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Title: Stress Physiology in Free-ranging Female African Buffalo (*Syncerus caffer*): Environmental Drivers, and Immunological and Infection Consequences.

Abstract approved:

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Anna E. Jolles

Fecal glucocorticoid metabolites (FGMs) are commonly used as indicators of an animal's stress response in behavioral and eco-physiological studies. Stress in wild animals represents an immediate measure of the physiological response to changes in the environment, and, potentially, a prospective assessment of the animal's health and well-being. In wild mammals, stress responses have been linked to changes in nutritional availability and predation, as well as anthropogenic disturbances including direct human contact and habitat loss. The recent global rise in infectious disease emergence in wildlife and humans, along with host stressors often implicated in adverse health consequences, raises the question whether stress has the potential to increase disease susceptibility, facilitate the spread of infections, and linking physiological effects of environmental change with disease dynamics. As such, a non-invasive measure of stress physiology may thus provide an integrative, mechanistically relevant measure of animal health at the interface of environmental drivers and immunological and infection consequences.

Here I use a well-studied model system, African buffalo (*Syncerus caffer*), to investigate links between physiological stress (measured as FGMs) for studying disease processes and immune-mediated interactions between hosts in natural populations, and parasite/pathogen existence. For chapter 1, an adrenocorticotropic

hormone (ACTH) experiment was performed on 12 buffalo to evaluate two commercially available FGM assays (radioimmunoassay and enzyme immunoassay) as a non-invasive tool for assessing stress in buffalo in field studies. For chapter 2 and 3, a longitudinal dataset of 200 buffalo (sampled every six months for four years) were used, to assess FGMs, and infections by viral and bacterial pathogens, gastrointestinal parasites, as well as a range of immune measures and physiological (hematological and biochemical) parameters.

The ACTH-induced plasma cortisol peak was detectable in FGMs at 10 – 20 hours post-injection (chapter 1). Acute viral infections rather than chronic infections contributed to stress in buffalo, and consequently increased the risk of obtaining some chronic bacteria (chapter 2). While accounting for fixed animal effects and current exposures, results support the hypothesis that buffalo with elevated FGMs had elevated pro-inflammatory immune responses (chapter 3: increased IFNγ, Il-12 responses, bacterial competency, the proportion of neutrophils), but weak evidence suppressing responses to chronical subclinical infections, such as strongyles (chapter 3). Together, findings of chapter 1-3, suggest that measuring FGMs as a stress marker can be used as an indicative tool associated with animals' environment, its physiological responses, some patterns of infectious diseases, and immunological consequences. Therefore, providing a widely used tool for scientists and wildlife managers interested in African buffalo management and conservation.

Stress Physiology in Free-ranging Female African Buffalo (*Syncerus caffer*): Environmental Drivers, and Immunological and Infection Consequences.

by
Johannie M. Spaan

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Chair of the Department of Integrative Biology

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Johannie M. Spaan, Author

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Chapter 1-3: Dr. Brianna Beechler was involved with the development of enzymeimmune assays used to measure fecal glucocorticoid metabolites and provided countless hours and feedback for chapter 1. For chapter 2-3 involvement in the development and running of assays for most physiological data (e.g., bacterial killing assays, biochemical parameters, etc.).

Chapter 2: Courtney Coon provided incidence data for all respiratory pathogens tested for in this study.

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INTRODUCTION

Any organism unable to maintain homeostasis undergoes stress. Animal stress physiology responds sensitively to changes in the social, biotic, and physical environments, and in turn, has been demonstrated to cause shifts in immunocompetence and infection risk in many animal species (Friedman and Lawrence 2002; Berger et al. 2005; Acevedo-Whitehouse and Duffus 2009). The distribution of parasites that threatens to shift due to environmental changes (e.g., climate change, land use changes and biodiversity loss) have caused global concerns (Patz et al. 2000a; Weiss and McMichael 2004b; Eisenberg et al. 2006; Parmesan 2006). The ability to predict and manage changes in infectious disease prevalence and severity depends critically on a mechanistic understanding linking anthropogenic environmental modifications to disease dynamic outcomes (Daszak et al. 2001). As such, a non-invasive measure of stress physiology may thus provide an integrative, mechanistically relevant measure of animal health at the interface of environmental drivers and immunological and infection consequences.

Hans (Selye 1936) defined "stress" eighty-two years ago as the: "non-specific response of the body to any demand made upon it". Till today, there is still no perfect definition of stress. In this study, we will refer to a stressor defined by (Creel 2001) as any stimulus, behavioral, environmental or demographic, that provokes a physiological stress response as measured by increased secretion of stress hormones. The stress response is the physiological reaction that occurs in response to a threat to survival, attack or harmful event (e.g. the fight or flight response), (Cannon 1916). Free-living animals regularly undergo both acute stressors, such as predator attacks and chronic stressors, such as food deprivation or climate change. These stressors can have detrimental impacts on the animal's health and survival (Mateo and Cavigelli 2005). Emphasizing the importance to be able to quantify the amount of stress an animal is experiencing.

Glucocorticoids are hormone steroids mediating stress responses in vertebrate animals. In ungulates, including African buffalo, the physiological stress response is mediated by the commonly measured glucocorticoid hormone, cortisol (Touma and

Palme 2005), and as seen in other ruminants, also includes corticosterone (Möstl and Palme 2002). The well-known pathway of the hypothalamic-pituitary-adrenal (HPA) axis activated by unpredictable or uncontrollable stressors have been well reviewed (Siegel 1980; Sapolsky et al. 2000; Tsigos and Chrousos 2002).

Measuring fecal glucocorticoid metabolites (FGMs) are non-invasive, efficient and have become desirable in many field-based wildlife studies (Graham and Brown 1996; Jurke et al. 1997; Wasser et al. 2000; Wielebnowski et al. 2002). FGMs are commonly used in many species including various ungulate species such as cattle (*Bos primigenius*) (Palme et al. 2000), North American and European populations of red deer (*Cervus elaphus*) (Wasser et al. 2000); (Huber et al. 2003). Chinnadurai and colleagues (2010) included species such as giraffe (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), nyala (*Tragelaphus buxtoni*), kudu (*Tragelaphus strepsiceros*) and blue wildebeest (*Connochaetes taurinus*). Despite the widespread use of FGM assays there are species-specific differences in hormone metabolite excretion, in terms of the amount and route of FGMs excreted as well as the types of metabolites formed (Palme 2005; Palme et al. 2005; Touma and Palme 2005; Hodges et al. 2010). Necessitating ACTH validation experiments in target species.

Therefore, objectives for chapter 1 were: 1) to evaluate 2 commercially available FGM assays (RIA and EIA) to confirm that FGMs reflect blood cortisol changes expected within buffalo of both sexes and across different age groups, that are experiencing stress; 2) to determine the lag time between the increase of circulating cortisol and the appearance of its metabolites in the feces of buffalo; and 3) to investigate whether immobilization and associated sampling stimulate a cortisol stress response in buffalo.

African buffalo is a well-studied model system for studying disease processes in natural populations (Sinclair 1977; Prins 1996; Winterbach 1998; Jolles 2003; Cross et al. 2005; Michel and Bengis 2012; Melletti and Burton 2014). Not only are they carriers of many pathogens and parasites, with the potential of some to spillover to livestock (Michel and Bengis 2012), but a great system to study immune-mediated interactions between host and parasite/pathogen existence (Jolles et al. 2008; Beechler et al. 2015; Ezenwa and Jolles 2015). Buffalo are ecologically important,

non-selective bulk grazers, which influence the function of savannah ecosystems by shaping plant communities and consequently affecting other herbivorous animals (Mcnaughton 1978; Winnie et al. 2008). Buffalo increase plant diversity (Huntley 1991) and have been used in the management and restoration of biodiversity, especially savannah grasslands (Olff and Ritchie 1998). They are also an important prey species for lions (*Panthera leo*) and have economic value as a key species in the hunting and tourism industries. Buffalo's size and fierce nature require chemical immobilization during any handling for management or research purposes. Hence, the reason for investigating a non-invasive tool such as measuring FGMs as a stress marker, to be used in a wider context including physiological health (e.g., immune, hematologic and biochemical) measures.

Glucocorticoids affect approximately 10% of genome expression, targeting a wide variety of genes that control metabolism, growth, reproduction fitness and immune function (Phuc Le et al. 2005). Wild animals experience unstable habitats resulting in physiological, morphological and behavioral modifications that allow them to adapt to predictable events (Möstl and Palme 2002). This emphasizes the importance of understanding the physiological adaptations in buffalo.

For chapter 2 and 3, a longitudinal dataset of 200 buffalo captured up to 9 times (sampled every six months for four years) were used, to assess FGMs, and infections by viral and bacterial pathogens, gastrointestinal parasites, as well as a range of immune measures and physiological (hematological and biochemical) parameters.

Infectious diseases have been implicated in wildlife population declines and extinctions; yet in many cases, it is likely that multiple stressors acting in synergy are responsible for these declines. Chapter 2 investigate how stress responses (measured as FGMs) and infectious diseases interact in a natural mammalian population, asking (i) How do infections by a diverse suite of parasites affect stress responses?; (ii) How does stress responses affect the incidence of these infections?; and ultimately, (iii) Is there evidence for positive feedback-cycles between stress responses and disease in a relatively undisturbed population of wild buffalo?

