lmer*, package "lme4"), we fitted haematocrit, haemoglobin, total protein, globulin, albumin, BUN, CREA, calcium, potassium, chloride, and sodium as dependent variables using a Gaussian distribution. Using generalized mixed models (*glmer*, package "lme4" by Bates, Mächler, Bolker, & Walker, 2015), we fitted AST, ALKP and CK as dependent variables using a Poisson error distribution and triglycerides and total white blood cell count as dependent variables using a gamma distribution with a log link function. Our main predictor variable of interest was the three-level parasite load fitted in each model as a fixed effect. Our models also controlled for any differences in the health parameters

due to age, sex, origin (captive or wild-caught), and the location (Kawlin, East and West Katha). We included individual identity as a random intercept, to avoid pseudo-replication issues.

We performed an AIC-based model selection. For each health parameter, we compared (1) a first base model including the confounding variables and the season of sampling (hot, dry, monsoon); (2) a second base model including the confounding variables, deworming time as a 2-level variable; (3) a third model including the confounding variables, parasitism as a 3-level variable (FEC) and the season of sampling (hot, dry, monsoon); (4) a fourth model including the confounding variables, parasitism as a 3-level variable (FEC) and deworming time as a 2-level variable. We selected the most likely models using the Akaike information criterion (AIC), considering each random effect as one parameter (Pinheiro, 2002). We retained the model with the lowest AIC as the best model. Where the difference in AIC between competing models was less than two, we retained the simplest model (Burnham & Anderson, 2002). We also calculated the Akaike weight (AICw) for each model to provide the relative likelihood that the model was the best among the candidate models. We report only the results for the best models, and we checked the robustness of them by reporting effect sizes and by bootstrapping the log likelihood of the models. Further details about model selection are presented in Appendices (Table 5.2). The figures presented represent count data and do not take into account model covariates.

Results

Repeatability of our measurements

Serological parameters as measured on the IDEXX VetTest® analyzer appeared to be robust, with high repeatability (>0.9) for BUN, CREA, TRIG and CK, moderately high repeatability (0.6 to 0.8) for ALB, GLOB, and ALKP, but rather poor repeatability (<0.6) for Ca, TP and AST.

Effect of parasites on overall health

The LDA shows a strong association between heavy parasite loads and deviations from standard health patterns (df_{health}=476, $df_{parasite}$=2, Pillai=0.13, F=2.12, p<0.01). On the other hand, health patterns of elephants with mild parasite infection do not appear to differ from those of parasite-free elephants, regardless of age or sex. The LDA clearly discriminates heavily infected individuals from those with mild or negligible infections. The main contributors to the two retained discriminant functions are four immunological parameters: the total white blood cell count, and the absolute count of heterophils, monocytes and lymphocytes, which showed the highest loadings (Table 1, Fig. 1), meaning that these parameters vary between heavily infected individuals, and the rest of our sample. Since these results are based on the residuals of a multivariate model fit, they are not confounded by the host's sex, age, origin or place and season of sample collection, or repeated sampling of the same individuals longitudinally. Overall, these results show that gastrointestinal parasite load, as measured by FEC, is associated with a modified host homeostasis, in particular in the immune system, and that this is particularly true in animals with heavy infections.

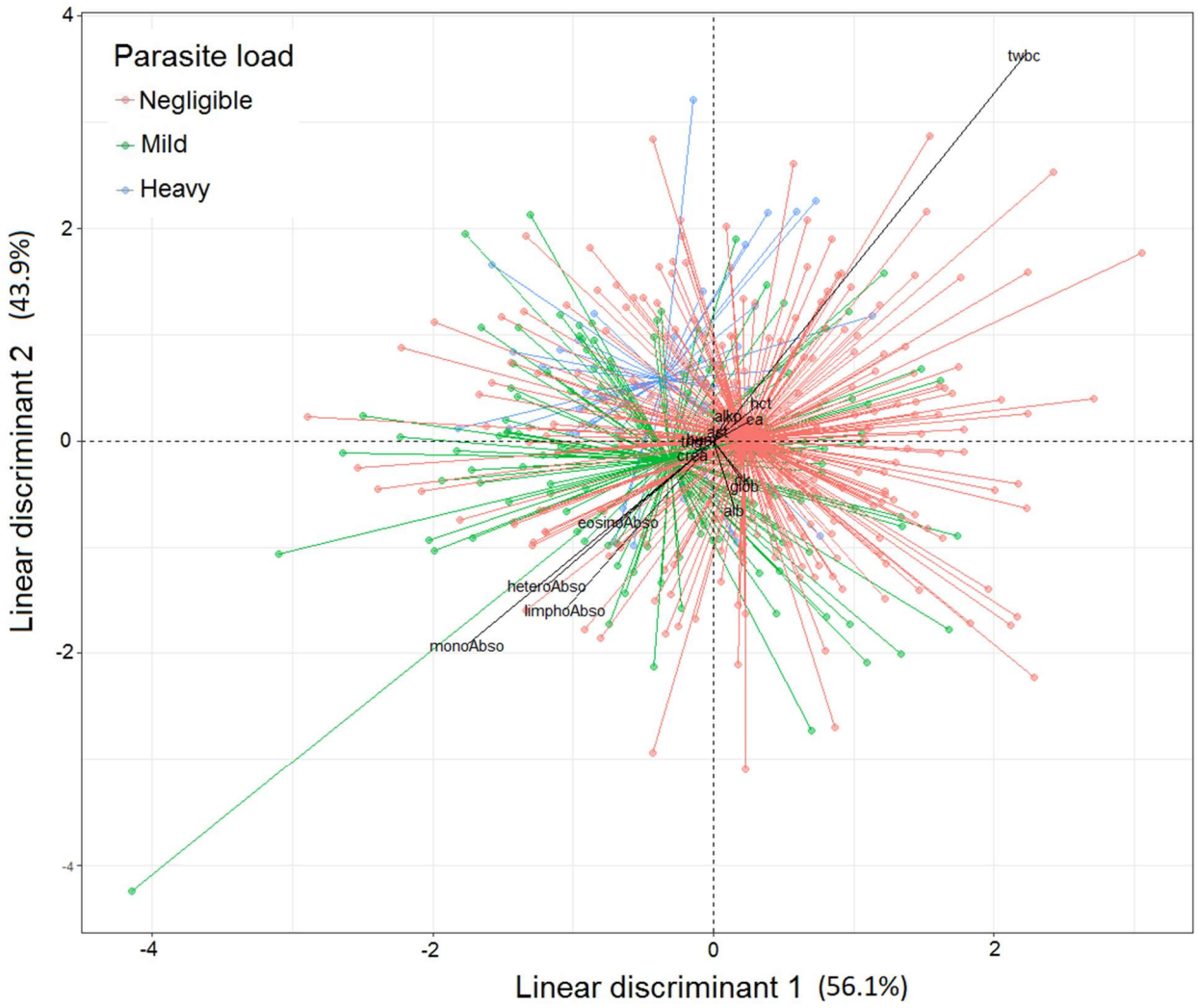


Figure 5.1. Biplot from the LDA showing clustering of “negligible”, “mild” and “heavy” parasite loads across 15 health parameters. The figure combines the standardised coefficients of health in black (i.e. canonical weights of the linear discriminant functions on the two axis of the linear discriminant analysis), and the projection of the health samples with gravity centres of each parasite load (“negligible” in red, “mild” in green, and “heavy” in blue).

Table 5.1. Standardized coefficients for the health parameters of the linear discriminant 1 (LD1) and the linear discriminant 2 (LD2). Health parameters with the highest loadings are highlighted in grey.

Health parameters	LD1	LD2
Haematocrit	0,344	0.362
Lymphocytes	-1,054	-1,582
Monocytes	-1,753	-1,915
Heterophils	-1,187	-1,353
Eosinophils	-0,679	-0,751
TWBC	2,227	3,639
Calcium	0,299	0,213
Albumin	-0,150	-0,649
Globulin	-0,229	-0,411
BUN	-0,096	-0,031
Creatinine	0,140	0,124
CK	0,216	-0,353
ALKP	-0,108	0,251
AST	-0,032	0,101
Triglycerides	-0,153	0,003

Nematode load and specific health parameters

After confirming that a particularly heavy parasite load was associated with a clear shift in overall health state using a multivariate approach, we investigated the association of parasite loads with all the specific underlying health parameters, and found support for the majority (19 out of 21) being significantly associated with gastrointestinal parasite infection.

Haematology

On average, our sampled elephants had 16,705 x 10^3 white blood cells/L but this ranged widely both between and within individuals sampled multiple times, from 7,722 to 29,656 x 10^3 white blood cells/L. We showed that TWBC counts were not statistically different with a heavy parasite infection compared to a negligible one (β = 0.024 ± 0.029, t = 0.844). This result was supported by the AIC-model selection process, since the best model included parasite load as a 3-level factor (AIC = 11,929.50, wAIC = 0.979; Appendices - Table 5.3). In the differential white blood cell counts with a Dirichlet distribution using a multivariate generalised model, mild infection had a significant difference compared to negligible shedders. Indeed, elephants displaying a mild parasite infection had 7% more lymphocytes (β = 0.169 ± 0.076, z = 2.221), 12% more heterophils (β = 0.259 ± 0.076, z = 3.407), 9% more monocytes (β = 0.243 ± 0.077, z = 3.156) and more eosinophils (β = 0.434 ± 0.084, z = 5.135) than negligible shedders (AIC = -11,431.92, wAIC = 1; Appendices - Table 5.4 and Figure 3).

Red blood cells were similarly affected by parasitism. On average, elephants presented haemoglobin levels of 11.95 ± 0.18 g/dL, with wide variance among samples (9.2-16.0). Mild shedders had 2.2% lower haemoglobin (β = -0.268 ± 0.107, t = -2.501) than negligible shedders. The AIC-model selection confirmed that the model including parasite load as a 3-level factor was the best at explaining variation in haemoglobin levels (AIC = 1,500.569, wAIC = 1; Appendices - Table 5.3). Moreover, on average, elephants presented 34.8 ± 0.5% of haematocrit, ranging from 28 to 44. Mild shedders had 1.7% lower haematocrit (β= -0.584 ± 0.248, t = -2.351) than negligible shedders. The AIC-model selection confirmed that the model including parasite load as a 3-level factor was the best at explaining variation in haematocrit (AIC = 3,263.655, wAIC = 1; Appendices - Table 5.3 and Figure 3).

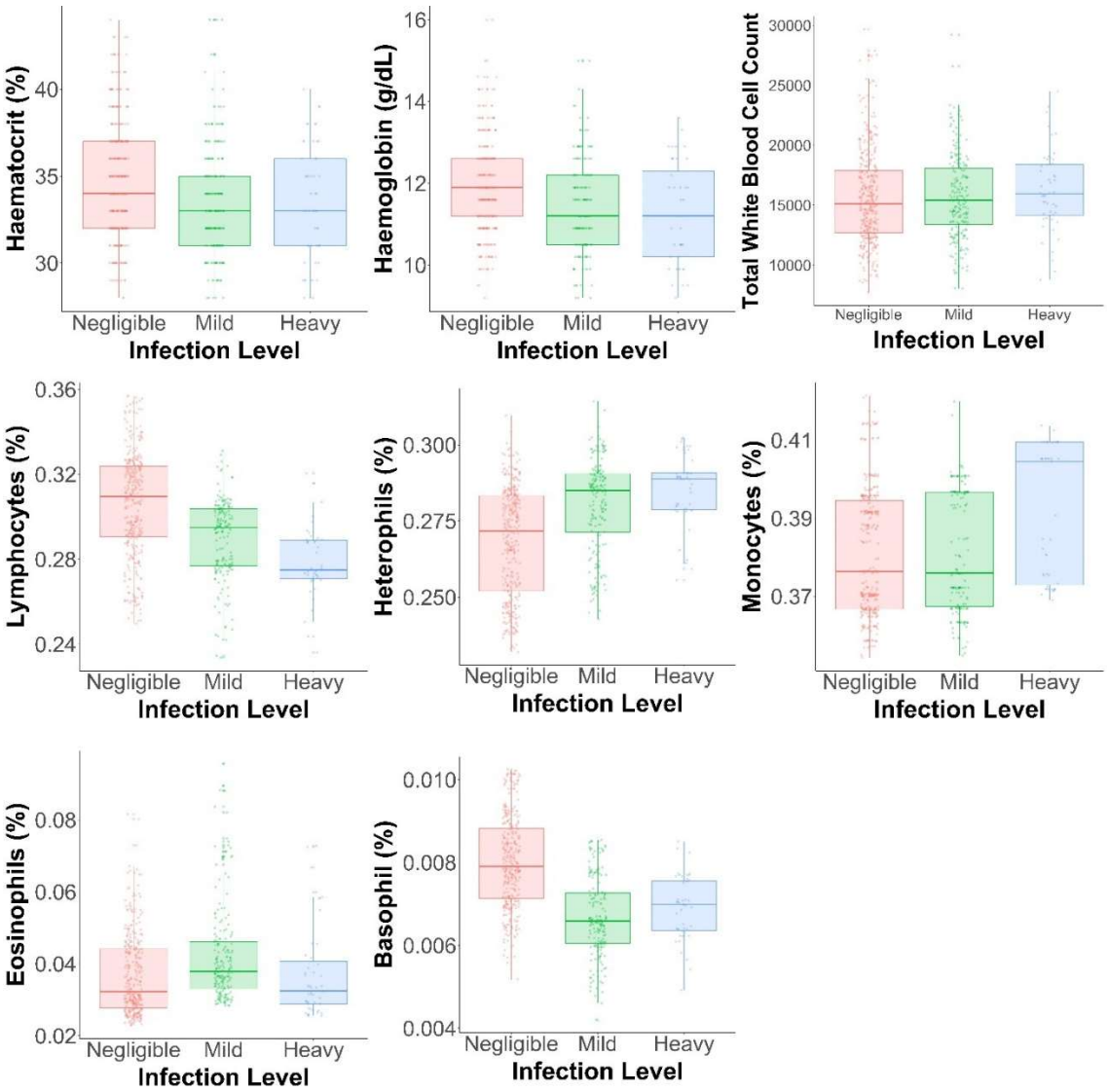


Figure 5.2. Parasite-related changes in haematocrit, haemoglobin, total white blood cell counts and differential cell counts. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentiles, and the whiskers indicate the highest and lowest values of the results.

Proteins

On average, elephants presented 7.64 ± 0.07 g/dL (ranging from 6.2 to 9.0) of total proteins including 3.14 ± 0.04 g/dL (ranging from 2.4 to 3.7) of albumin and 4.43 ± 0.06 g/dL (ranging from 3.6 to 5.8) of globulins, but parasite load influenced the three measures of proteins in different ways. Overall, we showed a 3.8% decrease of total

proteins from heavy to mild levels (β= -0.216 ± 0.069, t = 3.168) and 3.9% decrease from heavy to negligible levels (β= -0.300 ± 0.068, t = -4.389) as found by the AIC-model selection retaining the model including parasite as a 3-level factor (AIC = 880.681, wAIC = 0.999; Appendices - Table 5.5). Similar results were found for globulins for which elephants with heavy infections (β= -0.181 ± 0.047, t = -3.878) had 4.1% less globulins than those with negligible infections and having 3.7% less globulins than the mild shedders (β= -0.159 ± 0.047, t = -3.410; AIC = 437.506, wAIC = 0.790; see Appendices - Table 5.5 and Figure 4). However, an elephant's parasite infection was not associated with their albumin level (AIC = 62.252, wAIC = 0.990; Appendices - Table 5.5). Since the repeatability for albumin was moderate, this result may be an artefact.

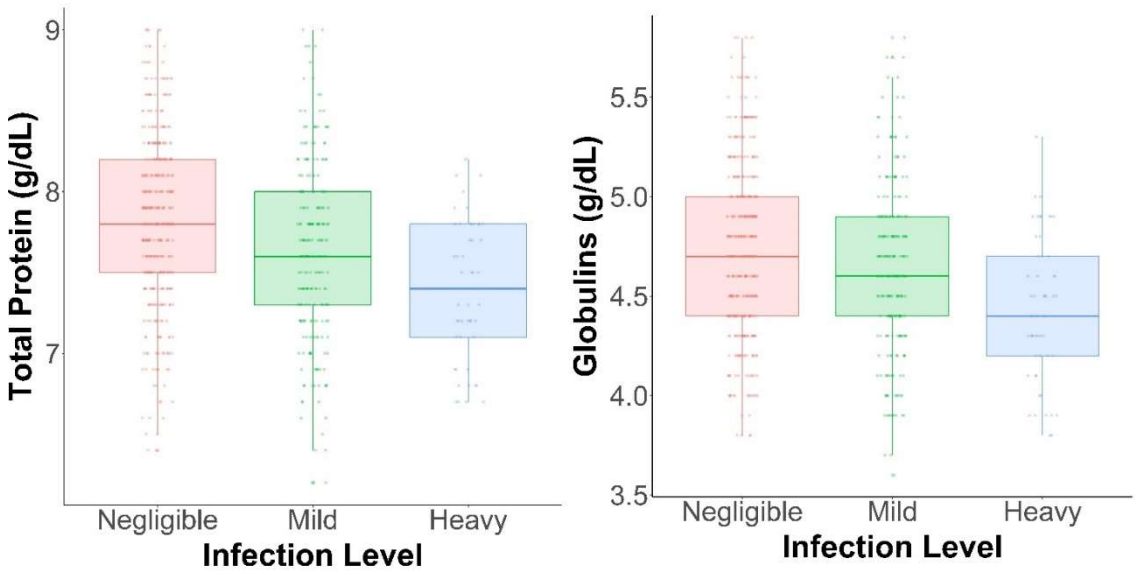


Figure 5.3. Parasite-related changes in protein activity. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentiles, and the whiskers indicate the highest and lowest values of the results.

Enzymes

We measured three enzymes, namely AST (26 U/L, on average and 0 to 144 range), ALKP (120 U/L, on average and 20 to 249 range), and CK (129 U/L, on average and 10 to 491 range) that are associated with liver (AST, ALKP) and muscular (CK) activity. Parasite load appears to be associated with the three enzymes as the best models included parasite load. Heavy (β= -0.210 ± 0.042, z = -4.956) shedders showed 6.5% lower AST levels, respectively, than negligible shedders (AIC = 10,216.90, wAIC = 1; Appendices - Table 5.6). Mild (β= -0.105 ± 0.011, z = -9.695) shedders exhibited 2.2% lower ALKP levels than negligible ones and we observed the same trend (0.8% decrease) from heavy to negligible shedders (β= -0.039 ± 0.018, z = -2.113; AIC = 8,596.613, wAIC = 1; Appendices - Table 5.6). Mild (β= -0.028 ± 0.009, z = -3.074) shedders showed 0.6% lower CK levels than negligible shedders (AIC = 21,619.53, wAIC = 1; Appendices - Table 5.6 and Figure 5).

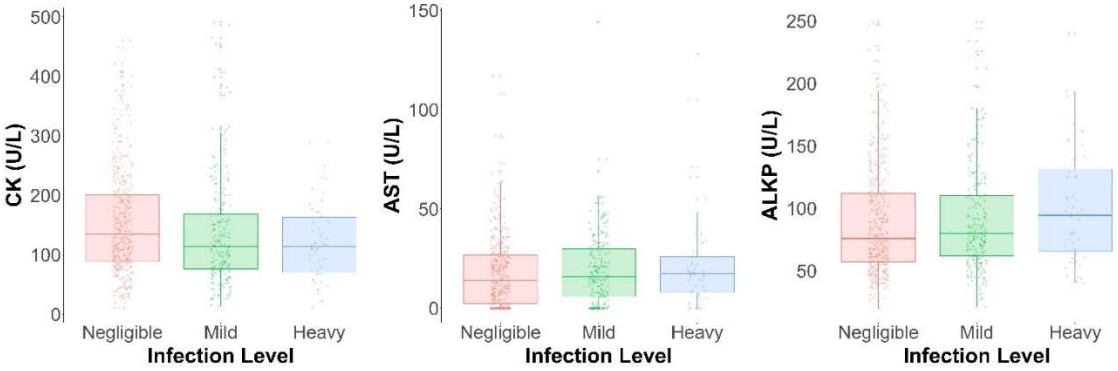


Figure 5.4. Parasite-related changes in enzyme activity. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentiles, and the whiskers indicate the highest and lowest values of the results

Kidney activity

We measured kidney activity using two parameters BUN (19.85 ± 0.59 mg/dL, on average, ranging from 3 to 34) and CREA (1.17 ± 0.03 mg/dL, on average, ranging from 0.6 to 1.7). Parasite load showed no association with creatinine but did associate with

BUN. Heavily infected elephants had 5.9% lower BUN levels, than negligibly infected elephants (β= -1.166 ± 0.556, t = -2.094; AIC = 3,684.463, wAIC = 1; Appendices - Table 5.7; figure 6). However, we did not detect any association of parasite load with creatinine levels (AIC = -146.542, wAIC = 1; Appendices - Table 5.7).

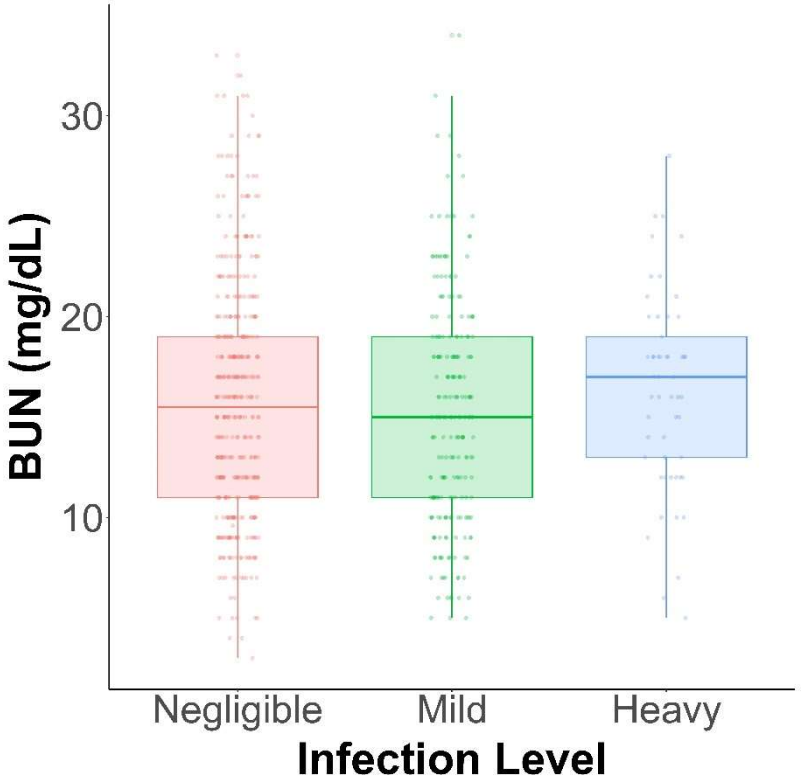


Figure 5.5. Parasite-related changes in BUN. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentiles, and the whiskers indicate the highest and lowest values of the results

Triglycerides

We measured fat storage using triglyceride levels, which averaged 11.59 mg/dL [10.07 – 13.33] across the samples but ranged from 0 to 88. We showed that parasite levels were associated with this variation. Mildly and heavy infected elephants showed no difference from negligible shedders (β_{mild}= -0.087 ± 0.096, t_{mild} = - 0.907; β_{heavy}= 0.006 ± 0.166, t_{heavy} = 0.035; AIC = 4,524.479, wAIC = 1; Appendices - Table 5.8 and figure 7).

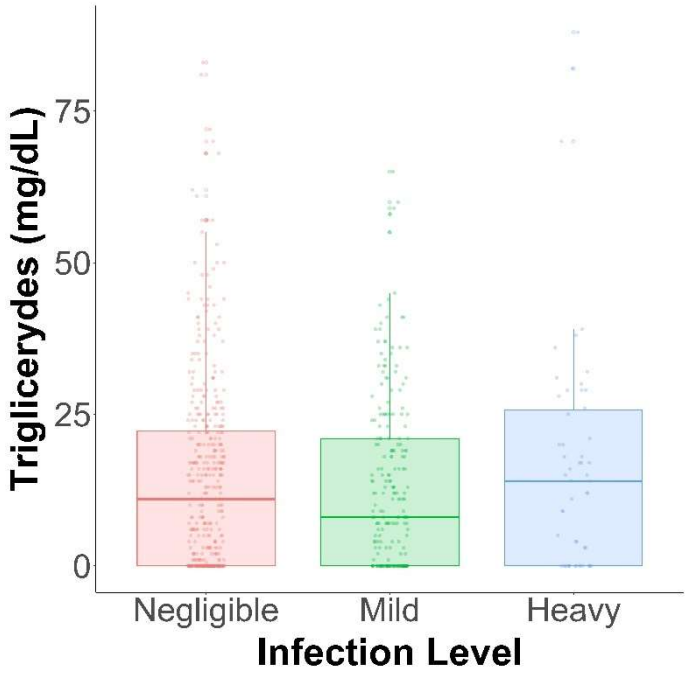


Figure 5.6. Parasite-related changes in triglycerides. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentiles, and the whiskers indicate the highest and lowest values of the results

Electrolytes

On average, elephants displayed 4.93 ± 0.06 mEq/L of potassium (range from 3.8 to 6.6), 129.82 ± 0.39 mEq/L of sodium (range from 123 to 134), 91.53 ± 0.37 mEq/L of chloride (range from 83 to 97), 10.19 ± 0.08 mg/dL of calcium (range from 7.7 to 11.2). The best models for each electrolyte included parasite load as a 3-level factor. Mildly and heavy infected elephants had lower potassium than negligible shedders despite being non-significant (AIC = 593.133 wAIC = 1; Appendices - Table 5.9). Sodium levels were decreased in individuals with a mild infection compared to those with a negligible one (β= -0.555 ± 0.233, t = -2.379; AIC = 2,169.864, wAIC = 1; Appendices - Table 5.9). Moreover, heavy shedders showed an increase of chloride compared to those with negligible infection despite being non-significant (AIC = 2,264.588, wAIC = 0.837; Appendices - Table 5.9). Finally, we found that mild (β= -0.214 ± 0.049, t = -4.355)

shedders showed a decrease of 2.1% in calcium levels compared to negligible shedders (AIC = 1,147.642, wAIC = 0.930; Appendices - Table 5.9 and Figure 8).

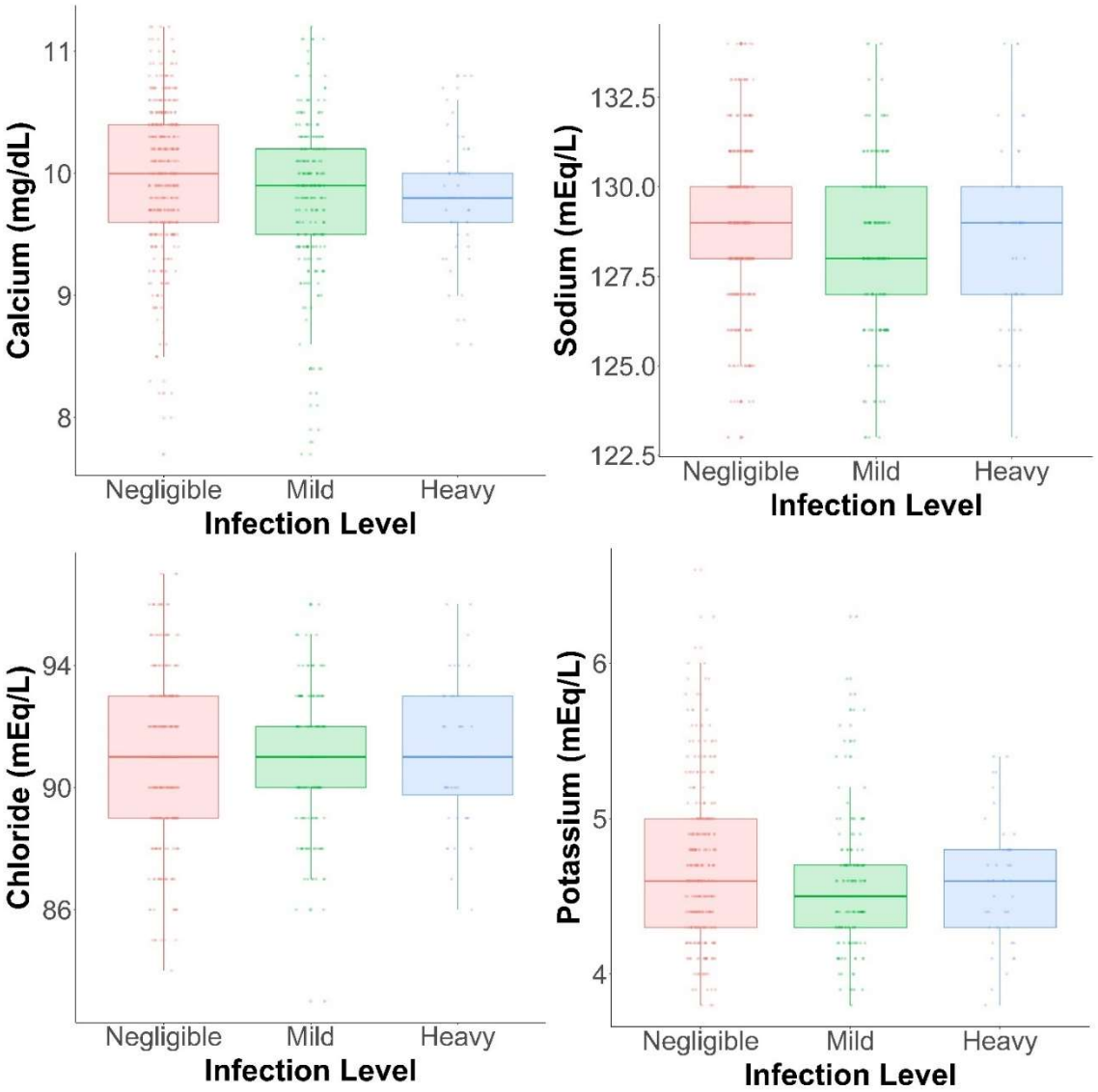


Figure 5.7. Parasite-related changes in enzyme activity. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentiles, and the whiskers indicate the highest and lowest values of the results.

Discussion

A large number of laboratory and field studies now show that parasites exert direct fitness costs on their hosts. Costs to survival and reproduction are predicted to arise through the influence of parasites on host physiology and disruption of homeostasis (i.e. health; Beldomenico et al., 2008; Monteiro et al., 2010). However, at present our understanding of the functional ecology of parasitism, particularly in natural populations of animals is limited and the handful of previous studies reported mixed results (Dazak et al., 2000; Stien et al., 2002; Zuk & Stoehr, 2002). In this study, we document the impact of gastrointestinal parasites on host health in one of the longest-living terrestrial species, the Asian elephant. We showed that our measure of gastrointestinal parasite infection, faecal egg count, was associated with overall health where heavily parasitized individuals showed health decline, as shown by the declines in a range of physiological parameters including haematological, immunological, muscular, kidney and liver functions, as well as protein and electrolyte balances. Studying a large number of animals, with repeated measures of various health parameters associated with different physiological functions, longitudinally over multiple years and seasons, allowed us to gain an integrated and global view of the role of parasites in elephant health. Our results highlight the need to determine the proximate effects of parasitism on physiological processes and more largely on health, which underlie the link between parasites and fitness outcomes among individuals.

At the population level, macroparasites, such as nematodes, are usually aggregated across hosts, whereby a small group of individuals usually carry the heaviest burdens and the majority of hosts have low or no observed nematode loads (Poulin, 2013). In some systems, heavily parasitized hosts also display clearly reduced health compared to less parasitized individuals, leading to difficulties in detecting health impacts under certain parasite loads (Dimitrijević et al., 2016). While the majority of previous

studies have investigated the effects of parasite load as a continuous variable (Marcus et al., 2015; Rouatbi et al., 2016), we used a categorical measure of parasite burden, following recommendations in the literature concerning heavy infection thresholds in terms of EPG in horses (Lyons, Tolliver, & Kuzmina, 2012; Nielsen et al., 2010). This approach of categorising parasite load allowed us to mitigate the effects of the intrinsically moderate repeatability of FEC measurements, and to maximize the detection of parasite effect on health in a semi-wild population setting. Consistent with the theory (Poulin, 2013), we found that only 8% of all samples in our population could be classed as heavily infected by gastrointestinal nematodes (EPG > 500). Our study highlights the mechanisms by which a high parasite load leads to ill health, which can be used as a basis for designing interventions. In our population, young animals and senior elephants present the highest FEC and the highest parasite driven mortality (Lynsdale, 2017; Lynsdale et al., 2017), creating the need for this more strategic approach. Also, it is known that despite parasite burdens being similar across the sexes, males present a higher mortality (Lynsdale, 2017) and we need to increase our understanding of how the health of these different demographic groups is affected by parasite infection. This is important, because elephant populations worldwide are threatened, and the semi-captive population in Myanmar is not self-sustaining in its current state due to high juvenile mortality (Choudhury et al., 2008; Jackson et al., 2019; Leimgruber et al., 2008). With the knowledge that only heavily parasitized elephants present deviation from the normal health parameters, a more strategic anthelminthic treatment protocol can be implemented with more emphasis on parasite surveillance and diagnosis (Nielsen et al., 2010).