The immune system provides adequate immunological protection against pathogens, whether it is viral, bacterial, protozoan or fungal (Carroll and Forsberg 2007). Although stress-immunity has been widely studied and concluded both immune enhancement and immune suppression, there are many factors that affect the enhanced or suppressed outcome of the stress-immune system. It is unclear, then, whether/when stress responses will increase vulnerability to infectious diseases, or decrease them, and therefore, at the population level, how stress responses are likely to affect disease dynamics. For chapter 3 this longitudinal study design, allowing for the capture of both "fixed"/repeatable and plastic components of the stress responses, asking: How does stress (i.e., measured as FGMs) affect immune responses that mediate acute and chronic infections? If the adaptive function of stress responses is to help animal cope with immediately harmful situations, then one might expect stress to upregulate immune responses to acute infections (especially microparasites, which can multiply quickly in the host), but suppress immune responses to less imminently risky infections, such as macroparasites (which don't reproduce within the host) and thus don't suddenly increase in severity and harmfulness. Lastly, to gain a more holistic insight into health effects, we looked at physiological outcomes of variation in FGM, in concert with the immunological outcomes.

CHAPTER 1

Noninvasive measures of stress response in African buffalo (*Syncerus caffer*) reveal an age-dependent stress response to immobilization

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1.1 Abstract

Fecal Glucocorticoid Metabolites (FGMs) are commonly used as indicators of an animal's stress response in behavioral and eco-physiological studies. FGM assays provide a non-invasive and efficient means of assessing adrenocortical activity. We used 12 African buffalo (*Syncerus caffer*) temporarily maintained in an enclosure to evaluate 2 commercially available FGM assays as tools for assessing stress levels in buffalo in field studies. We also used the experiment to assess potential adverse effects of immobilizations on the study animals. Buffalo responded rapidly to stimulation with adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to secrete glucocorticoids. ACTH-stimulated buffalo had higher plasma cortisol concentrations than saline-injected controls between 5 minutes and 1 hour after injection. The ACTH-induced plasma cortisol peak was detectable in FGMs at 10 - 20 hours post injection. Both of the commercial test kits we evaluated were capable of detecting the ACTH-induced peak in FGM. However, the radioimmunoassay (RIA) delivered more consistent detection across weeks than the enzyme immunoassay (EIA). We tested whether immobilization and handling elicited a stress response detectable by FGM, by comparing immobilized, saline-injected buffalo with controls that were not immobilized or handled. Adult buffalo mounted a stress response to immobilization and handling, whereas subadults did not, suggesting an age-related difference in response to chemical immobilization. Our study validates use of commercially available kits for quantifying FGMs under field conditions.

1.2 Introduction

Free-living animals regularly face both acute stressors, such as predator attacks, and chronic stressors, such as food deprivation or climate change (Morton and Sherman 1978; Kotrschal et al. 1998; Lima 1998; Cavigelli 1999; Creel 2001; Goymann et al. 2001; Abbott et al. 2003; Sands and Creel 2004). Chronic stressors can have detrimental impacts on the animals' health, reproductive performance, and survival (Mateo and Cavigelli 2005). Quantifying the stress that an animal is experiencing thus represents a measure of the physiological response to endogenous factors and changes in its environment that can complement behavioral and ecological approaches in assessments of animal population health.

The most direct measure of the physiological stress response is to quantify glucocorticoids in blood (Harlow et al. 1990; Widmaier et al. 1994; Wingfield et al. 1994; Hood et al. 1998; Gregory and Schmidt 2001). Using blood is not always practical in wildlife, because capture and collection of a blood sample may itself be stressful to the animal. The acute stress response to capture can thus hide the signal related to environmental stressors of interest. Capture and immobilization also disrupt the animal's normal behaviors, are expensive, and often may be hazardous for the observer (Hopster et al. 1999; Moberg and Mench 2000). Alternatively, physiological stress can be assessed non-invasively by measuring fecal glucocorticoid metabolites (FGMs) – a method often used in field-based wildlife studies (Touma and Palme 2005; Keay et al. 2006). FGMs have been assessed in many species including various ungulate species such as our target species, African buffalo (*Syncerus caffer*, Ganswindt et al. 2012), cattle (*Bos taurus*, Palme et al. 2000), North American and European populations of red deer (*Cervus elaphus*, Wasser et al. 2000; Huber et al. 2003); and Chinnadurai and colleagues (2009) included African ungulate species such as giraffes (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), nyala (*Tragelaphus buxtoni*), kudu (*Tragelaphus strepsiceros*), and blue wildebeest (*Connochaetes taurinus*).

FGMs are mainly determined by competitive immunoassays (Möstl et al. 2005), such as radioimmunoassays (RIAs) and enzyme immunoassays (EIAs), but more recently also by liquid chromatography-mass spectrometry (LC-MS). RIAs

have high precision, robustness, and good performance, but because cortisol and corticosterone are absent or rare in the feces of mammals and birds (Palme et al. 2005), these assays rely on the cross-reactivity and recognition by the antibody of the group of metabolites excreted (Möstl et al. 2005). Safety regulations associated with the use of radioactive isotopes, especially the disposal of radioactive material (Sheriff et al. 2011), make RIAs less accessible than EIAs. The group-specific antibodies that have been specifically designed to measure metabolite groups in EIAs allow for use in a broad range of species (Möstl et al. 2005; Touma and Palme 2005; Palme 2012).

Despite the widespread use of FGM assays there are species-specific differences in hormone metabolite excretion, in terms of the amount and route of FGMs excreted as well as the types of metabolites formed (Palme 2005; Palme et al. 2005; Touma and Palme 2005; Hodges et al. 2010). In ungulates, including African buffalo, the physiological stress response is mediated by the commonly measured glucocorticoid hormone, cortisol (Touma and Palme 2005), and as seen in other ruminants, also includes corticosterone (Möstl and Palme 2002). Validation experiments demonstrating that variation in FGMs reflect changes in cortisol concentration in the blood, and the time scale at which this occurs, are therefore necessary for each new target species. For African buffalo, a validation experiment was performed by Ganswindt et al. (2012), who used an in-house EIA protocol to demonstrate that FGMs correlate to glucocorticoid levels in plasma. However, validation of commercially available kits is useful, because they are accessible to researchers conducting ecological and behavioral field studies (who are not typically in a position to design their own endocrinological assays). Moreover, while the Ganswindt et al. (2012) study served as proof of concept for the use of FGMs in African buffalo, it was based on a single adult female and her calf. Typical ecological field studies include animals of both sexes, and across the full age spectrum for the species; as such, a validation study allowing broader inference to natural buffalo populations is desirable. Finally, an estimation of the timing of FGM excretion following a cortisol- corticosterone peak in the blood, and expected variability in this timing, is essential to designing sampling schedules in behavioral and ecological studies, and interpreting FGM data from field-collected samples.

Most often, validation experiments are performed in the target species by administering synthetic adrenocorticotropic hormone (ACTH) and monitoring cortisol production and subsequent peaks in FGM (e.g., see reviews by Touma and Palme 2005; Keay et al. 2006). Experimentally administered ACTH causes a rapid peak in blood cortisol levels followed by return to baseline after a few hours (Norris 2006). The same pattern also occurs in feces, with the onset of the peak excretion delayed by the species-specific excretion lag time (Palme et al. 1999, 2005; Palme 2005; Touma and Palme 2005; Hodges et al. 2010). Here, we report on an ACTH challenge experiment on temporarily confined wild buffalo, aimed at evaluating 2 commercially available kits (RIA and EIA) for FGM quantitation in buffalo.

Buffalo are ecologically important, non-selective bulk grazers, that influence the function of savanna ecosystems by shaping plant communities and consequently affecting other herbivores (Mcnaughton 1978). Buffalo increase plant diversity (Huntley 1991) and have been used in the management and restoration of savanna biodiversity (Olff and Ritchie 1998). They are also an important prey species for lions (*Panthera leo*), and have economic value as a key species in the hunting and tourism industries. Buffalo are highly gregarious and carry many pathogens and parasites, some of which can cause important diseases in livestock (Michel and Bengis 2012). As a result, ecology and behavior of African buffalo are studied in many contexts and a FGM assay will provide a valuable and potentially widely used tool for scientists and wildlife managers interested in this iconic species. Because of their size and fierce nature, any handling of African buffalo for management or research purposes typically involves chemical immobilization. As such, assessing whether immobilizations elicit a stress response is of immediate relevance to the husbandry of African buffalo populations.

Our study objectives were: 1) to evaluate 2 commercially available FGM assays (RIA and EIA) to confirm that FGMs reflect blood cortisol changes expected within buffalo of both sexes and across different age groups, that are experiencing stress; 2) to determine the lag time between the increase of circulating cortisol and the appearance of its metabolites in the feces of buffalo; and 3) to investigate whether

immobilization and associated sampling stimulate a cortisol stress response in buffalo.