We measured 21 health parameters that represented several bodily systems and functions (e.g. haematology, immunology, proteins, muscle, kidney, liver, electrolytes). Because most functions are interconnected within organisms (Trayhurn, 2005), we took

an innovative approach to take into account such interdependency between the systems and functions in the body. As expected (Dimitrijević et al., 2016; Monteiro et al., 2010), we found an overall effect of parasite infection on elephant health, especially in heavily parasitized animals. Evolutionarily, this result can be explained by selection favouring parasites with intermediate levels of virulence (Gandon & Michalakis, 2000). Indeed, parasites should maximize their reproductive success without compromising host survival, to guarantee future transmission (Gandon, Jansen, & Van Baalen, 2001). Interestingly, this global analysis indicated that white blood cells were most associated with high levels of gastrointestinal nematodes in Asian elephants. This result was also corroborated by the detailed independent analysis of each health parameter. Total white blood cell count and the immunological response is expected to increase with higher parasite infection (Day & Burns, 2003; Maizels, Hewitson, & Smith, 2012), and our results confirmed a stronger immunological response in heavily infected individuals. The increase in white blood cells is in line with parasite infection triggering immunological defence, namely the innate and adaptive response of different white blood cells (Day & Burns, 2003; Maizels et al., 2012). Some previous studies have also observed an increase in white blood cells with parasitic infection (López-Olvera et al., 2006; Marcus et al., 2015; Matanović et al., 2007; Nnabuchi et al., 2015; Shender et al., 2002) but results from different species are not yet conclusive concerning the response of white blood cells to the presence of parasites (Dimitrijević et al., 2016; López-Olvera et al., 2006). Interestingly, we showed that the high TWBC counts seen in heavily parasitized individuals was driven by higher heterophil, monocyte and eosinophil levels, while lymphocyte levels were lower in heavily infected individuals. This finding cannot be explained by immunosenescence with age alone, nor sex, which we included as covariates. In the presence of macroparasites like nematodes, the immune system responds by activating the Th2 cells which include the eosinophils and T cells (lymphocytes), while monocytes and heterophils are present in Th1 responses, especially to intracellular infections by bacteria and viruses (Díaz & Allen, 2007). This is

not in agreement with what was observed in our population. However, this can be the result of an immunomodulation process where parasites downregulate the immune system to avoid active clearance by a host's immune response (Maizels & Yazdanbakhsh, 2003). In our study we do not investigate the presence of other parasites that may also have an effect in this immunomodulation (Fenton, Lamb, & Graham, 2008). It is also important to bear in mind that for the differential white blood cell counts, a conditional counting is performed, meaning that when there is an increase of one kind of white blood cell line, other(s) would inevitably be lower since the total number of cells counted always equals 100 (Unkelbach, 1980). More investigations are required to identify the specific immune response of parasites in Asian elephants to further our understanding of the white blood cell profile in heavily parasitized animals.

In addition to immune function, gastrointestinal parasites also affected other physiological functions of the elephants. Red blood cell parameters, namely haematocrit and haemoglobin, decreased with increased parasite burden. This could be explained by the gut parasites consuming blood or by lowered production by the host and is in line with other studies (Dimitrijević et al., 2016; Rouatbi et al., 2016). Protein levels were lower in heavily infected hosts and this was mainly driven by loss of globulins, since no difference was seen in albumin. The loss of protein is due to parasite attachment to the gut wall with destruction of the mucosa that allows protein to cross the gut barrier to the lumen (Marcus et al., 2015). Fat storage is measured by triglyceride levels (Ferguson & Leese, 2006). In our study population, mild and heavy parasitism were associated with slight, non-significant decreases in triglyceride levels. This result contradicted the theory that high parasite burdens have negative effects on host nutritional status due to exploitation of the host's resources (Murray et al., 1998). Concerning kidney function, in our study we saw no influence of parasite infection on creatinine but BUN levels decreased with increased parasite levels. Since BUN is an end product of protein

catabolism by metabolization in the liver and excretion by the kidney, the decreased level of proteins may be responsible for the decrease in BUN (Nnabuchi et al., 2015). Contrary to other studies, parasitism was responsible for a decrease in enzyme (ALKP and AST) activity (López-Olvera et al., 2006; Nnabuchi et al., 2015). This could be due to the fact that this population is not heavily affected by hepatic parasites (Ahmed, Ambali, & Baba, 2006), although microfilaria loads have not been studied in depth in the Myanmar population (but see Min Oo, Kyaw, Nyunt, & Khaing, 2009). Electrolytes had mixed trends but the changes were very slight, confirming that electrolytes tend to be stable across and between individuals (Fowler & Mikota, 2006).

Studying the effect of parasites on host health in natural environments is difficult in wild populations due to several limitations. Consequently, using a semi-captive population such as the timber elephants of Myanmar has many strengths. These elephants display a range of key natural behaviours, like roaming, feeding and mating in the forest without human control, and sometimes interacting with wild conspecifics (Lynsdale et al., 2017). In this environment elephants are naturally infected by nematodes, and parasites are also an important driver of mortality in elephants of different demographic groups (Lynsdale et al., 2017). Importantly, however, the MTE elephants receive monthly check ups by trained veterinarians which enable these animals to be handled, identified and subjected to longitudinal health data collection not possible from wild populations. However, our study also has some limitations. Asian elephants are endangered, and ethical concerns precluded us from designing studies in which subjects would be experimentally manipulated to experience increased parasite loads. Instead the elephants were dewormed periodically, regardless of their parasite load, which allowed us to investigate how individual and population-level health varied longitudinally across time when individuals were exposed to different parasite levels. Another potential limitation is that, like many studies investigating gastrointestinal

parasites in long-lived hosts (Dimitrijević et al., 2016; López-Olvera et al., 2006; Monteiro et al., 2010), we used FEC as an indirect measure of parasite load without identification to a species level. This prevents us from determining if the observed effects on health are due to coinfection by multiple parasites, or are led by one species only. Another common concern for natural populations is whether parasites directly affect health, or whether fluctuations in health driven by other unmeasured causes, affect the number of parasites (Raberg, Graham, & Read, 2009). Periodical deworming reduces the likelihood that individual quality differences would be the main driver confounding associations between parasites and health. Furthermore, we followed the same individuals longitudinally over several years in which time parasite loads fluctuated strongly across seasons and years, allowing us to observe within-individual variation in health with different parasite levels.

Our study has at least three major implications. First, our results highlight the importance of investigating health globally to fully appreciate the multitude of effects that parasitic infections can have on individual fitness. However, such studies are currently rare particularly in semi-captive and natural populations, and we therefore call for future studies to address this gap across a range of ecologies and study systems. Second, our results demonstrate the importance of quantifying parasites in a nonlinear way, since sometimes effects exist only after a certain parasite load threshold. Parasite infection as estimated by FECs in our study were associated with host health especially in heavily infected animals, and only a small fraction of the population presented high parasite burdens with deviations from normal health values. Finally, our results have important management implications. For example, in human managed wildlife populations, it is important not to deworm all animals indiscriminately and instead keep a more specific treatment schedule of deworming that targets only the animals with high parasite burdens. Future studies should focus on classifying health and creating a health ranking

system that could help inform rational management choices for captive or wild populations (Monteiro et al., 2010)

Chapter 6

Thesis discussion - Life history Traits as Health Commanders and Conservation Issues

Discussion

In this thesis, I investigated the interaction between a large range of health parameters and several life-history traits and ecological factors in a long-lived species managed in semi-captivity. Asian elephants employed in Myanmar's timber industry. I began by determining what can considered the reference health parameters values for this specific population, and then analysed how different life-history traits such as sex and age, local ecology (season) and parasite burden were associated with differences in the physiological systems and functions of these animals. The research presented in this thesis contributes to our understanding of the physiological mechanisms of how individuals respond to external factors. It also provides an early example of how we may understand animal health as an interconnected net of systems and functions that affect each other. These findings contribute to the field of ecophysiology, but also have important conservation implications for this unsustainable population of an endangered species. In the long term, my findings and the application of established health screening methods provide tools for improving veterinary care, in order to consider seasonal, sex-specific or age-related health risks in strategic health care. In this chapter I will review the findings from each of the chapters and discuss them in a wider context. In doing so, I will summarize some of the novelties that this study brings, the limitations it faced, future directions and some practical applications for the results.

Who is healthy? Reference Intervals of Asian elephants in Myanmar

The reference values of health parameters are an increasingly essential tool for establishing and monitoring the health status of individuals and populations in the wild, and especially for endangered species (Catenacci et al., 2017; Deem et al., 2001). Although they are rarely used outside of veterinary science (but see e.g. May-Júnior et al., 2009; Nussey, Watt, Pilkington, Zamoyska, & McNeilly, 2012), reference intervals can provide the basis for assessing the mechanistic basis for many life-history trade-offs

and responses to environmental variation in evolutionary ecology and ecophysiology. In the case of Asian elephants, our knowledge of health in the wild is scarce and limited by small sample sizes and difficulties in obtaining repeated samples of known individuals (Gromadzka-Ostrowska et al., 1988; Niemuller et al., 1990; Silva & Kuruwita, 1993b), and we have little understanding of “normal” health variation in this species, or sex differences therein. This is reinforced by zoo populations suffering from well-known behavioural, reproductive and health problems, which considerably reduces lifespan when compared to the wild (Clubb et al., 2008), which therefore provide little aid in understanding natural health variation in Asian elephants.

Chapter 2 described for the first time, to my knowledge, the body condition score (BCS) in elephants outside of zoos and blood pressure with the use of a new method, together with a wide range of other health parameters routinely used in veterinary science. Contrary to the zoo populations that are generally on the BCS level ‘very fat’ (4) or ‘obese’ (5) (Morfeld et al., 2016), the Myanmar timber elephants were generally on the level 3 (normal) with very few exceptions. This result shows that when animals are given the chance to forage naturally and keep their natural exercise rhythm, providing climatic conditions are good, they tend to keep a healthy BCS. To my knowledge, blood pressure has not been reported in elephants until now, and the new method I established will hopefully be tested in other populations and have a clinical application, similarly to humans in which blood pressure is routinely used as an indicator of cardiovascular health (Kannel, 1996).

I also observed some sex differences in health parameters. For example, females had better body condition, as measured in both BCS and triglycerides. Several reasons may contribute to this finding, with females given rest during pregnancy and 2 years post-

partum, compared to the higher levels of testosterone in males - an anabolic for fat metabolism (De Maddalena et al., 2012). Although elephants do not experience physiological reproductive cessation (menopause) like humans, they also exhibit extended post-reproductive lifespans (Lahdenperä et al., 2014), and the higher fat levels in such females fit with studies on humans showing that after menopause, oestrogen levels in females tend to decrease, increasing the risk of fat storage (Brown & Clegg, 2010). In contrast, creatinine levels were higher in males than females, consistent with creatinine being a by-product of muscle metabolism and males having higher muscle mass than females on average (Braun et al., 2003).

More generally, this chapter contributes to the small but increasing number of studies that establish reference values for wild populations, allowing us to understand the physiological differences between the sexes and how physiological parameters affect or are affected by other life-history traits.

Nevertheless, there are some limitations concerning the study design that were inevitable due to field conditions and population limitations, and that should be considered when interpreting the reference values. First, glucose was measured in the laboratory rather than immediately after blood collection, and this approach requires accounting for the glycolysis *in vitro* that artificially decreases serum glucose with time (Fobker, 2014). Second, the majority of the elephants in this study were young, training-age elephants under 18 years old (60% of samples), due to the difficulty in accessing working animals in the forest. Moreover, in working and retired ages, the majority of the elephants sampled were females (80% in working and 78% in retired elephants), reflecting high male mortality (Lahdenperä et al., 2018) and reduced access to working-age males, which are normally working deeper in the forest. Working-aged females can also be more conveniently sampled in maternity camps given that they stop working from mid-pregnancy until the baby is two years old (Mar et al., 2012).

A limitation that I could not explore in this chapter was the repeatability for each of the parameters, which could be useful for stronger statistical analysis and also to validate the methods used, especially for the haematological analyses and serum chemistry. Nevertheless, all methods were executed by myself to reduce the human error associated with the methods.

Age-related declines in molecular and physiological health markers

Health is predicted to decline with age due to senescence where organisms accumulate damage over their lifetime, leading to the loss of function and eventually death (Monaghan et al., 2008). However, although the evidence for declining reproductive ability and survival with age in both laboratory and wild animals is now overwhelming (e.g. Nussey, Froy, Lemaitre, Gaillard, & Austad, 2013; Shanley & Kirkwood, 2001), knowledge of the mechanisms underpinning senescence and its associated health declines comes mainly from model organisms, whereas very little is known of animals living in natural conditions. I used the diverse physiological health data from the Asian elephants of known age and previous history obtained in Chapter 2 to compare overall health and its constituents between animals of different life-stages (age). This work is one of few studies to analyse health markers both individually but also globally in order to reveal physiological senescence patterns in a long-lived mammal. The global analysis showed that health declined with increased age in both sexes and was heavily influenced by four health markers that belong to different systems and functions, namely immune defense (*the total white blood cells and lymphocytes*), liver (*ALKP*), fat storage (*triglyceride*s), and proteins level (*albumin*). An independent analysis of the individual markers confirmed this pattern. These results are in accordance with results registered in humans (Gruver, Hudson, & Sempowski, 2007). The results from this analysis show the first evidence of a decline in molecular and physiological

functions with age in a long-lived mammal that lives largely in natural conditions. It is important to note that the age categories in which we observe the higher declines in the health markers correspond to the ones in which we see declines in reproduction and survival (Lahdenperä et al., 2014, 2018).

Our novel approach, which used a multivariate-health parameter analysis as well as a linear discriminant analysis (LDA) allowed us to understand that, with age, an overall decline in health is present with no difference between males and females, despite males having a higher mortality rate (Lahdenperä et al., 2018). The age-related decrease in the total white blood cells accompanied by an increase of globulins may be good markers for immunosenescence, and the finding is corroborated by cross-sectional data on wild sheep (Nussey et al., 2012). The fact that we observed the largest declines in physiological markers for retired elephants over the age of 50, when reproduction and survival are also observed to rapidly decline in the population (Lahdenperä et al., 2018, 2014), provides insight into the likely mechanisms for such patterns. This calls for further investigation with longitudinal data, that by itself is difficult to collect due to Asian elephants being such a long living animal, given only cross-sectional data was available to address this question. Furthermore, as is often the case for senescence studies on long-lived animals, the sample size of very old animals, particularly males, was low, because few individuals on the population level survive to such ages.

Similar challenges were faced in this work as described in chapter 2. Another limitation on this study concerns females. Different levels of investment in reproduction lead to different patterns of senescence and survivorship (Massot et al., 2011; Thompson et al., 2007) with healthier females maintaining high birth rates in life (Thompson et al., 2007). In this study, when looking at different sexes we did not analyse different investment into reproduction and whether healthier females had a longer reproductive life. This limitation can also be a path to be explored in future studies.

Hot, cold or rainy? Ecological drivers of health variation in a monsoon climate

Climatic variability is known to influence several life-history traits, such as body mass (Perret & Aujard, 2001), reproduction (Rosa & Bryant, 2003) and survival (Mumby et al., 2013a). With climate change and the subsequently changes to food availability, pathogen exposure and seasonal timing of biological events, understanding how physiological markers for health vary with ecological variation is crucial. The majority of studies comparing health traits across different seasons when animals are faced with different ecological challenges has focused on animals that live in high latitudes or that hibernate (e.g. Butler et al., 2006; DelGiudice et al., 1992; Yang et al., 2018). However, it has become important to also understand how animal health is influenced by seasonal variation in a monsoon tropical climate, given little is currently understood of the "ecological medicine" of such systems in general, and given that climate change is predicted to alter such climates in South East Asia by increasing the average temperature, and increasing rainfall during the monsoon and the occurrence of extreme events (Rao et al., 2013; Ye Htut, 2014). In Chapter 4, I addressed this question in a long-lived animal, the Asian elephant, that faces several challenges in coping with seasonal ecological variation, namely food availability and quality (decrease with low rainfall and increased with high rainfall), thermoregulation (high temperatures can lead to heat stroke due to difficulty in loosing heat (Weissenböck et al., 2011) or even body condition and stress levels (Mumby et al., 2015). In doing so, I evaluated a wide range of health markers both individually and globally that may underlie previously documented variation in mortality risk, conception rate of females, stress and body condition across the different seasons (Mumby et al., 2013a, 2013b, 2015).

My results revealed that even in this long-lived mammal adapted to survive for decades of annual cycles of local ecology in its lifetime, season was a major driver of

health variation. The biggest differences were seen between hot and monsoon seasons, suggesting that the difference in rainfall and water availability as well as food quantity and quality are more important for determining health parameters than temperature, given both hot and monsoon seasons are associated with high temperatures. Looking at the health markers individually, season had an important influence on almost all the parameters studied. Importantly, sex differences also emerged with these effects, similarly to what I found regarding overall health differences in Chapter 2. This study is among the first to my knowledge to be conducted on a natural animal population from a tropical monsoon climate. Understanding how environmental factors affect animal health is important not only to unravel the ecophysiological basis for the observed seasonal variation in mortality and fertility in the population (Mumby et al., 2013a, 2013b), but also a key factor to bear in mind both clinically and also in management of this world's largest semi-captive population.