1.3 Methods

Experimental design and sample collection.—An ACTH challenge was conducted using a holding facility operated by South African National Parks' Veterinary Wildlife Services (VWS) located in Skukuza, Kruger National Park (KNP), South Africa (Fig. 1.1). The buffalo (n = 12) originated from a population (n = 34) previously part of a Bovine Tuberculosis-free breeding project in an 800-ha enclosure in KNP and were housed in the holding facility for 2 weeks prior to induction of experiment to allow them to become habituated to their new environment. The buffalo were then subdivided into 3 groups of 4 animals 3 days prior to the start of the experiment. Ideally, experimental groups should match in age-sex composition of subjects; however, we were constrained in our allocation of buffalo to experimental groups by: 1) limited access to matching individuals (similar age and sex groups); 2) practical difficulty of partitioning buffalo into groups without additional immobilizations; and 3) concern about potential aggressive behavior of the older males towards the rest of the animals during confinement. As a result, our study groups are not matched for age and sex (Table 1.1), with the older males concentrated in a single group – which we designated as our control group (no immobilization). The lack of females in the control group is a limitation of our study design; however, we mitigated the imperfect matching in the other 2 groups by choosing a randomized crossover experimental design, in which each animal in turn acts as an experimental individual and as control. The buffalo were fed *Eragrostis tef* and provided with an ad libitum water supply.

A randomized crossover experimental design with an 8-day washout period between the 2 interventions (week 1 and week 2) was used. Groups 1 and 2 were treatment groups and the third group served as a control. During each trial, buffalo in groups 1 and 2 were immobilized and injected intramuscularly with either a saline solution or ACTH. Buffalo that received the saline solution during the first trial were switched to ACTH during the second trial, and vice versa (see Table 1.1). In both

trials, the control buffalo (group 3) were not immobilized and did not receive any treatment. The experiment was designed to: 1) measure the increase in plasma cortisol concentration in response to ACTH stimulation, by comparing plasma cortisol concentration when stimulated with ACTH versus saline treatment; 2) detect the ACTH-induced plasma cortisol spike in FGMs, and quantify the characteristic lag time between ACTH stimulation and elevated FGMs using 2 commercially available detection assays (RIA and EIA); and 3) investigate the possible effect of handling stress on FGM excretion, by comparing immobilized saline-injected buffalo against unhandled control animals (group 3).

Immobilization of the buffalo was conducted by VWS wildlife veterinarian, Dr. Peter Buss according to the South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. Each buffalo received a combination of etorphine hydrochloride (Novartis, Kempton Park, 1619, South Africa), azaperone (Janssen Parmaceutical Ltd., Halfway House, 1685, South Africa) and hyaluronidase (Kyron Laboratories, Benrose, 2001, South Africa). This drug combination was delivered into the muscle of the rump remotely using a dart propelled by a compressed air rifle (DAN-INJECT, International S.A., Skukuza, 1350, South Africa). The doses varied between 2-4 mg etorphine, 50-80 mg azaperone and 5000 i.u. hyaluronidase depending on the size of the animal.

At the first immobilization, the buffalo were weighed. A synthetic ACTH analogue (Norvartis, Synacthen® Depot Ampoule, 1 mg/ml) was administered intramuscularly at 0.01 ml/kg (Alam et al. 1986) and the animals treated with saline received an equivalent volume of saline. Blood was collected from the jugular vein into heparinized blood tubes prior to and at 5-minute intervals for 1-hour post-ACTH or saline administration. Blood samples were stored on ice and processed in the laboratory within 4-8 hours of collection. Samples were centrifuged for 10 minutes at 3,000 rpm and plasma harvested into 3.5-ml cryotubes. Plasma samples were then stored at -20°C until the cortisol analysis was performed.

Individual buffalo were identified with colored tape placed around the horns or in some cases by using distinguishing facial markings and horn shapes. All animals also had a numbered ear tag.

Animals between 2 and 5 years old were aged according to incisor emergence patterns (Grimsdell 1973). In older buffalo, the tooth wear of the first incisor was evaluated for age determination (Jolles 2007). The control animals that were not immobilized were aged according to (Grimsdell 1973) based on body size and horn development. For statistical analysis, all buffalo were categorized into subadults (≤ 3.5 years) or adults (>5 years) based on their reproductive status for separating the effect of reproductive hormones on glucocorticoids. Females can reach sexual maturity at 3.5 years (Pienaar 1969; Jolles 2007), whereas males can reach maturity as early as 2.5-3 years, but rarely breed until an age of 7-8 years (Pienaar 1969).

At the end of the procedure, naltrexone (the antidote to etorphine hydrochloride; 40 mg/ml, Kyron) was administered intravenously and the animal kept under observation until fully recovered.

Fecal samples were collected at 3 time periods: prior to a trial, during immobilization, and after each trial for 6 consecutive days (Table 1.1 shows the number of defecations per buffalo). During immobilization fecal samples were collected directly from the rectum, while the remaining samples were collected from the ground in the holding facility, using a scooping ladle mounted on a long pole (to avoid the observer having to enter the buffalos' enclosure; Fig. 1.1). The buffalo were observed continuously for the first 48 hours after each trial and thereafter only during daytime. Fecal samples were only collected if: 1) the identity of the buffalo was known, and 2) the location of the fecal sample was accessible (Fig. 1.1 shows enclosure layout and fecal collection procedure). All fecal samples (±30 g) were taken from the center of the dung pile within 30 minutes post defecation, immediately placed in a 50-ml conical tube on ice, and stored at -20°C within 1 hour until analysis.

All procedures conducted during this research followed the ASM guidelines (Sikes et al. 2016) and were approved by the South African National Parks Board (Reference No. CALJM728), and Oregon State University's Animal Care and Use Committee (OSU ACUP No. 3822).

Plasma cortisol RIA.—A coated tube cortisol RIA was used to determine plasma cortisol concentrations according to manufacturer's instructions (Siemens, PITKCO-8, 2009-07-16).

Fecal sample extraction for RIA.—To prepare fecal samples for the FGM extraction, each fecal sample was thawed at room temperature and thoroughly mixed. Then a sample of 1 g (± 0.0001 g) was placed in a tube and 3 ml of 80 % methanol (Palme and Möstl 1997) was added. This sample was rotated in a vertical plane for 8 hours at room temperature followed by 10-minute centrifugation at 1200xG. The resulting supernatant was withdrawn and stored in cryotubes at -20°C until analysis. To determine the moisture content of each fecal sample, a portion was weighed before and after being placed in a drying oven for 48 hours at 60°C.

Fecal glucocorticoid metabolite RIA.—The concentration of steroid metabolites in the extracted fecal samples was measured with a double antibody RIA (^{125}I Corticosterone RIA kit; MP Biomedicals LLC, Santa Ana, California) previously validated in several species, including cattle (*Bos taurus*, Morrow et al. 2002), African elephants (*Loxodonta africana*), Roosevelt elk (*Cervus canadensis*, Wasser et al. 2000), giraffes (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), and kudu (*Tragelaphus strepsiceros*, Wasser et al. 2000; Morrow et al. 2002; Chinnadurai et al. 2009). Sample dilutions were optimized for buffalo feces (the methanolic extracts were diluted 10 times with the manufacturer's steroid diluent, Cat. No. 07-166196). Manufacturer's instructions were followed except for the following step. To account for the 1:200 dilution prescribed for the rat plasma corticosterone, the 6 corticosterone calibrators included in the RIA kit (0–1000 ng/ml) were pre-diluted to extend the calibration range from 0-5 ng/ml, therefore lowering the actual concentration of each calibrator by 200 times. FGM concentrations were expressed as ng/g dry feces, by correcting for the moisture content of the feces and dilution ratios. Upon analysis, slopes ($r > 0.98$) for serial dilutions of samples remained parallel to the standard curve and within-assay variation was less than 7 %.

Fecal sample extraction for EIA.—To prepare fecal samples for the metabolite extraction, each fecal sample was thawed and > 2 g was dried at 25°C under forced air until dry (approximately 12 hours, Mateo and Cavigelli 2005). Once dry, 0.2 g

was placed in a 15-ml screw cap tube and 1.5 ml of 99% ethanol was added. The sample was vortexed for 3 seconds and centrifuged for 20 minutes at 1200xG (Palme et al. 2000; Mateo and Cavigelli 2005). The resulting supernatant was withdrawn and stored in 1.5-ml cryotubes at -20°C until final processing within 2 weeks. Due to logistic constraints, this extraction technique differs from the one used for the RIA and follows Mateo and Cavigelli's (2005) field-friendly extraction technique. Because the extraction technique and the detection method differ, we do not directly compare the EIA and RIA, but are instead asking if both are capable of detecting FGMs in African buffalo.

Fecal glucocorticoid metabolite EIA.—During final processing, the frozen supernatant was removed and the ethanol evaporated under forced air for 4-6 hours. Once all ethanol was evaporated, 1,000 μL of assay buffer was added to dilute the samples to achieve dilutions likely to fall within the range of the standard curve provided in the kit. The assay was performed using the EIA kit per manufacturer instructions (Cortisol ELISA kit; Catalog #: ADI-900-071; Enzo Life Sciences Inc, Farmingdale, New York).

Effect of ACTH treatment on plasma cortisol concentrations.—To test whether buffalo responded to ACTH stimulation by increasing plasma cortisol concentration, we compared ACTH-treated buffalo with placebo buffalo that received saline. We ran the experiment twice, with treatment buffalo in week 1 ($n = 4$) used as placebo animals in week 2, and placebo buffalo in week 1 ($n = 4$) treated with ACTH in week 2. To account for the fact that each buffalo was used once as a placebo animal and once as a treatment animal, we used linear mixed-effect models (LMEM), with individual ID included as a random effect (Bates et al. 2012). We included the following independent variables as possible predictors of plasma cortisol: treatment, time, week, age ($n = 2$ adults and $n = 6$ subadults), and sex ($n = 3$ males and $n = 5$ females). All 2-way interactions containing treatment were included in the model. Visual inspection of residual plots did not reveal any obvious deviations from normality or homoscedasticity. *P*-values were obtained using the lmerTest package (Kuznetsova et al. 2016), and considered statistically significant if $P \leq 0.05$. Plasma cortisol results were expressed as nanomoles per liter (nmol/L).

Effect of ACTH treatment on fecal glucocorticoid metabolites.—To test whether the ACTH-induced spike in plasma cortisol was detectable in feces and to determine its time lag, FGMs (for both RIA and EIA results) in ACTH–treated and saline-injected (placebo) buffalo were compared for each 10-hour time interval from ACTH or saline injection to 140 hours later. LMEM was used, including individual ID as a random effect, and treatment, time, week, age (n = 2 adults and n = 6 subadults), and sex (n = 3 males and n = 5 females) as explanatory variables. As above, all 2-way interactions containing treatment were included. Visual inspection of residual plots for normality and homoscedasticity revealed the necessity to log transform the response variable (FGM). FGM results were expressed as nanograms glucocorticoid metabolites per gram of dry feces (ng/g).

We used the raw data (i.e., data not lumped into 10-hour time intervals) to calculate the time to peak (in hours) and the mean peak FGM concentration (ng/g) for ACTH-treated buffalo from both RIA and EIA results. A Wilcoxon rank sum test was used to test for weekly differences in the mean peak time and peak FGM concentrations for both RIA and EIA results, as well as whether there was a significant difference in the peak time between assays.

Effect of immobilization on stress response in buffalo.—To test whether buffalo showed elevated FGM due to immobilization and handling stress, immobilized saline-injected (placebo) buffalo were compared against unhandled (group 3) controls. As above, LMEM was used to compare FGMs (for both RIA and EIA results) in saline-injected and control buffalo for each 10-hour time interval. Treatment, time, week, age (n = 4 adults and n = 8 subadults), and sex (n = 7 males and n = 5 females) were all considered as possible predictors of FGMs. All 2-way interactions containing treatment were included, except for treatment x sex (due to no control females). The response variable (FGM) was log transformed to ensure no violation of the model assumptions and P-values were obtained as explained above.

Initial levels.—Initial plasma cortisol level was calculated as the mean plasma cortisol measured at time point 0, which was the blood sample collected before ACTH or saline injection for each immobilized buffalo during week 1 (n = 8) and week 2 (n = 8). We acknowledge that this initial level may be affected by handling or

immobilization of the buffalo, but this is unavoidable for the time point in which a blood sample was obtained.

For the initial FGM level, time point "initial" was calculated as the mean of each individual from the following time points where applicable: 1) fecal samples collected prior to treatment application, and 2) rectal fecal sample during immobilization.

Initial levels are shown as mean ± standard deviation ($\bar{X} \pm SD$). To test for differences in the mean initial levels between week 1 and week 2, a Wilcoxon Signed Rank test for paired data was used.

The statistical program R was used for statistical analysis (R Core Team 2016), including packages: lme4 (Bates et al. 2012), lmerTest (Kuznetsova et al. 2016), and ggplot2 (Wickham 2009).

1.4 Results

ACTH treatment resulted in a rapid and marked cortisol release, measureable in plasma within 10 minutes post injection during both runs of the experiment (Fig. 1.2a,b, Supplementary Data SD1.1). The plasma cortisol concentrations in experimental animals differed between weeks 1 and 2.

A total of 457 fecal samples were analyzed to determine whether, and when, the ACTH-induced peak in plasma cortisol was detectable in feces. Because fecal samples were collected at variable time intervals (when defecation occurred), we binned samples into 10-hour time intervals to assess during which periods post-ACTH injection FGM departed from initial levels. The increase in plasma cortisol in ACTH-treated buffalo was detectable in feces between 10-20 hours during both weeks for both RIA (Fig. 1.3a,b) and EIA. However, the detectability was not as strong during week 1 for the EIA (Fig. 1.3c,d). After accounting for week, age, sex, and buffalo ID (random effect), the average FGM concentrations (for both weeks) at time point 10.1-20 hours ($\bar{X}$ = 258.75 ng/g, SD = 119.03 ng/g, LMEM, $t_{125.17}$ = 4.85, P <0.0001) was 5 times higher than initial levels ($\bar{X}$ = 54.94 ng/g, SD = 21.76 ng/g, LMEM, $t_{125.17}$ = 4.85, P <0.0001) for the RIA, and 14 times higher for the EIA (initial: $\bar{X}$ = 6.71 ng/g, SD = 6.88 ng/g; 10.1-20 hours: $\bar{X}$ = 90.40 ng/g, SD = 82.39,

LMEM, $t_{117.01} = 5.29$, $P < 0.0001$) (Supplementary Data SD1.2). The average FGM concentrations at time point 20.1-30 hours ($\bar{X} = 119.88$ ng/g, $SD = 61.65$ ng/g, LMEM, $t_{125.13} = 2.35$, $P = 0.02$) and 30.1-40 hours ($\bar{X} = 123.91$ ng/g, $SD = 115.19$ ng/g, LMEM, $t_{125.18} = 2.55$, $P = 0.01$) was 2.25 and 2 times higher than initial levels respectively for the RIA; and 3.67 and 3.75 times higher for the EIA (20.1-30 hours: $\bar{X} = 19.25$ ng/g, $SD = 13.11$ ng/g, LMEM, $t_{116.98} = 3.09$, $P = 0.002$; 30.1-40 hours: $\bar{X} = 20.23$ ng/g, $SD = 15.97$ ng/g, LMEM, $t_{117.32} = 2.79$, $P = 0.006$) (Supplementary Data SD1.2). Using the raw data (not the 10-hour time intervals) to obtain a finer-scale estimate of the lag time between ACTH injection and peak FGM excretion, there was no difference between weeks for either the RIA (week 1: $\bar{X} = 23$ hours, $SD = 15.21$ hours; week 2: $\bar{X} = 16.75$ hours, $SD = 1.5$ hours; Wilcoxon rank sum test, $W = 5$, $P = 0.37$; Fig. 1.4a) or the EIA (week 1: $\bar{X} = 28.5$ hours, $SD = 13.92$ hours; week 2: $\bar{X} = 16.5$ hours, $SD = 1.29$ hours; Wilcoxon rank sum test, $W = 3$, $P = 0.14$; Fig. 1.4b). There was no significant difference in peak time between the RIA and EIA (Wilcoxon rank sum test, $W = 33$, $P = 0.91$). The average time to peak (in hours) of 19.88 (95% CI between 12.57 and 27.18) for the RIA and 22.5 (95% CI between 14.76 and 30.24) for the EIA coincides with the lag time found by the LMEM results of 10-20 hours for both assays. However, using the raw data to obtain a finer-scale estimate of the peak FGM concentration, week 2 ($\bar{X} = 402.28$ ng/g, $SD = 100.25$ ng/g) was 2 times higher than week 1 ($\bar{X} = 181.4$ ng/g, $SD = 77.3$ ng/g; Wilcoxon rank sum test, $W = 15$, $P = 0.06$ close to significance) for the RIA (Fig. 1.4c), and 7 times higher for the EIA (week 1: $\bar{X} = 39.06$ ng/g, $SD = 23.28$ ng/g; week 2: $\bar{X} = 285.44$ ng/g, $SD = 162.89$ ng/g; Wilcoxon rank sum test, $W = 16$, $P = 0.03$; Fig. 1.4d).

The initial level of plasma cortisol was much higher in week 1 ($\bar{X} = 144.8$ nmol/L, $SD = 66.77$ nmol/L; Fig. 1.2a) than in week 2 ($\bar{X} = 27.6$ nmol/L, $SD = 22.45$ nmol/L; Fig. 1.2b), (Wilcoxon Signed Rank test, $V = 36$, $P = 0.008$, $n = 8$ pairs), and after accounting for treatment, time, age, sex, and the random effect of each animal, the plasma cortisol response was also significantly higher during week 1 than week 2 (Fig. 1.2; ß = 142.06, $SE = 44.11$, $t_{4.12} = 3.22$, $P = 0.03$; Supplementary Data SD1.1). This weekly difference in the average plasma cortisol levels was not reflected in FGM, as assessed via RIA (Table 1.2: log ß = 0.18, $SE = 0.14$, $t_{6.29} = 1.25$, $P = 0.26$;