Despite these novelties, my study had some limitations. The first one is the fact that logistically, I could collect samples only once in each season. This limits the possibility of observing trends during the full year and especially in determining how long the health parameters take to adapt to the variation from one season to the other. Nevertheless, being able to confirm the observed pattern repeatedly over several years of sampling suggests that the seasonal differences are robust and repeatable. Another major limitation was the fact that we could not compare our study to others conducted on elephants or other species experiencing similar ecological challenges over the annual cycle, because to-date collecting and comparing physiological health measures over different environmental conditions is limited to continental climate systems (Butler et al., 2006; Hissa et al., 1994) or oceanic systems (Norman et al., 2012; Trumble et al., 2006).

Health cost of heavy parasite burden

Parasites are ubiquitous across the tree of life and they pose fitness costs to their hosts. Gastrointestinal parasites are predicted to present several consequences for host health due to the damage they do to the gut wall (Coop & Kyriazakis, 2001). With this study I aimed to understand how gastrointestinal parasites, measured by the faecal egg count (a proxy for parasite burden) affect animal health at the molecular and physiological levels in a long-lived host living in natural settings. Parasite burden was divided into categories because I expected to observe larger effects in individuals with the highest burdens. As in the previous chapters, the interaction between parasite burden and health markers was done individually and globally. The global analysis detected differences between animals with mild and heavy burdens as compared to those with negligible burdens and that the heavy shedders stand out from the others. The pattern was driven especially by the immune system markers. A likely explanation is the selection that parasites exert at intermediate levels of virulence (Gandon & Michalakis, 2000), with heavily parasitized hosts having to mount a stronger immunological response (Maizels et al., 2012). Individual analysis of the health parameters revealed that parasite infection was associated with measures of several body functions, but the effect sizes were small.

The use of a cutoff value for heavy infection levels in this chapter followed the recommendations for horses (Lyons et al., 2012), allowing me to detect the interaction between health and gastrointestinal parasitic burden that may not be observable using FEC as a continuous variable. However, the way we measured gastrointestinal parasite burden could be a limitation because it only gives a proxy of the real parasite burden by detecting the number of eggs in the faecal sample. Nonetheless, this method is reliable, especially in challenging field conditions, and the method has been validated for this species (Lynsdale et al., 2015). A novelty in our study was the consideration of the

interconnection between body systems and functions using a multivariate approach. The use of a semi-captive population allowed us to have a better understanding of natural parasite infection rates in elephants without human manipulation. However, with this study design I could not disentangle if it is health driving parasite burden or vice-versa (Raberg et al., 2009). However, the fact that these animals are periodically dewormed reduces the chances that individual quality differences related to parasite aggregation would be the main driver confounding associations between parasites and health.

Future directions

Health is complex to define because it involves the interconnection between several body systems and functions. The knowledge on how the animal lifestyle affects its health is still limited and we need more studies in wild populations to strengthen the conclusions from the current study. Several fields would benefit from more studies on the interconnection between health, life-history and ecology. In this thesis, I used a unique study system of a very long-lived mammal, to improve our understanding of life history choices and their consequences. The results I obtained call for further work on holistic health measures in wild populations.

When determining health parameters (Chapter 2), the next step would be to compare female health when separating animals into active reproductive stages (pregnant or with calf under weaning age) from the other, currently non-reproductive females. It is known that investment in to reproduction has trade-offs with survival (Leivesley et al., 2019), fitness (Mills et al., 2010) or even with other physiological systems like the immune system (Cox et al., 2010; French, DeNardo, & Moore, 2007). In the timber elephant population, investment in early-life reproduction (before age 30) has been shown to reduce survival in later life (over 30) (Hayward et al., 2014) and

nursing calves, particularly at older ages, can reduce maternal survival in the short-term (Robinson et al., 2012). However, the mechanisms and underlying health problems for these costs of reproduction have so far remained elusive. More generally, it would be interesting to compare current health indexes of animals with different earlier life health profiles, to determine how earlier life adverse events affect health and senescence patterns later on. One interesting possibility in the timber elephant population would be to infer past life events from the individual animal logbooks that vets have maintained to detail previous health history and any treatments and interventions.

Chapter 3 provides the first evidence of immunosenescence in this long-lived mammal. A future direction, not addressed yet in this work, would be to understand which particular factors of the immune system are responsible for this decay in the immune function and the mechanisms behind it (Cheynel et al., 2017a; Nussey et al., 2012). More generally, it would also be of importance to understand how age-related health is related with other markers of ageing like oxidative stress, oxidative damage or stress markers such as corticosteroids. Finally, my results from chapter 5 call for a better understanding on the genetic mechanisms that could increase the resistance of elephants to parasites to see if they display an association with parasite burden and health. For example, preliminary work during this PhD also focused on the major histocompatibility complex variation between my study animals, because it is predicted that through heterozygous advantage or frequency-dependent selection, MHC variation can be associated with increased resistance to parasites and better health (Froeschke & Sommer, 2005; Harf & Sommer, 2005). Future studies should also formally identify the parasite fauna that infects the elephants to understand if the phenomenon of coinfection or the presence of one specific species would be more deleterious to host health.

Conservation and Management of Asian elephants

Asian elephants' range and numbers are decreasing all over southeast Asia and in Myanmar the MTE population is also not sustainable (Jackson et al., 2019). We crucially need more scientific knowledge, but also better practices in managing populations. My results will contribute to these topics, and have led to the following recommendations to the organisation and veterinarians in charge of the health care. First, with the knowledge of the reference values and methodology for collecting health data, veterinarians will have a tool for better diagnostics and treatment of elephants, now also always taking into account the sex and age of the elephant. When caring for retired elephants, special attention should be paid to their health, and re-locating their camps to areas with better food quantity and quality, good and clean water and more veterinary care. Adding to this, and since health is heavily affected by seasonality, special attention should be given to the elephants by their mahouts and veterinarians during the hot season when the conditions are harsh for the animals in temperature, lack of water and good-quality food. Camp relocation and supplementary food should be provided, especially to more debilitated animals. Regarding parasite level, currently the MTE deworm all animals twice per year, regardless of their infection level. My results suggest that it would be advisable to change to a more tactical deworming strategy, since only animals with the higher levels display health effects. Doing so would reduce antiparasitic drug resistance and also save money that could be essential for more expensive treatments. A detailed description of the results and their application has already been communicated to the Myanmar government and MTE officials so that measures can be applied in the field to help in the conservation effort of Asian elephants, and also to improve veterinary care and economic management of this population.

Conclusions

Health is complex to define because it is affected by interconnections and interdependencies between multiple systems and functions of an organism. Understanding the mechanisms that are responsible for changing health is a big challenge, especially in wild populations. The long-lived Asian elephant in Myanmar faces several challenges both ecologically and due to their unique management system. Consequently, determining the factors linked to compromised health and survival is needed to put in place the veterinary and management changes necessary to preserve this species. Longitudinal studies provide us with a chance to monitor and analyse the biology of an animal, creating a launching platform for a wider understanding of ecophysiology and ecology in wild species. The fact that the population studied in this thesis is semi-captive created the chance to collect repeated measures of health for known individuals over 3 years, and an opportunity to understand health and the mechanisms behind it. The findings presented in this thesis allow us to build an understanding of the ecological medicine of elephants, and to pursue not only a better understanding of these issues but also to conservation outcomes.

Appendices

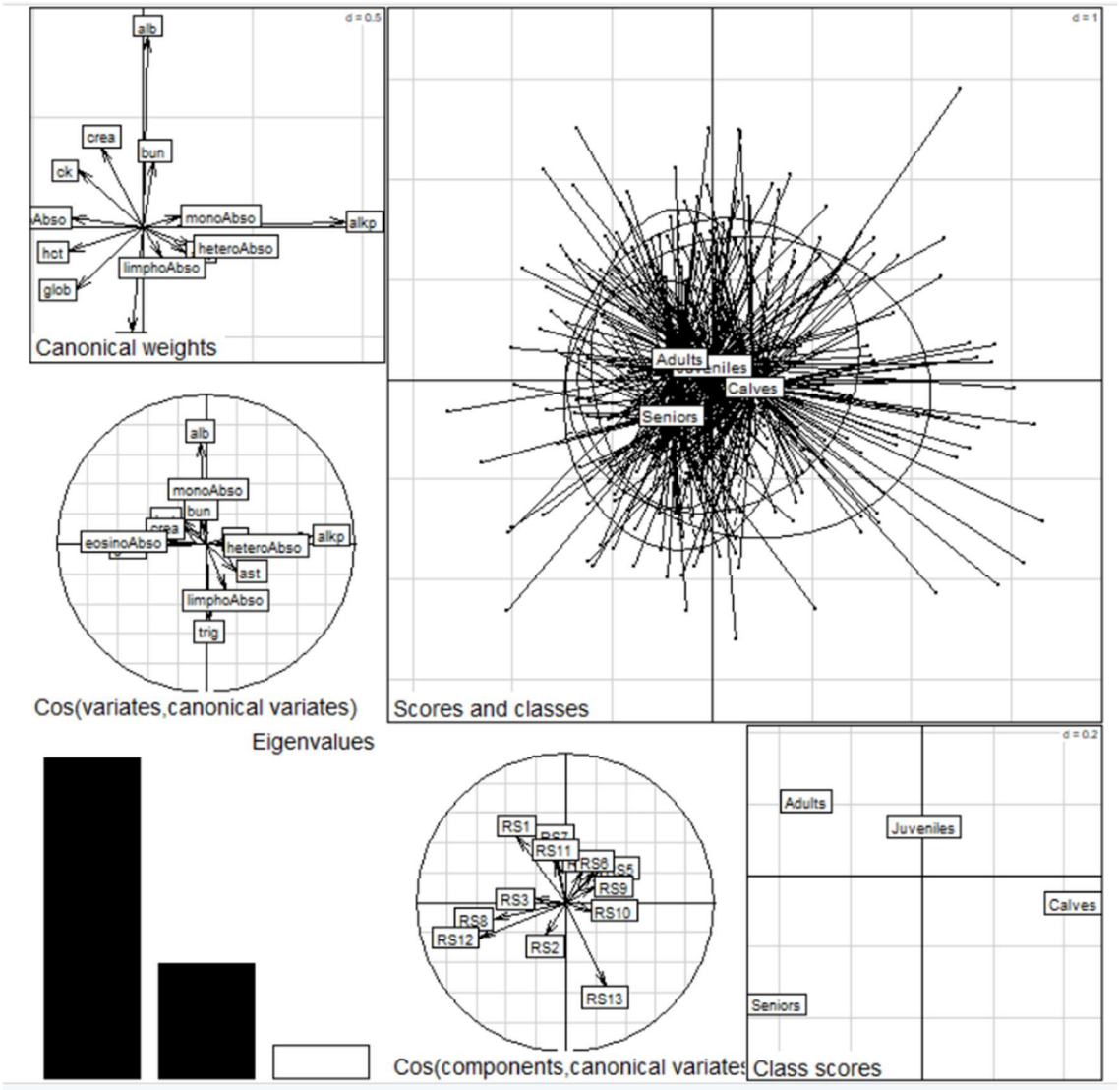


Figure 3.1. Scatterplots from the Linear Discriminant Analysis showing clustering of "calves", "juveniles", "adults", and "senior" elephants across 13 health parameters. This composed plot is divided into: 1- the health canonical weights (top left), 2- the health scores (middle left), 3- the projection of the rows with ellipses and gravity center of classes (main graph), 4- the eigenvalues bar chart (bottom left), 5- the plot of plain CA axes projected into BCA (bottom center), 6- the gravity centers of classes (bottom right).

Table 3.1. Summary of the statistical significance of age as a 4-level category, the interaction between age and sex, and each confounding variable for the models of hematocrit, hemoglobin and total white blood cells. We indicated the t-value (t). Estimates in bold correspond to the significant ones.

Model	Hematocrit	Hemoglobin	Total white blood cells
Additive age effect			
Intercept	β= 34.77±0.91, t=37.91	β= 12.02±0.33, t=36.67	β =16738.33±797.67, t=20.98
Age: juveniles	β= 0.97±0.52, t=1.87	β= 0.19±0.20, t=0.96	β= -86.89±487.49, t=-0.18
Age: adults	**β= 1.58±0.59, t=2.68**	β= 0.38±0.23, t=1.63	**β= -1619.55±599.97, t=-2.70**
Age: seniors	**β= 1.51±0.72, t=2.08**	β= 0.50±0.27, t=1.85	**β= -1869.1±784.02, t=-2.38**
Sex: males	β= -0.39±0.35, t=-1.11	β= -0.21±0.14, t=-1.47	β= -182.2±438.36, t=-0.42
Origin: wild	β= -0.53±0.53, t=-0.98	β= -0.25±0.22, t=-1.16	β= -860.64±657.31, t=-1.31
Season: hot	β= 0.30±0.27, t=1.09	β= 0.11±0.14, t=0.74	β= -163.99±273.45, t=-0.60
Season: monsoon	β= -0.46±0.28, t=-1.62	**β= -0.39±0.15, t=-2.59**	β= 524.02±285.43, t=1.84
Camp: Kawlin	**β= -1.01±0.42, t=-2.42**	β= -0.31±0.17, t=-1.85	β= -393.76±503.31, t=-0.78
Camp: West Katha	β= 0.25±0.58, t=0.44	β= 0.32±0.25, t=1.27	β= -1207.8±717.79, t=-1.68
Interaction age * sex			
Intercept	β= 35.04±0.95, t=36.93	β= 12.09±0.34, t=35.04	**β= 16721.6±864.51, t=19.34**
Age: juveniles	β= 0.29±0.67, t=0.43	β= 0.03±0.27, t=0.12	β= -91.61±705.21, t=-0.13
Age: adults	β= 1.24±0.71, t=1.75	β= 0.24±0.28, t=0.86	**β= -1565.26±755.37, t=-2.07**
Age: seniors	β= 1.40±0.81, t=1.73	β= 0.50±0.31, t=1.60	**β= -1868.35±898.43, t=-2.08**
Sex: males	β= -0.83±0.59, t=-1.40	β=- 0.34±0.25, t=-1.34	β= -153.69±725.44, t=-0.21
Origin: wild	β= -0.39±0.56, t=-0.71	β= -0.20±0.22, t=-0.88	β= -863.72±670.45, t=-1.29
Season: hot	β= 0.30±0.27, t=1.11	β= 0.11±0.14, t=0.78	β= -163.73±273.79, t=-0.60
Season: monsoon	β= -0.46±0.28, t=-1.63	**β= -0.39±0.15, t=-2.63**	β= 523.73±285.75, t=1.83
Camp: Kawlin	**β= -1.00±0.42, t=-2.38**	β= -0.31±0.17, t=-1.83	β= -392.95±506.66, t=-0.78
Camp: West Katha	β= 0.33±0.59, t=0.55	β= 0.36±0.25, t=1.42	β= -1219.93±726, t=-1.68
Age(juveniles):sex(males)	β= 1.29±0.80, t=1.63	β= 0.32±0.33, t=0.95	β= 28.62±932.22, t=0.03
Age(adults):sex(males)	β= 0.49±0.94, t=0.52	β= 0.26±0.39, t=0.68	β= -160.13±1126.46, t=-0.14
Age(seniors):sex(males)	β= -0.87±1.21, t=-0.72	β= -0.37±0.49, t=-0.76	β= 51.92±1489.91, t=0.03

Table 3.2. Summary of the statistical significance of age as a 4-level category, the interaction between age and sex, and each confounding variable for the models of total proteins, albumin, and globulin. We indicated the t-value (t). Estimates in bold correspond to the significant ones.