Supplementary Data SD1.2: log ß = 0.17, SE = 0.15, $t_{6.37}$ = 1.14, P = 0.30) and EIA (Table 1.2: log ß = -0.04, SE = 0.24, $t_{7.14}$ = -0.16, P = 0.88; Supplementary Data SD1.2: log ß = -0.03, SE = 0.25, $t_{6.88}$ = -0.10, P = 0.92). The initial FGM levels between weeks 1 and 2 also failed to detect the drop in the initial plasma cortisol levels in both RIA (week 1: $\bar{X}$ = 51.48 ng/g, SD = 23.83 ng/g; week 2: $\bar{X}$ = 58.4 ng/g, SD = 20 ng/g; Wilcoxon Signed Rank test, V = 39, P = 0.64) and EIA (week 1: $\bar{X}$ = 7.03 ng/g, SD = 8.17 ng/g; week 2: $\bar{X}$ = 6.38 ng/g, SD = 5.73 ng/g; Wilcoxon Signed Rank test, V = 36, P = 0.43). However, the magnitude by which the average plasma cortisol levels increased 10-60 minutes post ACTH injection from initial levels was lower during week 1 (week 1: $\bar{X}$ = 281.21 nmol/L, SD = 14.62 nmol/L, week 2: $\bar{X}$ = 117.68 nmol/L, SD = 25.77 nmol/L) than week 2 (2 and 4 fold, respectively; Fig 1.2a,b): The peak FGM concentration from initial levels was 3.5-fold higher during week 1 (peak: $\bar{X}$ = 181.4 ng/g, SD = 77.3 ng/g; initial: $\bar{X}$ = 51.48 ng/g, SD = 23.83 ng/g) and 7-fold higher during week 2 (peak: $\bar{X}$ = 402.28 ng/g, SD = 100.25 ng/g; initial: $\bar{X}$ = 58.4 ng/g, SD = 20 ng/g) for the RIA. The EIA showed a 5.5- and 44-fold increase during week 1 (peak: $\bar{X}$ = 39 ng/g, SD = 23.28 ng/g; initial: $\bar{X}$ = 7.03 ng/g, SD = 8.17 ng/g) and week 2 (peak: $\bar{X}$ = 285.64 ng/g, SD = 162.89 ng/g; initial: $\bar{X}$ = 6.38 ng/g, SD = 5.73 ng/g), respectively. We found that higher initial plasma cortisol concentrations (week 1) were associated with a reduced response to ACTH stimulation, compared to week 2. This suggests an upper boundary to plasma cortisol concentrations.

Overall, immobilized saline-injected buffalo showed no significant difference in FGM concentrations compared to unhandled controls for both RIA (log ß = -0.31, SE = 0.33, $t_{13.13}$ = -0.93, P = 0.37) and EIA (log ß = -0.98, SE = 0.57, $t_{14.85}$ = -1.71, P = 0.11; Table 1.2). Interestingly, both datasets reveal an interaction effect of age x treatment, with adult animals responding to immobilization with elevated FGM, whereas subadults did not (RIA: log ß = 0.55, SE = 0.25, $t_{5.25}$ = 2.18, P = 0.08; EIA: log ß = 1.25, SE = 0.43, $t_{6.19}$ = 2.87, P = 0.03; Fig. 1.5).

1.5 Discussion

In this study, we measured FGMs as a non-invasive alternative to measuring plasma cortisol in male and female wild African buffalo aged between 2-9 years. The setting and logistics of our study resembled a typical eco-physiological field study, including sample handling, and delays between sample collection, FGM extraction, and detection.

Stimulation of buffalo with ACTH resulted in elevated plasma cortisol concentrations in comparison to saline-injected controls. This plasma cortisol peak was detectable in FGMs 10-20 hours later, which is concordant with previous findings in an adult female of this species and her calf (Ganswindt et al. 2012). We focused on methodological approaches that could be replicated in ecological and behavioral field studies, utilizing 2 commercially available kits for FGM detection, including an RIA and an EIA kit. Both approaches yielded qualitatively similar results in terms of timing and detectability of the experimentally induced FGM peak, but the RIA delivered more consistent results across our 2 experimental periods.

Overall, 3 minor differences were observed between the 2 approaches. Firstly, the magnitude in change from initial levels to FGM peak was higher in the EIA than the RIA. Secondly, the EIA detected age-related effects more strongly than the RIA. Thirdly, the absolute levels of FGMs detected were higher for the RIA than the EIA, most likely due to the different antibody sets used in the 2 immunoassays and variation in cross-reactivity with excreted FGMs from buffalo. We used different metabolite extraction methods with the 2 kits (80% methanol for RIA, 99% ethanol for EIA) due to logistical constraints, which may also have contributed to quantitative differences in detected FGMs (Palme et al. 2013). However, studies comparing extraction methods for FGMs suggest that variation due to different extraction protocols are probably minor (Mateo and Cavigelli 2005). We conclude that variation in plasma cortisol can be detected, with a delay of 10-20 hours, in FGM of male and female African buffalo of various ages, and that both kits we evaluated are suitable for assessing adrenocortical activity in this species under field conditions. Re-emphasizing that we do not directly compare the RIA and EIA due to different extraction techniques and detection methods, the EIA might be more sensitive in that it revealed a higher magnitude of ACTH-induced change in FGM from initial levels,

and distinguished responses by different age groups of buffalo more clearly than the RIA. This sensitivity could result in positive outcomes (e.g., detect biologically important differences at a finer scale), but might also introduce more noise in variable datasets, potentially obscuring real differences among groups or time periods. As such, the RIA tended to return more consistent findings across weeks, which may help compensate for the logistical challenges associated with the use of radioactive materials. Moreover, the RIA detected higher FGM concentrations overall, which could be an advantage in situations where FGM levels approach the EIA's lower detection limit.

The passage rate of digesta plays an important role in determining the time course of the excretion of steroids (Palme et al. 1996; Wasser et al. 2000). Seasonal changes in feed intake and pasture digestibility can affect the transit time of digesta passing between the bile duct and the rectum (Möstl et al. 1999; Palme et al. 2000). For example, in an ACTH stimulation experiment in cattle, the median gut passage time was 16.6 hours during autumn and 9.8 hours during spring, reflected in an ACTH peak in FGMs at 14-18 hours in autumn and 8-9 hours in spring (Morrow et al. 2002). The faster rate of passage in spring (wet season) was associated with higher consumption of feed, increased pasture digestibility, increased fecal output, and decreased dry matter content (Morrow et al. 2002). In free-ranging buffalo in the dry season, one might thus expect the time delay from plasma cortisol to FGMs to be longer than observed in our study, because moisture content of forage and water intake in the dry season are lower than those experienced by our experimental animals. In the wet season, free-ranging buffalo may have a quicker gut passage time, and shorter lag between cortisol release and detectable FGM peak than observed here.

Initial and stimulated plasma cortisol concentrations were much higher in week 1 of this study than in week 2. Even though we allowed a 2-week period for the animals to adjust to their confinement prior to initiating the experiment, it is possible that the process of habituation to their situation was ongoing during the experiment, resulting in lower plasma cortisol concentrations in week 2. In addition, they may have been responding to increased human presence during the experiment in week 1, but may have felt less perturbed by our observer by week 2. Alternatively, the

observed higher stress levels in week 1 may have been associated with high burdens of coccidian parasites detected in the buffalo at the time. We treated this infection by adding cocciastatic drugs to the buffalos' drinking water, and by week 2 of the experiment, parasite burdens were greatly reduced.

Animals with elevated baseline glucocorticoid concentrations often show a weaker acute glucocorticoid response (Creel 2001; Romero et al. 2009). We observed a similar pattern, where the higher initial plasma cortisol concentrations during week 1 were associated with a lower change in magnitude post ACTH injection compared to week 2. Our results are thus consistent with the idea that animals experiencing constitutively less stressful conditions are more able to respond to acute stressors.

The differences in overall plasma cortisol concentrations between week 1 and week 2 were not detected in the FGMs, because the magnitude of differences was small or the variability among animals was large. In this study, the experimental manipulation was detectable, because changes were consistent across animals (e.g., experimental: treatment response across similar conditions), and they were large (change in magnitude from initial levels to peak concentrations), whereas the initial differences between week 1 and 2 were not detectable with FGMs, because they were variable across animals, but relatively small.

Capture, confinement and handling typically increase endogenous glucocorticoid levels in wild animals (Hamilton and Weeks 1985; Cook et al. 1996; Hopster et al. 1999; Harper and Austad 2000; Millspaugh et al. 2001). Indeed, avoiding the immediate stress response due to capture and handling is one of the reasons for measuring stress responses non-invasively in fecal material rather than blood. In this study, we showed that responses to immobilization and handling are age-dependent in African buffalo: immobilized, saline-injected adults showed elevated FGMs compared to control animals that were not immobilized, whereas subadults mounted no detectable stress response to immobilization and handling. The adult buffalo used in this experiment had been captured using chemical immobilization on 8-10 previous occasions (Peter Buss, [SANPARKS, Veterinary Wildlife Services, Kruger National Park, South Africa], personal communication, [October 2016]), whereas the subadults were naïve to the experience. It is therefore

possible that prior capture experience may moderate how stressful a capture event is perceived by buffalo. Similar results were found by Moore et al. (2000), where mature male red-sided garter snakes (*Thamnophis sirtalis parietalis*) showed greater elevation following capture stress than younger individuals. The relationship between age and FGM concentrations also varies considerably across studies, from no effect in Alaskan brown bears (*Ursus arctos horribilis*, von der Ohe et al. 2004), African elephants (*Loxodonta africana*, Viljoen et al. 2008), Egyptian spiny mice (*Acomys cahirinus*, (Nováková et al. 2008), and meerkats (*Suricata suricatta*, Braga Goncalves et al. 2016) to a positive correlation in elk (*Cervus elaphus*, Creel et al. 2002) and a negative correlation in male Alpine chamois (*Rupicapra* spp., Corlatti et al. 2014). Therefore, the observed differences in response to capture may simply reflect age-related differences in behavior and stress physiology that are unrelated to the animals' experience of capture and immobilization.

This study confirms that stress responses can be assessed non-invasively via analysis of glucocorticoid metabolites in feces in wild African buffalo of both sexes and across a broad spectrum of ages. Both of the commercially available FGM assay kits we evaluated were suitable for FGM detection in a typical ecological field study context. Our work also points to age or experience–related variation among buffalo in stress response to chemical immobilization. We believe that our findings will provide a practical contribution facilitating eco-physiological research and conservation management of this key grazer species in sub-Saharan African savanna biomes.

1.6 Tables and Figures

Table 1.1 Summary of the African buffalo (*Syncerus caffer*) group composition (group, sex, age, treatment trial, body weight, treatment dose and number of fecal samples collected).

Animal ID	Sex	Age (yrs.) *	Treatment trial 1 (Week 1)	Treatment trial 2 (Week 2)	Body weight (kg)	Treatment dose (ml)	# Fecal samples collected
GROUP 1							
B266	Female	9	Saline	ACTH	486	4.86	48
B252	Female	8	ACTH	Saline	490	4.90	33
G46	Female	2.5	ACTH	Saline	328	3.28	49
G53	Male	2	Saline	ACTH	262	2.62	36
GROUP 2							
G30	Female	2.5	Saline	ACTH	306	3.06	45
G44	Female	2	ACTH	Saline	278	2.78	45
G28	Male	3	ACTH	Saline	378	3.78	34
G29	Male	2	Saline	ACTH	300	3.00	41
GROUP 3							
B163	Male	9	Control	Control	NA	NA	32
B203	Male	8.5	Control	Control	NA	NA	29
G35	Male	3.5	Control	Control	NA	NA	36
G36	Male	3.5	Control	Control	NA	NA	29

* For analyses, all buffalo were categorized into subadults (≤3.5 years) or adults (>5 years).

Table 1.2 Summary of linear mixed effects model for the FGMs (for both assays) comparing control and saline treated African buffalo (*Syncerus caffer*), accounting for time, age, sex, and random effects of individual buffalo (n = 12) during both trials (weeks) of the validation experiment. Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: treatment = saline; week = week 2; age = adult; sex = female; time = initial FGM level. Sample size for age category = 4 adults and 8 subadults. Sample size for sex category = 7 males and 5 females. FGM = fecal glucocorticoid metabolite; EIA = enzyme immunoassay; RIA = radioimmunoassay.

Predictor	Effect[a]	RIA (n = 162)			EIA (n = 144)		
		Log FGM (ß) Estimate ± SE (ng/g)	t-value	P-value	Log FGM (ß) Estimate ± SE (ng/g)	t-value	P-value
Fixed effects							
(Intercept)		**3.93 ± 0.20**	**19.33**	**< 0.0001**	**2.48 ± 0.33**	**7.46**	**<0.0001**
Treatment: control		-0.31 ± 0.33	-0.93	0.3699	-0.98 ± 0.57	-1.71	0.1078
Time: 0 – 10 hours		0.01 ± 0.24	0.06	0.9537	-0.04 ± 0.38	-0.10	0.9221
Time: 10.1 – 20 hours		0.10 ± 0.19	0.53	0.5948	0.05 ± 0.30	0.16	0.8710
Time: 20.1 – 30 hours		0.07 ± 0.20	0.37	0.7089	-0.23 ± 0.31	-0.72	0.4711
Time: 30.1 – 40 hours		-0.07 ± 0.20	-0.36	0.7178	-0.13 ± 0.33	-0.39	0.7007
Time: 40.1 – 50 hours		0.04 ± 0.19	0.19	0.8468	0.22 ± 0.30	0.73	0.4663
Time: 50.1 – 60 hours		-0.21 ± 0.22	-0.95	0.3455	-0.66 ± 0.38	-1.77	0.0805
Time: 60.1 – 70 hours		0.20 ± 0.20	1.02	0.3084	-0.26 ± 0.33	-0.80	0.4258
Time: 70.1 – 80 hours		0.02 ± 0.19	0.12	0.9026	0.08 ± 0.33	0.25	0.8006
Time: 80.1 – 90 hours		-0.12 ± 0.24	-0.46	0.6473	0.11 ± 0.38	0.30	0.7620
Time: 90.1 – 100 hours		-0.25 ± 0.20	-1.23	0.2199	-0.29 ± 0.31	-0.91	0.3641
Time: 100.1 – 110 hours		-0.14 ± 0.24	-0.59	0.5596	-0.58 ± 0.38	-1.54	0.1272
Time: 110.1 – 120 hours	↓	**-0.56 ± 0.22**	**-2.54**	**0.0125**	-0.04 ± 0.38	-0.10	0.9202
Time: 120.1 – 130 hours		0.05 ± 0.27	0.19	0.8533	-0.21 ± 0.42	-0.50	0.6165
Time: 130.1 – 140 hours		-0.15 ± 0.42	-0.36	0.7189	-0.60 ± 0.68	-0.89	0.3750
Time: 140.1 – 150 hours		-0.37 ± 0.42	-0.87	0.3865			
Week: 1		0.18 ± 0.14	1.25	0.2573	-0.04 ± 0.24	-0.16	0.8772
Age: subadult	↓	-0.14 ± 0.18	-0.77	0.4673	**-1.15 ± 0.31**	**-3.73**	**0.0068**
Sex: male		0.07 ± 0.17	0.40	0.6999	-0.05 ± 0.28	-0.19	0.8546

[a] Effect column indicates the direction of the slope predicted by the model.

Table 1.2 *Continued…*

Predictor	Effect[a]	RIA (*n* = 162)			EIA (*n* = 144)		
		Log FGM (ß) Estimate ± SE (ng/g)	*t*-value	*P*-value	Log FGM (ß) Estimate ± SE (ng/g)	*t*-value	*P*-value
Interaction terms							
Treatment (control):Time (0 – 10 hours)		-0.19 ± 0.34	-0.56	0.5760	-0.30 ± 0.56	-0.54	0.5910
Treatment (control):Time (10.1 – 20 hours)		0.14 ± 0.29	0.49	0.6251	-0.28 ± 0.49	-0.57	0.5683
Treatment (control):Time (20.1 – 30 hours)		-0.34 ± 0.29	-1.15	0.2531	0.01 ± 0.50	0.02	0.9842
Treatment (control):Time (30.1 – 40 hours)		-0.31 ± 0.30	-1.02	0.3113	0.15 ± 0.53	0.28	0.7784
Treatment (control):Time (40.1 – 50 hours)		-0.32 ± 0.28	-1.15	0.2525	-0.42 ± 0.47	-0.90	0.3721
Treatment (control):Time (50.1 – 60 hours)	🡻	**-0.80 ± 0.39**	**-2.06**	**0.0411**	0.78 ± 0.64	1.22	0.2270
Treatment (control):Time (60.1 – 70 hours)	🡻	**-0.72 ± 0.32**	**-2.28**	**0.0243**	-0.20 ± 0.52	-0.39	0.6979
Treatment (control):Time (70.1 – 80 hours)		-0.29 ± 0.28	-1.03	0.3049	-0.89 ± 0.51	-1.76	0.0815
Treatment (control):Time (80.1 – 90 hours)		-0.34 ± 0.32	-1.06	0.2909	-0.70 ± 0.53	-1.31	0.1915
Treatment (control):Time (90.1 – 100 hours)		-0.17 ± 0.29	-0.59	0.5571	-0.35 ± 0.50	-0.71	0.4774
Treatment (control):Time (100.1 – 110 hours)		-0.66 ± 0.48	-1.36	0.1772	0.63 ± 0.77	0.81	0.4183
Treatment (control):Time (110.1 – 120 hours)		0.15 ± 0.30	0.49	0.6222	-0.44 ± 0.53	-0.82	0.4121
Treatment (control):Time (120.1 – 130 hours)	🡻	**-0.81 ± 0.35**	**-2.32**	**0.0221**	-0.26 ± 0.57	-0.46	0.6489
Treatment (control):Week (1)		0.23 ± 0.17	1.31	0.2136	0.08 ± 0.30	0.26	0.7966
Treatment (control):Age (subadult)	🡹	0.55 ± 0.25	2.18	0.0781	**1.25 ± 0.43**	**2.87**	**0.0273**
Random effects							
		Variance	*SD*		Variance	*SD*	
Animal ID (intercept)		0.023	0.151		0.068	0.260	
Residuals		0.149	0.385		0.365	0.604	

[a] Effect column indicates the direction of the slope predicted by the model.