Model	Total proteins	Albumin	Globulin
Additive age effect			
Intercept	β= 7.61±0.16, t=48.33	β= 3.18±0.09, t=36.01	β= 4.41±0.08, t=54.90
Age: juveniles	**β= 0.15±0.06, t=2.28**	β= 0.03±0.04, t=0.99	**β= 0.13±0.05, t=2.71**
Age: adults	**β= 0.53±0.08, t=6.84**	β= 0.05±0.04,t=1.19	**β= 0.50±0.06, t=8.19**
Age: seniors	**β= 0.46±0.10, t=4.72**	β= -0.07±0.05, t=-1.54	**β= 0.54±0.08, t=6.85**
Sex: males	**β= -0.12±0.05, t=-2.31**	**β= -0.06±0.02, t=-2.81**	β= -0.05±0.04, t=-1.25
Origin: wild	β= 0.03±0.08, t=0.32	β= -0.02±0.03, t=-0.72	β= 0.07±0.07, t=1.01
Season: hot	β= -0.02±0.04, t=-0.62	**β= -0.06±0.02, t=-3.21**	β= 0.04±0.03, t=1.39
Season: monsoon	β= -0.02±0.04, t=-0.59	**β= -0.10±0.02, t=-4.99**	β= 0.06±0.03, t=1.89
Camp: Kawlin	β= -0.03±0.06, t=-0.52	**β= -0.09±0.03, t=-3.53**	β= 0.06±0.05, t=1.13
Camp: West Katha	β= -0.05±0.08, t=-0.53	β= 0.001±0.04, t=0.03	β= -0.04±0.07, t=-0.52
Interaction age * sex			
Intercept	β= 7.58±0.16, t=46.82	β= 3.18±0.09, t=35.54	β= 4.40±0.09, t=50.81
Age: juveniles	**β= 0.22±0.09, t=2.43**	β= 0.04±0.05, t=0.83	**β= 0.17±0.07, t=2.37**
Age: adults	**β= 0.51±0.1, t=5.31**	β= 0.03±0.05, t=0.68	**β= 0.47±0.08, t=6.12**
Age: seniors	**β= 0.52±0.11, t=4.65**	β= -0.07±0.05, t=-1.24	**β= 0.56±0.09, t=6.21**
Sex: males	β= -0.08±0.09, t=-0.92	β= -0.07±0.04, t=-1.84	β= -0.05±0.07, t=-0.71
Origin: wild	β= 0.03±0.08 ,t=0.39	β= -0.02±0.03, t=-0.62	β= 0.07±0.07, t=1.04
Season: hot	β= -0.02±0.04, t=-0.58	**β= -0.06±0.02, t=-3.22**	β= 0.04±0.03, t=1.42
Season: monsoon	β= -0.02±0.04, t=-0.58	**β= -0.10±0.02, t=-4.98**	β= 0.06±0.03, t=1.89
Camp: Kawlin	β= -0.03±0.06, t=-0.55	**β= -0.09±0.03, t=-3.48**	β= 0.06±0.05, t=1.10
Camp: West Katha	β= -0.04±0.09, t=-0.44	β= 0.008±0.04, t=0.19	β= -0.03±0.07, t=-0.43
Age(juveniles):sex(males)	β= -0.14±0.12, t=-1.21	β= -0.003±0.05, t=-0.06	β= -0.07±0.09, t=-0.76
Age(adults):sex(males)	β= 0.14±0.14, t=0.96	β= 0.05±0.06, t=0.88	β= 0.12±0.11, t=1.04
Age(seniors):sex(males)	β= -0.2±0.18, t=-1.09	β= -0.05±0.08, t=-0.65	β= -0.09±0.15,t=-0.58

Table 3.3. Summary of the statistical significance of age as a 4-level category, the interaction between age and sex, and each confounding variable for the models of triglycerides. We indicated the t-value (t). Estimates in bold correspond to the significant ones.

Model of triglycerides	Additive age effect	Interaction age * sex
Additive age effect		
Intercept	β= 2.62±0.15, t=17.69	β= 2.5±0.16, t=15.13
Age: juveniles	β= -0.11±0.11, t=-0.99	β= -0.03±0.16, t=-0.22
Age: adults	β= -0.03±0.14, t=-0.22	β= 0.03±0.17, t=0.16
Age: seniors	β= 0.23±0.17, t=1.41	**β= 0.43±0.19, t=2.29**
Sex: males	**β= -0.19±0.09, t=-2.07**	β= -0.04±0.16, t=-0.29
Origin: wild	β= -0.29±0.14, t=-1.98	β= -0.25±0.15, t=-1.65
Season: hot	**β= 0.32±0.10, t=3.08**	**β= 0.34±0.11, t=3.19**
Season: monsoon	**β= 0.88±0.12, t=7.41**	**β= 0.91±0.12, t=7.51**
Camp: Kawlin	**β= -0.33±0.11, t=-2.95**	**β= -0.31±0.11, t=-2.73**
Camp: West Katha	β= -0.12±0.17, t=-0.73	β= -0.1±0.17, t=-0.58
Age(juveniles):sex(males)	-	β= -0.12±0.23, t=-0.51
Age(adults):sex(males)	-	β= -0.05±0.28, t=-0.18
Age(seniors):sex(males)	-	**β= -1.06±0.32, t=-3.33**

Table 3.4. Summary of the statistical significance of age as a 4-level category, the interaction between age and sex, and each confounding variable for the models of BUN and creatinine. We indicated the t-value (t). Estimates in bold correspond to the significant ones.

Model	BUN	Creatinine
Additive age effect		
Intercept	β= 3.07±0.08, t=39.69	β= 1.06±0.09, t=11.17
Age: juveniles	β= -0.04±0.04, t=-0.89	β= 0.03±0.03, t=1.16
Age: adults	β= -0.03±0.06, t=-0.62	β= 0.05±0.03, t=1.31
Age: seniors	β= -0.07±0.07, t=-0.94	β= 0.03±0.04, t=0.59
Sex: males	β= -0.02±0.04, t=-0.61	β= 0.03±0.02, t=1.25
Origin: wild	β= 0.01±0.06, t=0.16	β= -0.06±0.04, t=-1.56
Season: hot	**β= -0.44±0.02, t=-18.92**	β= -0.01±0.02, t=-0.62
Season: monsoon	**β= 0.07±0.02, t=2.97**	β= -0.01±0.02, t=-0.43
Camp: Kawlin	**β= -0.25±0.05, t=-5.27**	**β= -0.06±0.03, t=-2.05**
Camp: West Katha	β= 0.11±0.06, t=1.79	β= -0.03±0.04, t=-0.77
Interaction age * sex		
Intercept	β= 3.10±0.08, t=37.01	β= 1.07±0.10, t=11.00
Age: juveniles	β= -0.04±0.07, t=-0.67	β= 0.04±0.04, t=1.06
Age: adults	β= -0.12±0.07, t=-1.74	β= 0.01±0.04, t=0.24
Age: seniors	β= -0.10±0.08, t=-1.08	β= 0.01±0.05, t=0.27
Sex: males	β= -0.10±0.07, t=-1.44	β= 0.008±0.04, t=0.18
Origin: wild	β= 0.01±0.06, t=0.17	β= -0.06±0.04, t=-1.70
Season: hot	**β= -0.44±0.02, t=-18.99**	β= -0.01±0.02, t=-0.57
Season: monsoon	**β= 0.07±0.02, t=2.96**	β= -0.01±0.02, t=-0.45
Camp: Kawlin	**β= -0.25±0.05, t=-5.31**	**β= -0.06±0.03, t=-2.12**
Camp: West Katha	**β= 0.13±0.06, t=2.06**	β= -0.03±0.04, t=-0.68
Age(juveniles):sex(males)	β= 0.005±0.08, t=0.06	β= -0.03±0.06, t=-0.49
Age(adults):sex(males)	**β= 0.25±0.10, t=2.51**	β= 0.12±0.07, t=1.75
Age(seniors):sex(males)	β= -0.01±0.14, t=-0.08	β= 0.04±0.08, t=0.51

Table 3.5. Summary of the statistical significance of age as a 4-level category, the interaction between age and sex, and each confounding variable for the models of AST, ALKP, and CK. We indicated the t-value (t). Estimates in bold correspond to the significant ones.

	Liver activity		Muscular activity
Model	**AST**	**ALKP**	**CK**
Additive age effect			
Intercept	β= 2.59±0.28, t=9.36	β= 4.54±0.08, t=55.64	β= 4.71±0.14, t=33.39
Age: juvenile	**β= -0.27±0.08, t=-3.47**	β= -0.02±0.03, t=-0.72	β= -0.004±0.03, t=-0.14
Age: adults	**β= -0.77±0.24, t=-3.12**	**β= -0.43±0.06, t=-7.43**	β= 0.07±0.12, t=0.57
Age: seniors	β= -0.54±0.30, t=-1.77	**β= -0.66±0.07, t=-8.73**	β= 0.05±0.13, t=0.37
Sex: males	β= 0.006±0.12, t=0.05	**β= 0.1±0.04, t=2.85**	β= 0.06±0.05, t=1.12
Origin: wild	β= 0.03±0.26, t=0.11	β= -0.01±0.06, t=-0.23	β= -0.042±0.10, t=-0.43
Season: hot	**β= 0.36±0.03, t=12.69**	**β= 0.12±0.01, t=10.1**	**β= 0.50±0.01, t=49.90**
Season: monsoon	**β= 0.62±0.03, t=22.09**	**β= -0.09±0.01, t=-7.31**	**β= -0.03±0.01, t=-2.89**
Camp: Kawlin	β= -0.04±0.16, t=-0.22	β= -0.04±0.04, t=-0.98	β= -0.14±0.08, t=-1.86
Camp: West Katha	β= -0.30±0.22 ,t=-1.39	β= -0.07±0.06, t=-1.15	β= -0.04±0.10, t=-0.38
Interaction age * sex			
Intercept	β= 3±0.19, t=15.71	β= 4.567±0.08, t=54.38	β= 4.70±0.14, t=33.68
Age: juveniles	**β= -0.5±0.11, t=-4.38**	**β= -0.12±0.04, t=-2.69**	β= 0.07±0.04, t=1.66
Age: adults	**β= -0.86±0.21, t=-4.01**	**β= -0.42±0.07, t=-6.28**	β= 0.02±0.12, t=0.14
Age: seniors	**β= -0.68±0.28, t=-2.45**	**β= -0.65±0.08, t=-7.97**	β= 0.006±0.13 ,t=0.05
Sex: males	β= -0.12±0.17, t=-0.68	β= 0.09±0.05, t=1.85	β= 0.03±0.06, t=0.42
Origin: wild	β= -0.27±0.23, t=-1.17	β= 0.004±0.06, t=0.06	β= -0.06±0.11, t=-0.56
Season: hot	**β= 0.23±0.03, t=8.59**	**β= 0.12±0.01, t=10.13**	**β= 0.50±0.01, t=49.84**
Season: monsoon	**β= 0.61±0.03 ,t=21.95**	**β= -0.09±0.01, t=-7.31**	**β= -0.03±0.01, t=-2.88**
Camp: Kawlin	β= -0.14±0.17, t=-0.84	β= -0.04±0.04, t=-1.04	β= -0.14±0.08, t=-1.77
Camp: West Katha	β= -0.37±0.24, t=-1.56	β= -0.07±0.06, t=-1.18	β= -0.02±0.10, t=-0.20
Age(juveniles):sex(males)	β= 0.06±0.14, t=0.45	**β= 0.16±0.05, t=3.08**	**β= -0.12±0.05, t=-2.28**
Age(adults):sex(males)	β= 0.14±0.32, t=0.44	β= -0.09±0.09, t=-1.05	β= 0.24±0.14, t=1.75
Age(seniors):sex(males)	β= 0.51±0.47, t=1.08	β= -0.13±0.12, t=-1.04	β= 0.17±0.20, t=0.82

Table 3.6. Summary of the statistical significance of age as a 4-level category and each confounding variable for the models of differential white blood cells. We indicated the t-value (t). Estimates in bold correspond to the significant ones.

Model	Lymphocytes	Heterophils	Monocytes
Intercept	β= 2.59±0.13, t=20.61	β= 2.31±0.13, t=18.18	β= 2.79±0.12, t=22.52
Age: juveniles	**β= 0.20±0.09, t=2.11**	**β= 0.25±0.09, t=2.70**	**β= 0.19±0.09, t=2.08**
Age: adults	β= 0.12±0.11, t=1.05	β= 0.21±0.11, t=1.81	β= 0.13±0.11, t=1.18
Age: seniors	β= 0.11±0.14, t=0.80	β= 0.18±0.14, t=1.33	β= 0.10±0.13, t=0.73
Sex: males	β= 0.09±0.07, t=1.19	β= 0.14±0.08, t=1.86	β= 0.05±0.07, t=0.61
Origin: wild	β= -0.06±0.12, t=-0.52	β= 0.01±0.12, t=0.12	β= -0.06±0.11, t=-0.49
Season: hot	**β= 0.27±0.10, t=2.78**	β=0.06±0.10, t=0.64	β= -0.14±0.10, t=-1.39
Season: monsoon	**β= 0.22±0.10, t=2.27**	β= 0.12±0.09, t=1.28	β= -0.03±0.09, t=-0.35
Camp: Kawlin	β= -0.10±0.09, t=-1.15	β= 0.11±0.09, t=1.24	β= 0.01±0.09, t=0.08
Camp: West Katha	β= 0.13±0.14, t=0.92	β= 0.08±0.14, t=0.58	β= 0.13±0.14, t=0.93
Year 2017	**β= -0.26±0.08, t=-3.18**	β= -0.07±0.08, t=-0.86	β= 0.11±0.08, t=1.30
Year 2018	**β= -0.64±0.12, t=-5.30**	**β= -0.52±0.12, t=-4.39**	β= -0.02±0.12, t=-0.13

Model	Eosinophils	Basophiles
Intercept	β= -0.10±0.14 t=-0.72	β= -1.23±0.15, t=-8.25
Age: juveniles	**β= 0.77±0.11, t=7.34**	β= 0.10±0.11, t=0.84
Age: adults	**β= 0.91±0.13, t=7.30**	β= -0.02±0.14, t=-0.17
Age: seniors	**β= 0.90±0.16, t=5.74**	β= -0.02±0.16, t=-0.14
Sex: males	β= 0.02±0.08, t=0.19	β= 0.01±0.09, t=0.11
Origin: wild	β= -0.10±0.13, t=-0.75	β= -0.03±0.14, t=-0.19
Season: hot	β= -0.11±0.10, t=-1.08	β= -0.15±0.11, t=-1.35
Season: monsoon	β= 0.24±0.10, t=2.33	β= -0.08±0.11, t=-0.69
Camp: Kawlin	β= 0.06±0.10, t=0.56	β= 0.09±0.11, t=0.89
Camp: West Katha	β= -0.03±0.15, t=-0.20	β= 0.13±0.16, t=0.79
Year 2017	**β= 0.26±0.09, t=2.96**	β= 0.03±0.10, t=0.37
Year 2018	**β= -0.22±0.13, t=-1.67**	**β= 0.30±0.14, t=2.11**

Table 4.1 – Summary of NA's, percentage of outliers removed and sample size for each health parameter

Health parameter	NA	Outliers removed (% total)	Total without outliers
Hematocrit	13	2.5	765
Hemoglobin	187	1.3	603
Total white blood cell count	14	1.8	749
Heterophils	129	1.3	660
Lymphocytes	129	1.0	662
Eosinophils	129	0.3	667
Monocytes	129	0.1	668
Basophils	129	0	669
Systolic Pressure	544	3.9	244
Diastolic Pressure	544	4.7	242
Calcium	8	2.9	767
BUN	8	0.8	784
Creatinine	8	3.7	761
CK	9	3.5	761
Albumin	10	5.3	746
Globulins	10	2.2	771
AST	8	1.0	782
ALKP	8	1.4	779
Triglycerides	8	0	782
Total Proteins	17	2.2	772
Sodium	187	3.6	589
Potassium	187	1.6	601
Chloride	188	0.7	606

Table 4.2 – Summary of individual health parameters model selection and distributions

Health parameter	Distribution	model	AIC	ΔAIC	AIC weights
Total white blood cells	Gamma	Season	14079.37	0.000	0.623
		Season*Sex	14080.51	1.143	0.352
		Base	14085.75	6.383	0.026
Hematocrit	Gaussian	Season * Sex	3806.503	0.000	0.736
		Season	3808.554	2.051	0.264
		Base	3842.713	36.211	<0.001
Hemoglobin	Gaussian	Season	1810.522	0.000	0.778
		Season * Sex	1813.026	2.504	0.222
		Base	1851.997	41.475	<0.001
Systolic Pressure	Gaussian	Season * Sex	2002.262	0.000	0.952
		Season	2008.263	6.001	0.047
		Base	2017.461	15.200	<0.001
Diastolic Pressure	Gaussian	Season * Sex	1975.204	0.000	0.980
		Season	1983.401	8.197	0.016
		Base	1986.520	11.316	0.003
Total Protein	Gaussian	Base	1009.757	0.000	0.998
		Season	1021.901	12.144	<0.05
		Season * Sex	1032.009	22.253	<0.001
Albumin	Gaussian	Base	26.544	0.000	1
		Season	42.049	15.505	<0.001
		Season * Sex	52.058	25.514	<0.001
Globulins	Gaussian	Base	479.614	0.000	0.999
		Season	492.851	13.237	<0.05
		Season * Sex	505.099	25.485	<0.001
BUN	Gaussian	Season * Sex	4316.746	0.000	0.987
		Season	4325.478	8.731	0.013
		Base	4783.781	467.035	<0.001
Creatinine	Gaussian	Season	-134.158	0.000	0.655
		Base	-132.861	1.296	0.342
		Season * Sex	-123.395	10.763	<0.05
Triglycerides	Gamma	Season	5780.397	0.000	0.514
		Season * Sex	5780.509	0.112	0.486
		Base	5.834.073	53.676	<0.001

Table 4.2 (continuation) – Summary of individual health parameters model selection and distributions

Health parameter	Distribution	model	AIC	ΔAIC	AIC weights
AST	Poisson	Season * Sex	11933.80	0.000	0.965
		Season	11940.41	6.607	0.035
		Base	12221.00	287.202	<0.001
ALKP	Poisson	Season * Sex	10142.99	0.000	1
		Season	10182.65	39.662	<0.001
		Base	10398.70	255.717	<0.001
CK	Poisson	Season * Sex	25767.59	0.000	1
		Season	25816.74	49.142	<0.001
		Base	28895.48	3127.884	<0.001
Potassium	Gamma	Season	596.126	0.000	0.591
		Season * Sex	596.861	0.735	0.409
		Base	698.868	102.742	<0.001
Sodium	Gaussian	Season	2667.559	0.000	0.834
		Season * Sex	2670.793	3.234	0.166
		Base	2693.975	26.416	<0.001
Chloride	Gaussian	Base	2771.599	0.000	0.810
		Season	2774.811	3.211	0.163
		Season * Sex	2778.404	6.805	0.027
Calcium	Gamma	Season	1208.970	0.000	0.774
		Season * Sex	1211.444	2.474	0.225
		Base	1222.762	13.793	<0.001
Differential White Blood Cells	Dirichlet	Season	-12830.25	0.000	0.977
		Season * Sex	-12822.76	7.485	0.023
		Base	-12788.65	41.600	<0.001

Table 4.3 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of total white blood cells. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	Total White Blood Cells
Additive Effect Season	
Intercept	β= 9.711±0.046, t=209.688
Age	β= -0.002±0.001, t=-1.811
Season: Hot	**β= -0.030±0.015, t=-2.003**
Season: Monsoon	β= 0.021±0.016, t=1.335
Place: Kawlin	β= 0.025±0.037, t=0.671
Place: West Katha	β= -0.011±0.056, t=-0.192
Sex: Male	β= -0.011±0.035, t=-0.327
Origin: Wild	β= -0.067±0.055, t=-1.219
Interaction Season*Sex	
Intercept	β= 9.723±0.047, t=207.937
Age	β= -0.002±0.001, t=-1.829
Season: Hot	**β= -0.049±0.019, t=-2.591**
Season: Monsoon	β= 0.008±0.020, t=0.404
Sex: Male	β= -0.041±0.039, t=-1.046
Place: Kawlin	β= 0.024±0.038, t=0.633
Place: West Katha	β= -0.009±0.056, t=-0.163
Origin: Wild	β= -0.064±0.055, t=-1.168
Season: Hot* Sex: M	β= 0.051±0.031, t=1.651
Season: Monsoon* Sex: M	β= 0.035±0.032, t=1.087

Table 4.4 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of differential white blood cells. We indicated the z-value (z). Estimates in bold correspond to the significant ones

Model	Lymphocytes	Heterophils	Monocytes	Eosinophils	Basophils
Additive Effect Season					
Intercept	β=2.414±0.099, z=24.346	β=2.173±0.097, z=22.391	β=2.636±0.094, z=27.903	β=0.040±0.104, z=0.381	β=-1.354±0.118, z=-11.438
Age	β=0.002±0.002, z=0.856	**β=0.003±0.002, z=2.065**	β=0.003±0.002, z=1.551	**β=0.017±0.002, z=6.889**	β=0.000±0.003, z=0.020
Season: Hot	β=-0.089±0.075, z=-1.197	**β=-0.203±0.074, z=-2.726**	**β=-0.254±0.074, z=-3.431**	**β=-0.172±0.082, z=-2.086**	β=-0.008±0.088, z=-0.091
Season: Monsoon	**β=0.173±0.085, z=2.048**	β=0.124±0.084, z=1.464	β=0.004±0.084, z=0.059	**β=0.324±0.092, z=3.542**	β=-0.007±0.101, z=-0.074
Place: Kawlin	β=-0.131±0.078, z=-1.676	β=0.064±0.078, z=0.817	β=-0.014±0.078, z=-0.174	β=-0.126±0.086, z=-1.476	β=0.065±0.093, z=0.694
Place: West Katha	β=-0.061±0.115, z=-0.529	β=-0.103±0.116, z=0.817	β=-0.004±0.114, z=-0.034	β=-0.249±0.127, z=-1.953	β=0.078±0.138, z=0.569
Sex: Male	**β=0.151±0.069, z=2.197**	**β=0.200±0.070, z=2.864**	β=0.151±0.069, z=2.202	β=0.070±0.076, z=0.915	β=0.055±0.083, z=0.669
Origin: Wild	β=-0.020±0.109, z=-0.183	β=-0.023±0.111, z=-0.209	β=-0.005±0.107, z=-0.042	β=-0.128±0.127, z=-1.009	β=-0.038±0.133, z=-0.289
Interaction Season*Sex					
Intercept	β=2.388±0.105, z=22.816	β=2.154±0.102, z=21.044	β=2.589±0.101, z=25.737	β=0.010±0.109, z=0.092	β=-1.395±0.125, z=-11.173
Age	β=0.002±0.002, z=1.031	**β=0.005±0.002, z=2.244**	β=0.004±0.002, z=1.755	**β=0.018±0.002, z=7.119**	β=0.000±0.003, z=0.061
Season: Hot	β=-0.045±0.094, z=-0.476	β=-0.145±0.094, z=-1.550	β=-0.172±0.093, z=-1.843	β=-0.061±0.102, z=-0.601	β=0.039±0.112, z=0.347
Season: Monsoon	**β=0.217±0.106, z=2.047**	β=0.128±0.106, z=1.205	β=0.080±0.105, z=0.764	**β=0.321±0.115, z=2.792**	β=-0.084±0.127, z=0.666
Sex: Male	**β=0.242±0.106, z=2.271**	**β=0.275±0.107, z=2.571**	**β=0.293±0.106, z=2.758**	β=0.184±0.118, z=1.551	β=0.166±0.129, z=1.287
Place: Kawlin	β=-0.144±0.079, z=-1.832	β=0.048±0.078, z=0.613	β=-0.026±0.078, z=-0.330	β=-0.151±0.086, z=-1.750	β=0.066±0.093, z=0.709
Place: West Katha	β=-0.070±0.115, z=-0.607	β=-0.119±0.116, z=-1.022	β=-0.016±0.115, z=-0.139	**β=-0.281±0.128, z=-2.191**	β=0.076±0.138, z=0.552
Origin: Wild	β=-0.031±0.109, z=-0.287	β=-0.032±0.111, z=-0.292	β=-0.020±0.107, z=-0.183	β=-0.139±0.127, z=-1.094	β=-0.044±0.133, z=-0.334
Season: Hot* Sex: M	β=-0.124±0.150, z=-0.829	β=-0.152±0.150, z=-1.009	β=-0.215±0.149, z=-1.442	β=-0.295±0.167, z=-1.768	β=-0.130±0.181, z=-0.715
Season: Monsoon* Sex: M	β=-0.114±0.171, z=-0.666	β=-0.014±0.171, z=-0.080	β=-0.191±0.167, z=-1.123	β=0.019±0.186, z=-0.101	β=-0.243±0.208, z=-1.173

Table 4.5 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of haematology. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	Haematocrit	Haemoglobin
Additive Effect Season		
Intercept	β=34.634±0.444, t=78.001	β=11.836±0.171, t=69.107
Season: Hot	**β=0.899±0.228, t=3.936**	**β=0.414±0.101, t=4.092**
Season: Monsoon	**β=-0.618±0.232, t=-2.661**	**β=-0.282±0.094, t=-3.003**
Age	**β=0.032±0.011, t=2.817**	**β=0.009±0.004, t=2.171**
Place: Kawlin	**β=-1.147±0.364, t=-3.151**	**β=-0.348±0.137, t=-2.547**
Place: West Katha	β=-0.036±0.523, t=-0.069	β=0.066±0.199, t=0.330
Sex: Male	β=-0.429±0.334, t=-1.285	β=-0.173±0.126, t=-1.378
Origin: Wild	β=-0.922±0.526, t=-1.753	β=-0.356±0.198, t=-1.793
Interaction Season*Sex		
Intercept	β=34.846±0.452, t=77.014	β=11.924±0.176, t=67.687
Season: Hot	β=0.488±0.287, t=1.702	β=0.255±0.126, t=20.25
Season: Monsoon	**β=-0.829±0.289, t=-2.872**	**β=-0.376±0.116, t=-3.235**
Sex: Male	**β=-1.021±0.432, t=-2.363**	**β=-0.423±0.178, t=-2.374**
Age	**β=0.032±0.011, t=2.815**	**β=0.009±0.004, t=2.181**
Place: Kawlin	**β=-1.162±0.362, t=-3.209**	**β=-0.360±0.136, t=-2.649**
Place: West Katha	β=0.009±0.521, t=0.018	β=0.073±0.197, t=0.372
Origin: Wild	β=-0.897±0.523, t=-1.715	β=-0.344±0.197, t=-1.743
Season: Hot* Sex: M	**β=1.121±0.475, t=2.360**	**β=0.445±0.211, t=2.110**
Season: Monsoon* Sex: M	β=0.595±0.486, t=-1.225	β=0.273±0.197, t=1.384

Table 4.6 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of blood pressure. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	Systolic Pressure	Diastolic Pressure
Additive Effect Season		
Intercept	β=136.975±3.397, t=40.327	β=94.382±3.059, t=30.856
Season: Hot	**β=-4.535±2.207, t=-2.055**	β=0.559±2.069, t=0.270
Season: Monsoon	β=-5.390±2.728, t=-1.976	β=1.077±2.626, t=0.410
Age	β=0.011±0.087, t=0.125	β=0.063±0.080, t=0.784
Place: Kawlin	β=-2.226±2.933, t=-0.759	β=-2.012±2.636, t=-0.763
Place: West Katha	β=-3.296±4.195, t=-0.786	β=0.804±3.858, t=0.208
Sex: Male	β=0.109±2.348, t=0.047	β=2.262±2.060, t=1.098
Origin: Wild	β=5.601±4.128, t=1.357	β=7.189±3.792, t=1.896
Interaction Season*Sex		
Intercept	β=136.867±3.560, t=38.446	β=95.366±3.175, t=30.038
Season: Hot	β=-4.184±3.001, t=-1.394	β=-2.383±2.799, t=-0.851
Season: Monsoon	β=-5.395±3.609, t=-1.495	β=0.072±3.541, t=0.020
Sex: Male	β=0.382±3.157, t=0.121	β=-0.533±2.865, t=-0.186
Age	β=0.011±0.087, t=0.127	β=0.062±0.079, t=0.788
Place: Kawlin	β=-2.232±2.950, t=-0.757	β=-1.837±2.616, t=-0.702
Place: West Katha	β=-3.268±4.217, t=-0.775	β=0.566±3.854, t=0.147
Origin: Wild	β=5.564±4.157, t=1.338	**β=7.664±3.768, t=2.034**
Season: Hot* Sex: M	β=-0.764±4.465, t=-0.171	β=6.600±4.175, t=1.581
Season: Monsoon* Sex: M	β=0.016±5.553, t=-0.003	β=2.374±5.298, t=0.448

Table 4.7 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of protein levels. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	Total Protein	Albumin	Globulins
Additive Effect Season			
Intercept	β=7.596±0.071, t=106.548	β=3.156±0.034, t=93.297	β=4.406±0.055, t=80.177
Season: Hot	β=0.047±0.036, t=1.314	β=-0.001±0.020, t=-0.050	β=0.035±0.025, t=1.433
Season: Monsoon	β=0.025±0.036, t=0.701	β=-0.016±0.020, t=-0.760	β=0.011±0.025, t=0.426
Age	**β=0.012±0.0.002, t=6.564**	β=-0.001±0.001, t=-0.873	**β=0.014±0.001, t=9.648**
Place: Kawlin	**β=-0.133±0.058, t=-2.286**	**β=-0.099±0.028, t=-3.590**	β=-0.033±0.045, t=-0.732
Place: West Katha	β=-0.003±0.084, t=-0.031	β=0.006±0.040, t=0.149	β=0.017±0.065, t=0.259
Sex: Male	β=-0.097±0.053, t=-1.822	β=-0.040±0.025, t=-1.583	β=-0.034±0.041, t=-0.815
Origin: Wild	β=-0.100±0.085, t=-1.178	β=-0.034±0.040, z=-0.852	β=-0.076±0.066, t=-1.156
Interaction Season*Sex			
Intercept	β=7.607±0.073, t=104.172	β=3.167±0.035, t=90.920	β=4.410±0.056, t=78.617
Season: Hot	β=0.022±0.045, t=0.489	β=-0.028±0.025, t=-1.111	β=0.032±0.031, t=1.010
Season: Monsoon	β=0.020±0.046, t=0.436	β=-0.022±0.026, t=-0.844	β=0.002±0.031, t=0.067
Sex: Male	β=-0.126±0.069, t=-1.841	**β=-0.071±0.035, t=-2.045**	β=-0.044±0.051, t=-0.875
Age	**β=0.012±0.002, t=6.551**	β=-0.001±0.001, t=-0.889	**β=0.014±0.001, t=9.634**
Place: Kawlin	**β=-0.134±0.058, t=-2.299**	**β=-0.099±0.027, t=-3.607**	β=-0.033±0.045, t=-0.731
Place: West Katha	β=0.006±0.084, t=0.071	β=0.010±0.040, t=0.252	β=0.017±0.065, t=0.257
Origin: Wild	β=-0.098±0.085, t=-1.158	β=-0.032±0.039, t=-0.814	β=-0.075±0.066, t=-1.140
Season: Hot* Sex: M	β=0.065±0.074, t=0.880	β=0.071±0.041, t=1.731	β=0.010±0.051, t=0.198
Season: Monsoon* Sex: M	β=0.014±0.076, t=0.190	β=0.015±0.042, t=0.362	β=0.023±0.052, t=0.450

Table 4.8 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of kidney function. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	BUN	Creatinine
Additive Effect Season		
Intercept	β=19.129±0.571, t=33.503	β=1.142±0.032, t=35.956
Season: Hot	β=-4.985±0.298, t=-16.709	**β=-0.062±0.017, t=-3.629**
Season: Monsoon	**β=2.878±0.303, t=9.484**	β=0.006±0.018, t=0.318
Age	β=-0.013±0.015, t=-0.895	β=-0.000±0.001, t=-0.092
Place: Kawlin	**β=-3.888±0.466, t=-8.350**	**β=-0.088±0.026, t=-3.382**
Place: West Katha	**β=2.154±0.679, t=3.174**	**β=-0.087±0.037, t=-2.332**
Sex: Male	β=0.715±0.426, t=1.680	**β=0.054±0.024, t=2.263**
Origin: Wild	β=-0.150±0.675, t=-0.222	β=-0.042±0.037, t=-1.128
Interaction Season*Sex		
Intercept	β=18.718±0.584, t=32.037	β=1.136±0.033, t=34.854
Season: Hot	β=-4.230±0.374, t=-11.301	β=-0.043±0.022, t=-1.969
Season: Monsoon	**β=3.356±0.377, t=8.911**	β=0.003±0.022, t=0.123
Sex: Male	**β=1.877±0.558, t=3.365**	**β=0.070±0.032, t=2.225**
Age	β=-0.013±0.015, t=-0.866	β=-0.000±0.001, t=-0.090
Place: Kawlin	**β=-3.880±0.465, t=-8.347**	**β=-0.087±0.026, t=-3.364**
Place: West Katha	**β=2.061±0.678, t=3.039**	**β=-0.090±0.037, t=-2.410**
Origin: Wild	β=-0.206±0.675, t=-0.306	β=-0.042±0.037, t=-1.133
Season: Hot* Sex: M	**β=-2.036±0.615, t=-3.313**	β=-0.051±0.035, t=-1.436
Season: Monsoon* Sex: M	**β=-1.316±0.629, t=-2.094**	β=0.008±0.036, t=0.216

Table 4.9 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of enzyme activity. We indicated the z-value (z). Estimates in bold correspond to the significant ones