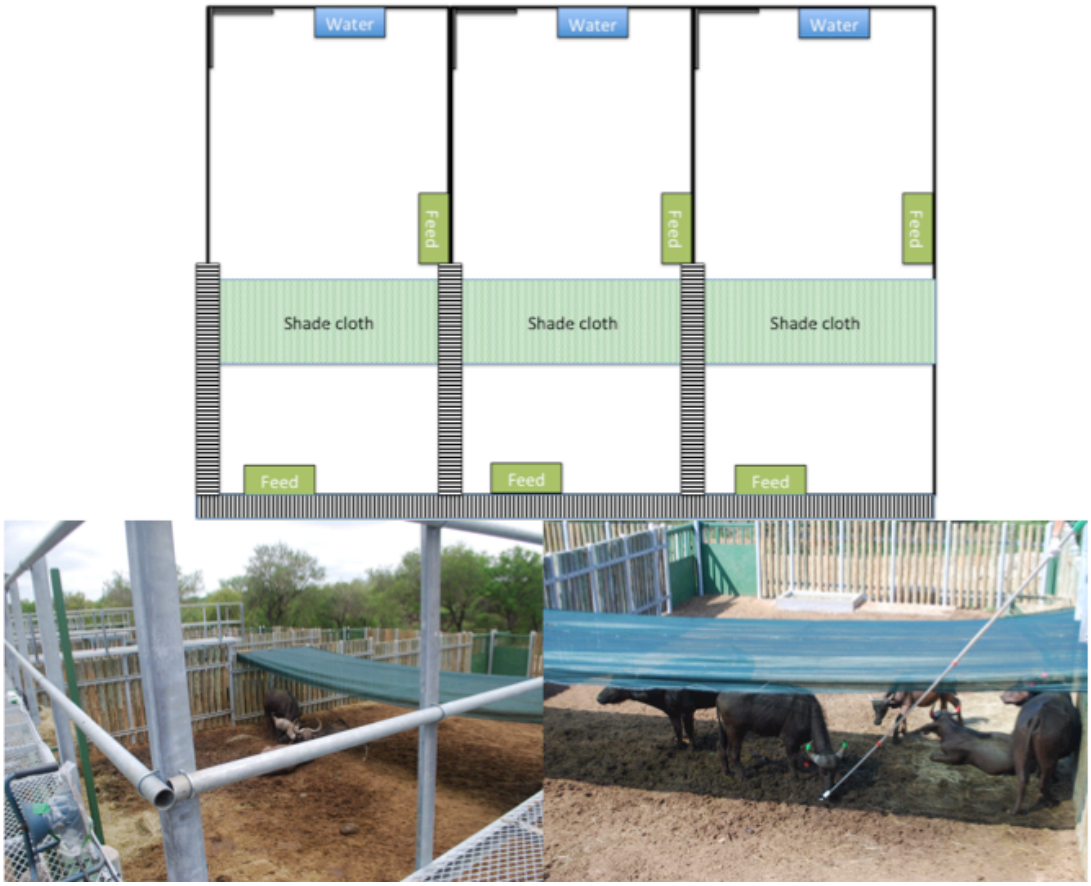

Fig. 1.1 Layout of the holding facility during the observation and sample collection of the ACTH validation challenge.

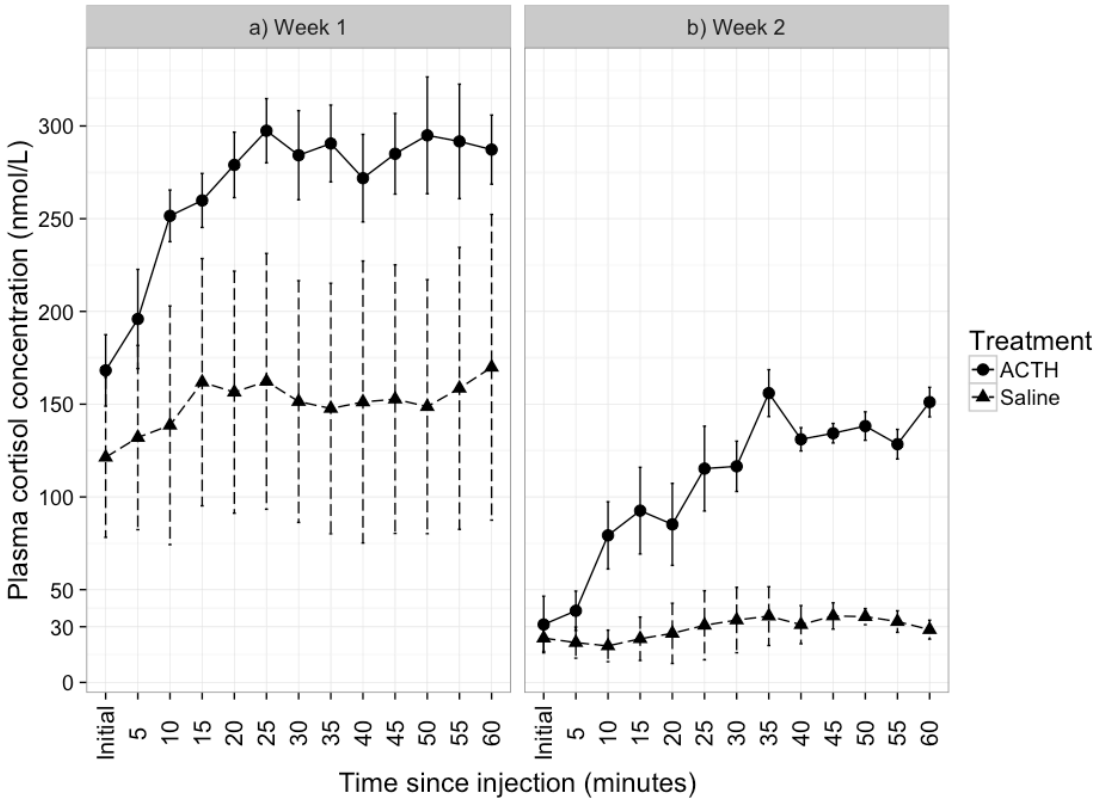

Fig. 1.2 a) The mean plasma cortisol concentration as a function of time post ACTH injection during week 1, and b) week 2 for ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups (n = 4 per group). Time point "0" indicates initial level. ACTH-stimulated African buffalo (*Syncerus caffer*) had significantly higher plasma cortisol concentrations than saline-injected buffalo at all time points between 5 minutes and 1 hour since application, after accounting for age, sex, week, and the random effects of individual buffalo (Supplementary Data SD1.1). Error bars represent *SE*.

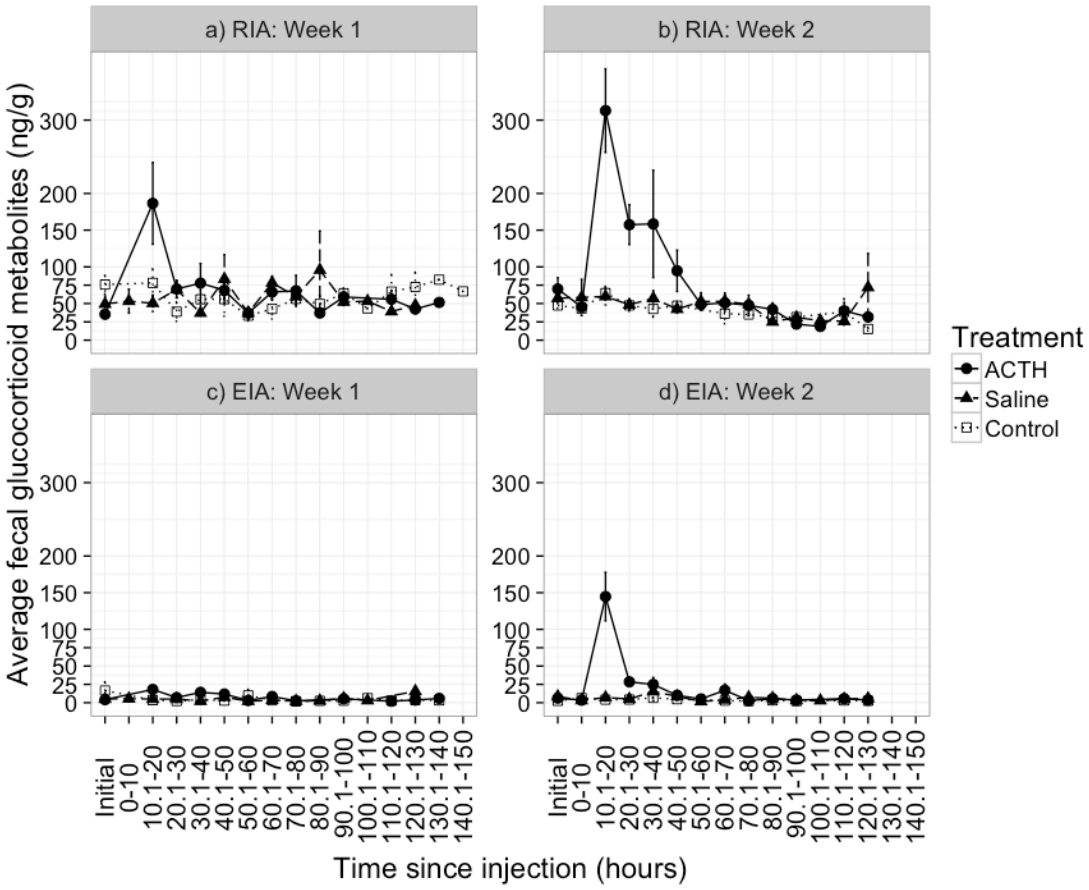

Fig. 1.3 a) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups of African buffalo (*Syncerus caffer*), as well as for the control (open squares, dotted line) group (n = 4 per group) during week 1, and b) week 2 from the RIA results. c) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups, as well as for the control (open squares, dotted line) group (n = 4 per group) during week 1, and d) week 2 from the EIA results. Error bars represent *SE*. The RIA detected the ACTH-induced peak in FGM consistently in both weeks. Overall, the EIA recovered less FGM than the RIA. The EIA did detect the ACTH-induced peak in FGM in both weeks, but in week 1, detection levels in all groups were comparatively low (Supplementary Data SD1.3).