Model	AST	ALKP	CK
Additive Effect Season			
Intercept	β=3.320±0.141, z=23.540	β=4.724±0.046, z=102.702	β=4.913±0.064, z=76.885
Season: Hot	**β=-0.061±0.023, z=-2.661**	**β=0.082±0.010, z=8.465**	**β=0.313±0.008, z=40.019**
Season: Monsoon	**β=0.340±0.021, z=15.930**	**β=-0.069±0.010, z=-6.930**	**β=-0.129±0.008, z=-15.309**
Age	**β=-0.025±0.004, z=-6.434**	**β=-0.017±0.001, z=-13.448**	β=-0.002±0.002, z=-0.974
Place: Kawlin	β=-0.109±0.121, z=-0.896	β=0.045±0.040, z=1.133	β=-0.074±0.057, z=-1.297
Place: West Katha	β=-0.069±0.179, z=-0.384	β=-0.002±0.059, z=--0.036	β=-0.055±0.091, z=--0.606
Sex: Male	β=-0.135±0.097, z=-1.395	**β=0.114±0.033, z=3.397**	β=0.083±0.043, z=1.948
Origin: Wild	β=0.162±0.181, z=0.895	β=0.107±0.058, z=1.843	β=0.058±0.085, z=0.680
Interaction Season*Sex			
Intercept	β=3.352±0.142, z=23.632	β=4.739±0.046, z=102.908	β=4.915±0.064, z=76.724
Season: Hot	β=0.003±0.029, z=0.107	**β=0.038±0.013, z=2.953**	**β=0.296±0.010, z=29.775**
Season: Monsoon	**β=0.311±0.026, z=11.776**	**β=-0.058±0.013, z=-4.588**	**β=-0.102±0.010, z=--9.868**
Sex: Male	**β=-0.211±0.101, z=-2.099**	**β=0.082±0.035, z=2.332**	**β=0.090±0.044, z=2.060**
Age	**β=-0.025±0.004, z=-6.448**	**β=-0.017±0.001, z=-13.524**	β=-0.002±0.002, z=-1.015
Place: Kawlin	β=-0.116±0.122, z=-0.955	β=0.042±0.039, z=1.066	β=-0.078±0.057, z=-1.366
Place: West Katha	β=-0.067±0.180, z=-0.371	β=0.005±0.058, z=0.080	β=-0.052±0.085, z=-0.568
Origin: Wild	β=0.172±0.182, z=0.945	β=0.109±0.058, z=1.880	β=0.057±0.085, z=0.674
Season: Hot* Sex: M	**β=0.154±0.047, z=3.244**	**β=0.100±0.020, z=5.102**	**β=0.042±0.016, z=2.600**
Season: Monsoon* Sex: M	β=0.082±0.045, z=1.833	β=-0.028±0.020, z=-1.373	**β=-0.087±0.018, z=-4.835**

Table 4.10 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of fat storage. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	Triglycerides
Additive Effect Season	
Intercept	β=2.434±0.115, t=21.239
Season: Hot	**β=0.252±0.088, t=2.867**
Season: Monsoon	**β=0.735±0.091, t=8.041**
Age	β=-0.004±0.003, t=1.482
Place: Kawlin	β=-0.124±0.090, t=-1.374
Place: West Katha	β=-0.021±0.130, t=-0.162
Sex: Male	**β=-0.248±0.079, t=-3.127**
Origin: Wild	β=-0.080±0.127, t=-0.633
Interaction Season*Sex	
Intercept	β=2.466±0.120, t=20.495
Season: Hot	β=0.138±0.111, t=1.251
Season: Monsoon	**β=0.759±0.114, t=6.663**
Sex: Male	**β=-0.335±0.133, t=-2.526**
Age	β=0.004±0.003, t=1.513
Place: Kawlin	β=-0.132±0.090, t=-1.468
Place: West Katha	β=0.000±0.130, t=0.001
Origin: Wild	β=-0.086±0.127, t=-0.681
Season: Hot* Sex: M	β=0.294±0.181, t=1.625
Season: Monsoon* Sex: M	β=-0.073±0.189, t=-0.383

Table 4.11 - Summary of the statistical significance of season as a 3-level category and each confounding variable for the models of electrolytes. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	Potassium	Sodium	Chloride	Calcium
Additive Effect Season				
Intercept	β=1.593±0.016, t=100.963	β=129.683±0.343, t=377.572	β=91.587±0.372, t=246.243	β=2.303±0.010, t=240.347
Season: Hot	**β=-0.077±0.009, t=-8.871**	β=0.068±0.230, t=0.296	β=0.203±0.227, t=0.898	**β=0.010±0.004, t=2.285**
Season: Monsoon	**β=-0.079±0.008, t=-9.834**	**β=-1.025±0.217, t=-4.727**	β=0.395±0.210, t=1.877	**β=-0.009±0.005, t=-2.078**
Age	**β=0.001±0.000, t=2.328**	β=-0.009±0.008, t=-1.152	β=-0.005±0.009, t=-0.508	β=-0.000±0.000, t=-1.218
Place: Kawlin	β=-0.012±0.013, t=-0.980	β=-0.307±0.270, t=-1.136	β=-0.914±0.297, t=-3.082	β=0.009±0.007, t=1.151
Place: West Katha	**β=-0.040±0.019, t=-2.148**	β=0.094±0.386, t=0.244	β=-0.145±0.432, t=-0.335	β=0.007±0.011, t=0.576
Sex: Male	β=-0.013±0.012, t=-1.082	β=-0.331±0.245, t=-1.351	β=-0.277±0.273, t=-1.014	β=0.001±0.007, t=0.155
Origin: Wild	**β=-0.044±0.019, t=-2.350**	β=-0.056±0.384, t=-0.145	β=0.019±0.429, t=0.044	β=-0.015±0.011, t=-1.329
Interaction Season*Sex				
Intercept	β=1.599±0.016, t=98.609	β=129.621±0.361, t=359.410	β=91.545±0.386, t=237.138	β=2.303±0.010, t=235.328
Season: Hot	**β=-0.088±0.011, t=-8.239**	β=0.189±0.288, t=0.656	β=0.281±0.284, t=0.991	β=0.008±0.006, t=1.504
Season: Monsoon	**β=-0.083±0.010, t=-8.409**	**β=-0.973±0.269, t=-3.616**	β=0.433±0.262, t=1.653	β=-0.007±0.006, t=-1.209
Sex: Male	β=-0.028±0.016, t=-1.791	β=-0.164±0.383, t=-0.427	β=-0.167±0.394, t=-0.424	β=0.002±0.009, t=0.178
Age	**β=0.001±0.000, t=2.319**	β=-0.009±0.008, t=-1.149	β=-0.005±0.009, t=-0.506	β=-0.000±0.000, t=-1.211
Place: Kawlin	β=-0.013±0.012, t=-1.055	β=-0.297±0.271, t=-1.095	**β=-0.907±0.297, t=-3.052**	β=0.009±0.008, t=1.141
Place: West Katha	**β=-0.040±0.019, t=-2.114**	β=0.089±0.387, t=0.229	β=-0.147±0.432, t=-0.341	β=0.007±0.012, t=0.610
Origin: Wild	**β=-0.043±0.018, t=-2.303**	β=-0.060±0.384, t=-0.155	β=0.015±0.429, t=0.034	β=-0.015±0.011, t=-1.343
Season: Hot* Sex: M	β=0.032±0.018, t=1.801	β=-0.335±0.481, t=-0.697	β=-0.214±0.473, t=-0.453	β=0.004±0.009, t=0.473
Season: Monsoon* Sex: M	β=0.014±0.017, t=0.835	β=-0.151±0.456, t=-0.330	β=-0.108±0.442, t=-0.244	β=-0.007±0.009, t=-0.784

Table 5.1 - Summary of NA's, percentage of outliers removed and sample size for each health parameter

Health Parameter	NA	Outliers removed (% total)	Total without outliers
Hematocrit	8	1.5	711
Hemoglobin	180	1.4	557
Total white blood cell count	26	2.0	697
Heterophils	111	1.4	617
Lymphocytes	111	0.3	624
Eosinophils	111	0.2	625
Monocytes	111	0.2	625
Basophils	111	0	626
Calcium	4	2.9	712
BUN	4	0.8	728
Creatinine	4	3.8	705
CK	5	3.7	705
Albumin	6	3.0	709
Globulins	6	2.2	715
AST	4	1.0	726
ALKP	4	1.5	722
Triglycerides	4	0	733
Total Proteins	5	2.3	715
Sodium	180	3.8	536
Potassium	180	1.3	550
Chloride	181	0.7	552

Table 5.2 – Summary of individual health parameters model selection, distributions and effect size

Health parameter	Distribution	model	AIC	ΔAIC	AIC weights	Effect Size	Observations/ ID
		Parasite + Deworming	11929.50	0.000	0.979		636/194
		Parasite + Season	11937.19	7.689	<0.05		
		Base Deworming	12495.50	565.998	<0.001		
Total White Blood Cells	Gamma	Base Season	12501.35	571.848	<0.001		
		Parasite + Season	-11431.92	0.000	1		587/193
		Base Season	-11418.38	13.544	<0.05		
White Blood Cells	Dirichlet	Base Deworming	-11403.33	28.593	<0.001		
Differential	Distribution	Parasite + Deworming	-11403.33	28.593	<0.001		
		Parasite + Season	1500.569	0.000	1	0.261	493/181
		Parasite + Deworming	1522.139	21.570	<0.001		
		Base Season	1564.901	64.332	<0.001		
Hemoglobin	Gaussian	Base Deworming	1598.894	98.325	<0.001		
		Parasite + Season	3263.655	0.000	1	0.071	653/197
		Parasite + Deworming	3275.863	12.208	<0.05		
		Base Season	3393.494	129.839	<0.001		
Hematocrit	Gaussian	Base Deworming	3417.538	153.883	<0.001		
		Parasite + Season	593.133	0.000	1	0.518	493/181
		Base Season	524.894	31.761	<0.001		
		Parasite + Deworming	673.914	80.781	<0.001		
Potassium	Gaussian	Base Deworming	704.642	111.509	<0.001		
		Parasite + Season	2169.864	0.000	1	0.107	478/176
		Parasite + Deworming	2187.453	17.589	<0.001		
		Base Season	2286.514	116.650	<0.001		
Sodium	Gaussian	Base Deworming	2310.971	141.107	<0.001		

Table 5.2 (continuation) – Summary of individual health parameters model selection, distributions and effect size

Health parameter	Distribution	model	AIC	ΔAIC	AIC weights	Effect Size	Observations/ ID
		Parasite + Deworming	2264.588	0.000	0.837	0.038	495/176
		Parasite + Season	2267.867	3.279	0.163		
		Base Season	2388.534	123.946	<0.001		
Chloride	Gaussian	Base Deworming	2389.636	125.047	<0.001		
		Parasite + Deworming	1147.642	0.000	0.930	0.654	652/194
		Parasite + Season	1152.829	5.187	0.070		
		Base Deworming	1185.442	37.800	<0.001		
Calcium	Gaussian	Base Season	1189.479	41.837	<0.001		
		Base Deworming	62.252	0.000	0.990		677/195
		Base Season	72.364	10.113	<0.05		
		Parasite + Deworming	73.224	10.973	<0.05		
Albumin	Gaussian	Parasite + Season	87.185	24.933	<0.001		
		Parasite + Deworming	437.506	0.000	0.790	0.974	656/198
		Base Deworming	440.299	2.794	0.195		
		Base Season	446.873	9.367	<0.05		
Globulins	Gaussian	Parasite + Season	446.951	9.444	<0.05		
		Parasite + Season	3684.463	0.000	1	0.612	674/194
		Base Season	3828.463	143.607	<0.001		
		Parasite + Deworming	4118.866	434.402	<0.001		
Blood Urea Nitrogen	Gaussian	Base Deworming	4253.736	569.273	<0.001		
		Base Deworming	-146.542	0.000	1		676/195
		Parasite + Deworming	-127.131	19.411	<0.001		
		Base Season	-125.622	20.920	<0.001		
Creatinine	Gaussian	Parasite + Season	-100.292	46.250	<0.001		

Table 5.2 (continuation) – Summary of individual health parameters model selection, distributions and effect size

Health parameter	Distribution	model	AIC	ΔAIC	AIC weights	Effect Size	Observations/ ID
Total proteins	Gaussian	Parasite + Deworming	880.681	0.000	0.999	1.155	655/195
		Parasite + Season	894.279	13.598	<0.05		
		Base Deworming	911.946	31.265	<0.001		
		Base Season	921.776	41.095	<0.001		
Aspartate Aminotransferase	Poisson	Parasite + Season	10216.90	0.000	1		668/194
		Parasite + Deworming	10410.85	193.950	<0.001		
		Base Season	10613.30	396.398	<0.001		
		Base Deworming	10819.03	602.127	<0.001		
Alkaline phosphatase	Poisson	Parasite + Season	8596.613	0.000	1		666/200
		Parasite + Deworming	8794.595	197.982	<0.001		
		Base Season	9028.844	432.231	<0.001		
		Base Deworming	9210.926	614.313	<0.001		
Creatinine kinase	Poisson	Parasite + Season	21619.53	0.000	1		649/191
		Base Season	23208.17	1588.639	<0.001		
		Parasite + Deworming	24858.40	3238.875	<0.001		
		Base Deworming	26253.19	4633.666	<0.001		
Triglycerides	Gamma	Parasite + Season	4524.479	0.000	1		706/204
		Parasite + Deworming	4542.384	17.905	<0.001		
		Base Season	4733.745	209.265	<0.001		
		Base Deworming	4761.557	237.078	<0.001		

Table 5.3 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of haematology. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	*Haematocrit*	*Haemoglobin*	*Total White Blood Cells*
Additive FEC effect + season			
Intercept	β=34.847±0.476, t=73.225	β=11.952±0.184, t=65.082	β= 9.748±0.046, t=212.002
Age	**β=0.034±0.012, t=2.877**	β=0.009±0.004, t=1.964	**β= -0.003±0.001, t=-2.588**
FEC: mild	**β=-0.584±0.248, t=-2.351**	**β=-0.268±0.107, t=-2.501**	β= -0.013±0.017, t=-0.755
FEC: heavy	β=-0.689±0.442, t=-1.558	β=-0.348±0.180, t=-1.938	β= 0.019±0.029, t=0.642
Season: Hot	**β=0.770±0.249, t=3.086**	**β=0.335±0.114, t=2.930**	β= -0.027±0.016, t=-1.694
Season: Monsoon	**β=-0.634±0.263, t=-2.410**	**β=-0.310±0.108, t=-2.875**	β= 0.017±0.018, t=0.961
Place: Kawlin	**β=-1.310±0.382, t=-3.427**	**β=-0.358±0.147, t=-2.436**	β= 0.002±0.037, t=0.059
Place: West Katha	β=0.026±0.564, t=0.046	β=0.143±0.212, t=0.673	β= -0.061±0.061, t=-1.018
Sex: Male	β=-0.322±0.345, t=-0.932	β=-0.211±0.131, t=-1.615	β= -0.023±0.035, t=-0.649
Origin: Wild	β=-1.010±0.561, t=-1.803	β=-0.269±0.212, t=-1.272	β= -0.066±0.056, t=-1.171
Additive FEC effect + deworm >4months			
Intercept	β=34.863±0.469, t=74.327	β=11.925±0.179, t=66.481	β= 9.723±0.046, t=212.637
Age	**β=0.032±0.012, t=2.712**	**β=0.010±0.005, t=2.145**	**β= -0.004±0.001, t=-2.772**
FEC: mild	**β=-0.783±0.249, t=-3.140**	**β=-0.346±0.110, t=-3.154**	β= -0.009±0.016, t=-0.523
FEC: heavy	**β=-1.403±0.438, t=-3.204**	**β=-0.600±0.181, t=-3.300**	β= 0.024±0.029, t=0.844
Deworm >4months	**β=0.755±0.220, t=3.439**	**β=0.259±0.104, t=2.495**	**β= 0.049±0.014, t=3.466**
Place: Kawlin	**β=-1.418±0.386, t=-3.676**	**β=-0.490±0.154, t=-3.175**	β= -0.006±0.038, t=-0.156
Place: West Katha	β=-0.099±0.567, t=-0.175	β=0.031±0.214, t=0.146	β= -0.049±0.061, t=-0.808
Sex: Male	β=-0.306±0.348, t=-0.879	β=-0.218±0.133, t=-1.647	β= -0.021±0.035, t=-0.592
Origin: Wild	β=-1.004±0.565, t=-1.779	β=-0.278±0.214, t=-1.296	β= -0.059±0.057, t=-1.036

Table 5.4 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of differential white blood cell count. We indicated the z-value (z). Estimates in bold correspond to the significant ones

Model	*Lymphocytes*	*Heterophils*	*Monocytes*	*Eosinophils*	*Basophils*
Additive FEC effect + season					
Intercept	β=2.413±0.111, z=21.786	β=2.090±0.108, z=19.356	β=2.572±0.105, z=24.583	β=-0.083±0.113, z=-0.728	β=-1.327±0.132, z=-10.078
Age	β=0.001±0.002, z=0.582	**β=0.006±0.002, z=2.338**	β=0.004±0.002, z=1.813	**β=0.019±0.003, z=7.183**	β=0.000±0.002, z=0.081
FEC: mild	**β=0.169±0.076, z=2.221**	**β=0.259±0.076, z=3.407**	**β=0.243±0.077, z=3.156**	**β=0.434±0.084, z=5.135**	β=0.053±0.092, z=0.574
FEC: heavy	β=-0.011±0.131, z=-0.083	β=0.096±0.131, z=0.736	β=0.104±0.129, z=0.801	β=0.150±0.145, z=1.028	β=-0.045±0.159, z=-0.284
Season: Hot	β=-0.112±0.079, z=-1.417	**β=-0.226±0.079, z=-2.859**	**β=-0.271±0.078, z=-3.477**	**β=-0.188±0.087, z=-2.148**	β=-0.044±0.092, z=-0.483
Season: Monsoon	β=0.142±0.094, z=1.514	β=0.108±0.094, z=1.147	β=-0.009±0.096, z=-0.095	**β=0.301±0.102, z=2.956**	β=-0.014±0.112, z=-0.126
Place: Kawlin	β=-0.138±0.086, z=-1.605	β=0.061±0.086, z=0.706	β=-0.032±0.087, z=-0.370	β=-0.162±0.094, z=-1.719	β=0.070±0.102, z=0.683
Place: West Katha	β=-0.035±0.127, z=-0.277	β=-0.027±0.127, z=-0.216	β=0.061±0.126, z=0.484	β=-0.105±0.139, z=-0.755	β=0.103±0.152, z=0.679
Sex: Male	β=0.115±0.072, z=1.602	**β=0.161±0.073, z=2.207**	β=0.113±0.072, z=1.575	β=0.001±0.081, z=0.015	β=0.039±0.087, z=0.448
Origin: Wild	β=-0.005±0.118, z=-0.045	β=-0.021±0.117, z=-0.182	β=0.006±0.115, z=0.049	β=-0.160±0.133, z=-1.203	β=-0.035±0.142, z=-0.246

Table 5.4 (continuation) - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of differential white blood cell count. We indicated the z-value (z). Estimates in bold correspond to the significant ones

Model	*Lymphocytes*	*Heterophils*	*Monocytes*	*Eosinophils*	*Basophils*
Additive FEC effect + deworm >4months					
Intercept	β=2.390±0.104, z=23.069	β=2.028±0.104, z=19.531	β=2.449±0.104, z=23.650	β=-0.169±0.112, z=-1.506	β=-1.343±0.127, z=-10.570
Age	β=0.001±0.002, z=0.314	**β=0.005±0.002, z=2.001**	β=0.003±0.002, z=1.444	**β=0.018±0.003, z=6.754**	β=0.000±0.003, z=0.011
FEC: mild	**β=0.178±0.075, z=2.359**	**β=0.272±0.076, z=3.596**	**β=0.239±0.076, z=3.150**	**β=0.503±0.083, z=6.080**	β=0.046±0.091, z=0.505
FEC: heavy	β=0.009±0.129, z=0.068	β=0.148±0.129, z=1.143	β=0.121±0.128, z=0.946	β=0.286±0.144, z=1.988	β=-0.050±0.156, z=-0.320
Deworm >4months	β=0.120±0.070, z=1.716	β=0.111±0.069, z=1.602	**β=0.184±0.069, z=2.669**	**β=0.195±0.077, z=2.547**	β=0.035±0.083, z=0.415
Place: Kawlin	**β=-0.217±0.086, z=-2.533**	β=-0.028±0.086, z=-0.333	β=-0.131±0.085, z=-1.535	**β=-0.242±0.094, z=-2.586**	β=0.044±0.102, z=0.437
Place: West Katha	β=-0.015±0.124, z=-0.123	β=-0.034±0.126, z=-0.274	β=0.068±0.124, z=0.543	β=-0.043±0.139, z=-0.310	β=0.101±0.151, z=0.667
Sex: Male	β=0.095±0.072, z=1.323	β=0.138±0.073, z=1.887	β=0.091±0.071, z=1.269	β=-0.020±0.080, z=-0.250	β=0.032±0.087, z=0.374
Origin: Wild	β=0.013±0.119, z=0.108	β=0.002±0.118, z=0.016	β=0.020±0.116, z=0.173	β=-0.159±0.134, z=-1.181	β=-0.029±0.142, z=-0.206

Table 5.5 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of protein activity. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	*Globulins*	*Total Protein*	*Albumin*
Additive FEC effect + season			
Intercept	β=4.429±0.059, t=75.549	β=7.668±0.077, t=99.600	β=3.226±0.039, t=82.879
Age	**β=0.013±0.002, t=8.622**	**β=0.012±0.002, t=5.980**	β=-0.001±0.001, t=-1.210
FEC: mild	β=-0.013±0.027, t=-0.476	**β=-0.078±0.039, t=-2.005**	**β=-0.076±0.021, t=--3.595**
FEC: heavy	**β=-0.152±0.048, t=-3.176**	**β=-0.279±0.070, t=-3.980**	**β=-0.147±0.038, t=-3.872**
Season: Hot	β=0.026±0.027, t=0.951	β=0.000±0.039, t=0.008	β=-0.018±0.021, t=-0.835
Season: Monsoon	β=-0.013±0.028, t=-0.443	β=0.015±0.041, t=0.359	β=-0.003±0.023, t=-0.148
Place: Kawlin	β=-0.002±0.048, t=-0.049	β=-0.119±0.062, t=-1.926	**β=-0.119±0.031, t=-3.832**
Place: West Katha	β=0.028±0.071, t=0.401	β=0.018±0.092, t=0.195	β=-0.011±0.046, t=-0.246
Sex: Male	β=-0.048±0.043, t=-1.123	**β=-0.134±0.056, t=-2.396**	**β=-0.056±0.028, t=-2.043**
Origin: Wild	β=-0.089±0.070, t=-1.267	β=-0.121±0.091, t=-1.337	β=-0.036±0.045, t=-0.799
Additive FEC effect + deworm >4months			
Intercept	β=4.426±0.057, t=77.099	β=7.644±0.074, t=102.876	β=3.203±0.037, t=85.513
Age	**β=0.013±0.002, t=8.526**	**β=0.011±0.002, t=5.914**	β=-0.001±0.001, t=-1.302
FEC: mild	β=-0.022±0.027, t=-0.817	**β=-0.084±0.039, t=-2.180**	**β=-0.077±0.021, t=-3.694**
FEC: heavy	**β=-0.181±0.047, t=-3.878**	**β=-0.300±0.068, t=-4.389**	**β=-0.151±0.037, t=-4.059**
Deworm >4months	**β=0.047±0.023, t=2.013**	**β=0.092±0.034, t=2.703**	**β=0.047±0.019, t=2.526**
Place: Kawlin	β=-0.009±0.048, t=-0.179	**β=-0.132±0.061, t=-2.140**	**β=-0.126±0.031, t=-4.075**
Place: West Katha	β=0.031±0.071, t=0.440	β=0.033±0.091, t=0.359	β=-0.001±0.046, t=-0.024
Sex: Male	β=-0.048±0.043, t=-1.118	**β=-0.131±0.056, t=-2.365**	**β=-0.056±0.028, t=-2.039**
Origin: Wild	β=-0.088±0.070, t=-1.252	β=-0.119±0.090, t=-1.319	β=-0.033±0.045, t=-0.729

Table 5.6 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of protein activity. We indicated the z-value (z). Estimates in bold correspond to the significant ones

Model	*AST*	*ALKP*	*CK*
Additive FEC effect + season			
Intercept	β=3.250±0.166, z=19.515	β=4.785±0.047, z=101.214	β=4.858±0.069, z=70.306
Age	**β=-0.030±0.005, z=-6.313**	**β=-0.018±0.001, z=-14.057**	β=0.001±0.002, z=0.720
FEC: mild	**β=-0.065±0.025, z=-2.636**	**β=-0.105±0.011, z=-9.695**	**β=-0.028±0.009, z=-3.074**
FEC: heavy	**β=-0.210±0.042, z=-4.956**	**β=-0.039±0.018, z=-2.113**	**β=-0.023±0.017, z=-1.350**
Season: Hot	β=0.041±0.025, z=1.610	**β=0.068±0.011, z=6.429**	**β=0.302±0.008, z=35.686**
Season: Monsoon	**β=0.334±0.024, z=13.973**	**β=-0.102±0.011, z=-9.157**	**β=-0.259±0.010, z=-26.676**
Place: Kawlin	β=-0.279±0.151, z=-1.852	β=0.039±0.041, z=0.968	β=-0.033±0.061, z=-0.540
Place: West Katha	**β=-0.549±0.237, z=-2.318**	β=-0.032±0.062, z=-0.515	β=0.036±0.104, z=0.346
Sex: Male	**β=0.481±0.103, z=4.672**	**β=0.136±0.034, z=3.955**	**β=0.126±0.045, z=2.819**
Origin: Wild	β=0.458±0.229, z=1.999	β=0.118±0.061, z=1.952	β=-0.072±0.094, z=-0.765
Additive FEC effect + deworm >4months			
Intercept	β=3.338±0.172, z=19.374	β=4.810±0.046, z=103.726	β=4.908±0.066, z=73.850
Age	**β=-0.033±0.005, z=-6.533**	**β=-0.0175±0.001, z=-13.736**	**β=0.008±0.001, z=4.381**
FEC: mild	β=-0.038±0.024, z=-1.573	**β=-0.124±0.011, z=-11.608**	**β=-0.080±0.009, z=-8.928**
FEC: heavy	**β=-0.171±0.041, z=-4.149**	**β=-0.088±0.018, z=-4.984**	**β=-0.234±0.017, z=-14.065**
Deworm >4months	**β=0.106±0.021, z=5.037**	**β=-0.038±0.009, z=-4.078**	**β=-0.096±0.008, z=-12.591**
Place: Kawlin	β=-0.306±0.156, z=-1.964	β=0.038±0.040, z=0.955	β=-0.071±0.060, z=-1.191
Place: West Katha	**β=-0.499±0.245, z=-2.035**	β=-0.060±0.061, z=-0.982	β=-0.033±0.100, z=-0.327
Sex: Male	**β=0.536±0.104, z=5.149**	**β=0.125±0.034, z=3.695**	**β=0.132±0.044, z=3.038**
Origin: Wild	**β=0.534±0.238, z=2.242**	β=0.098±0.060, z=1.652	**β=-0.274±0.091, z=-3.009**

Table 5.7 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of protein activity. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	*BUN*	*Creatinine*
Additive FEC effect + season		
Intercept	β=19.849±0.590, t=33.645	β=1.146±0.034, t=33.654
Age	β=-0.023±0.015, t=-1.547	β=-0.000±0.001, t=-0.265
FEC: mild	β=-0.497±0.313, t=-1.589	β=0.019±0.018, t=1.062
FEC: heavy	**β=-1.166±0.556, t=-2.094**	β=0.040±0.032, t=1.239
Season: Hot	**β=-5.170±0.313, t=-16.494**	**β=-0.050±0.018, t=-2.722**
Season: Monsoon	**β=3.245±0.332, t=9.788**	β=0.003±0.019, t=0.164
Place: Kawlin	**β=-3.902±0.473, t=-8.253**	**β=--0.097±0.027, t=-3.531**
Place: West Katha	**β=1.893±0.704, t=2.690**	**β=-0.087±0.040, t=-2.158**
Sex: Male	β=0.193±0.425, t=0.454	β=0.045±0.025, t=1.828
Origin: Wild	β=-0.068±0.686, t=-0.099	β=-0.065±0.040, t=-1.635
Additive FEC effect + deworm >4months		
Intercept	β=18.129±0.686, t=26.440	β=1.150±0.032, t=35.455
Age	β=-0.020±0.017, t=-1.218	β=-0.000±0.001, t=-0.145
FEC: mild	β=0.333±0.441, t=0.755	β=0.030±0.018, t=1.683
FEC: heavy	**β=1.565±0.770, t=2.032**	**β=0.079±0.031, t=2.550**
Deworm >4months	β=-0.366±0.396, t=-0.926	**β=-0.088±0.016, t=-5.609**
Place: Kawlin	**β=-3.711±0.559, t=-6.637**	**β=-0.083±0.027, t=-3.085**
Place: West Katha	**β=3.305±0.817, t=4.047**	**β=-0.092±0.039, t=-2.339**
Sex: Male	β=0.037±0.486, t=0.075	β=0.043±0.024, t=1.783
Origin: Wild	β=0.133±0.786, t=0.170	β=-0.064±0.039, t=-1.653

Table 5.8 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of protein activity. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	***Triglycerides***
Additive FEC effect + season	
Intercept	β=2.442±0.141, t=17.346
Age	β=0.004±0.003, t=1.152
FEC: mild	β=-0.087±0.096, t=-0.907
FEC: heavy	β=0.006±0.166, t=0.035
Season: Hot	**β=0.256±0.100, t=2.564**
Season: Monsoon	**β=0.680±0.108, t=6.274**
Place: Kawlin	β=-0.108±0.108, t=-0.999
Place: West Katha	β=-0.022±0.157, t=-0.139
Sex: Male	**β=-0.256±0.090, t=-2.827**
Origin: Wild	β=-0.075±0.150, t=-0.502
Additive FEC effect + deworm >4months	
Intercept	β=2.644±0.135, t=19.643
Age	β=0.005±0.003, t=1.757
FEC: mild	β=-0.026±0.096, t=-0.270
FEC: heavy	β=0.199±0.164, t=1.211
Deworm >4months	β=-0.127±0.087, t=-1.450
Place: Kawlin	β=0.050±0.110, t=0.453
Place: West Katha	β=0.066±0.157, t=0.422
Sex: Male	**β=-0.280±0.091, t=-3.077**
Origin: Wild	β=-0.161±0.151, t=-1.070

Table 5.9 - Summary of the statistical significance of FEC as a 3-level category and each confounding variable for the models of protein activity. We indicated the t-value (t). Estimates in bold correspond to the significant ones

Model	*Calcium*	*Chloride*	*Potassium*	*Sodium*
Additive FEC effect + season				
Intercept	β=10.121±0.075, t=135.048	β=91.329±0.414, t=220.746	β=4.931±0.064, t=76.631	β=129.817±0.391, t=331.619
Age	**β=-0.005±0.002, t=-2.684**	β=-0.003±0.010, t=-0.255	**β=0.006±0.001, t=4.502**	β=-0.010±0.009, t=-1.096
FEC: mild	**β=-0.207±0.049, t=-4.195**	β=0.002±0.230, t=0.009	β=-0.079±0.043, t=-1.851	**β=-0.555±0.233, t=-2.379**
FEC: heavy	β=-0.152±0.090, t=-1.677	β=0.295±0.402, t=0.734	β=-0.119±0.071, t=-1.660	β=-0.152±0.391, t=-0.388
Season: Hot	β=0.057±0.051, t=1.120	β=0.088±0.245, t=0.358	**β=-0.410±0.047, t=-8.745**	β=0.067±0.254, t=0.262
Season: Monsoon	β=-0.102±0.056, t=-1.835	β=0.281±0.236, t=1.188	**β=-0.389±0.045, t=-8.626**	**β=-0.923±0.244, t=-3.788**
Place: Kawlin	β=0.078±0.059, t=1.305	β=-0.483±0.331, t=-1.462	β=-0.072±0.049, t=-1.451	β=-0.259±0.305, t=-0.850
Place: West Katha	β=0.091±0.085, t=1.059	β=0.322±0.488, t=0.659	**β=-0.142±0.070, t=-2.021**	β=0.002±0.432, t=0.004
Sex: Male	β=-0.085±0.050, t=-1.707	β=-0.347±0.295, t=-1.175	β=-0.043±0.043, t=-1.026	β=-0.269±0.265, t=-1.016
Origin: Wild	β=-0.127±0.081, t=-1.558	β=-0.260±0.479, t=-0.544	**β=-0.252±0.068, t=-3.684**	β=-0.374±0.435, t=-0.860
Additive FEC effect + deworm >4months				
Intercept	β=10.175±0.072, t=141.222	β=91.524±0.399, t=229.463	β=4.640±0.065, t=71.916	β=129.636±0.380, t=340.863
Age	**β=-0.005±0.002, t=-2.737**	β=-0.003±0.010, t=-0.305	**β=0.006±0.001, t=4.311**	β=-0.010±0.009, t=-1.146
FEC: mild	**β=-0.214±0.049, t=-4.355**	β=0.044±0.228, t=0.193	**β=-0.123±0.046, t=-2.662**	**β=-0.672±0.237, t=-2.835**
FEC: heavy	β=-0.172±0.089, t=-1.932	β=0.392±0.394, t=0.995	β=-0.098±0.077, t=-1.277	β=-0.434±0.393, t=-1.104
Deworm >4months	**β=-0.123±0.045, t=-2.574**	β=-0.281±0.218, t=-1.288	β=0.078±0.046, t=1.699	β=0.044±0.228, t=0.193
Place: Kawlin	β=0.090±0.060, t=1.507	β=-0.348±0.341, t=-1.023	β=-0.110±0.056, t=-1.984	β=-0.341±0.322, t=-1.061
Place: West Katha	β=0.047±0.085, t=0.549	β=0.339±0.484, t=0.700	β=-0.107±0.073, t=-1.460	β=-0.216±0.436, t=-0.496
Sex: Male	β=-0.088±0.050, t=-1.744	β=-0.346±0.294, t=-1.174	β=-0.051±0.044, t=-1.145	β=-0.285±0.269, t=-1.061
Origin: Wild	β=-0.127±0.081, t=-1.557	β=-0.273±0.477, t=-0.572	**β=-0.222±0.071, t=-3.118**	β=-0.337±0.442, t=-0.763

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