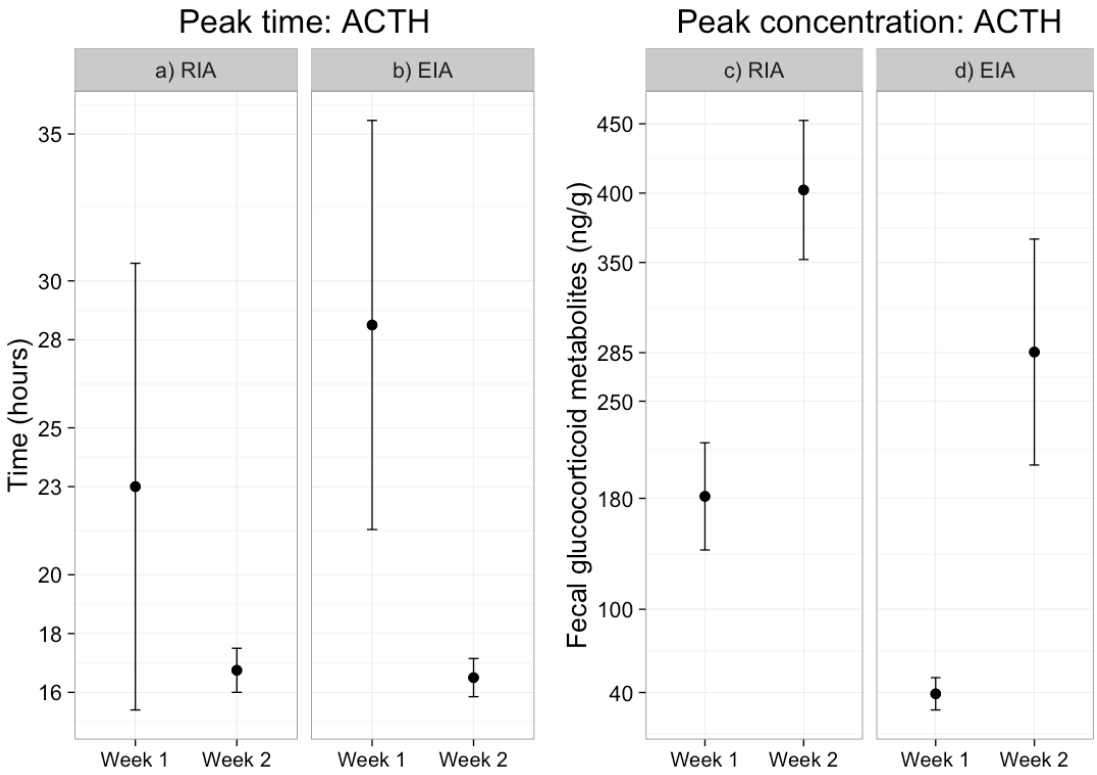

Fig. 1.4 a) The average time to peak for ACTH-stimulated African buffalo (*Syncerus caffer*) by week for the RIA (n = 8) and b) EIA (n = 8) results. Time to peak is not statistically different between weeks for both the RIA and EIA, as well as between assays. c) The average peak FGM concentration for ACTH stimulated buffalo by week for the RIA (n = 8) and d) EIA (n = 8). Error bars represent *SE*. The RIA detected a higher peak concentration of FGMs than the EIA in both weeks, and peak FGM was lower in week 1 than week 2.

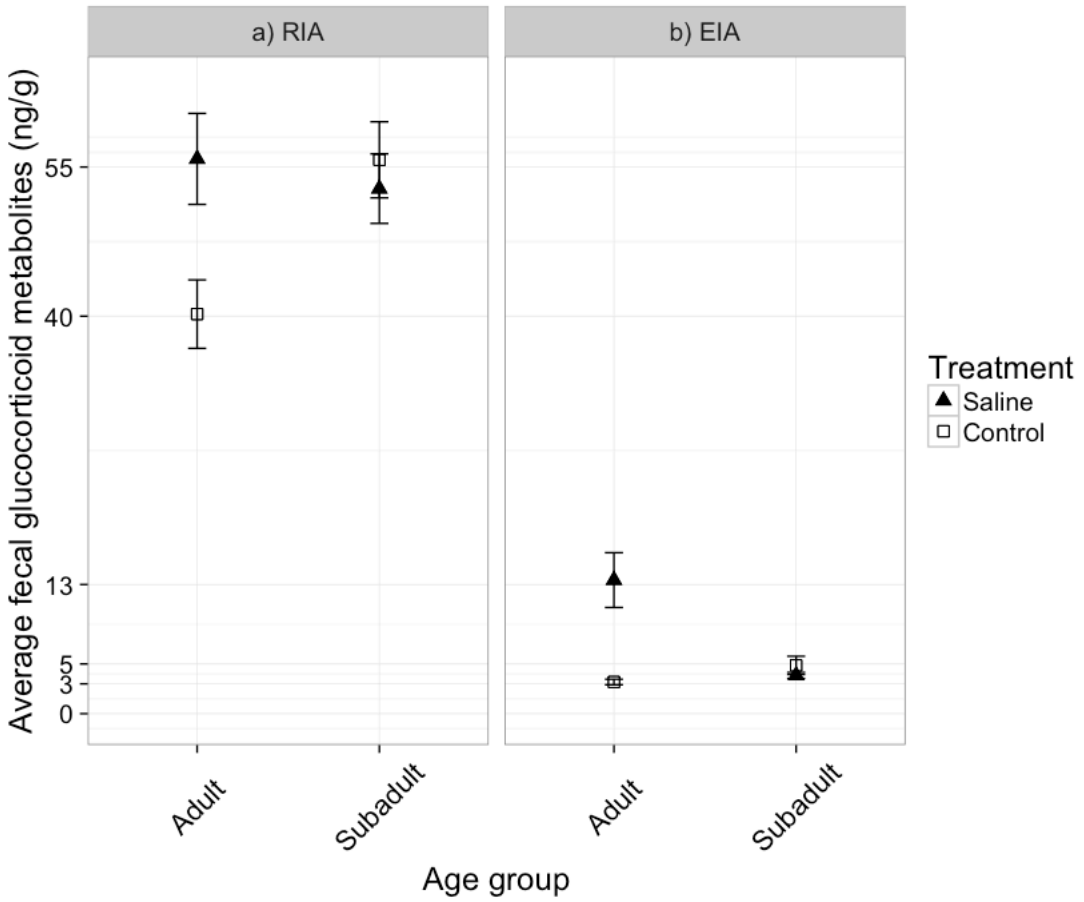

Fig. 1.5 a) Summary of the average FGM concentrations of immobilized, saline-treated African buffalo (*Syncerus caffer*) (triangles) and unhandled controls (open squares) across different age groups from the RIA results, and b) the EIA results shows the significant interaction term between age and treatment (Table 1.2). Adults, but not subadults, mounted a stress response to immobilization. Error bars represent *SE*.

CHAPTER 2

Feedback between stress responses and infectious diseases in a wild mammalian population

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Currently *in preparation* for Functional Ecology.

2.1 Abstract

Stress in wild animals represents an immediate measure of the physiological response to changes in the environment, and, potentially, a prospective assessment of the animal's health and well-being. In wild mammals, stress responses have been linked to changes in nutritional availability and predation, as well as anthropogenic disturbances including direct human contact and habitat loss. The recent global rise in infectious disease emergence in wildlife and humans raises the question whether such stress responses may play a role in facilitating the spread of infections, linking physiological effects of environmental change with disease dynamics. While stress responses have been linked to increased disease susceptibility in humans and domestic animals, its role in shaping or its response to, disease patterns in natural populations has been less clear. Here we tested whether infectious diseases affected stress responses (measured as fecal glucocorticoid metabolites, FGMs), and vice versa, in a free-ranging population of African buffalo. We measured FGMs, and infections by viral and bacterial pathogens, as well as gastrointestinal parasites in a longitudinal study including 200 individually marked hosts that were sampled every six months, for four years. Acute infections, particular infections by acute viral pathogens contributed to elevated FGMs in the buffalo, whereas chronic infections did not. In turn, increased FGMs were associated with subsequent chronic bacterial infections, suggesting a feedback cycle between disease (acute virus and chronic bacteria) and stress responses in this wild population. Our study thus points to the utility of FGMs as an integrative measure connecting the animal's environment, its physiological responses, and patterns of infectious disease. More alarmingly, our findings suggest a stress-mediated vortex of ill-health that may exacerbate the vulnerability of already struggling wildlife populations.

2.2 Introduction

Animals respond to exposure to unpredictable events by mounting a physiological stress response, which activates the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky 2002), followed by a cascade of effects, orchestrated by stress hormones including epinephrine and glucocorticoids (e.g., predominantly cortisol (ruminants, primates, horses, pigs) or corticosterone (mice, birds, reptiles), (Siegel 1980; Sapolsky et al. 2000; Tsigos and Chrousos 2002; Palme et al. 2005).

Cortisol levels can become elevated in response to a broad range of disturbances and as such, may serve as an integrative measure of animal health (McEwen 2004; Mormède et al. 2007; Palme 2012). In studies involving wild animals that requires immobilization, stress response is most commonly measured using non-invasive methods, such as detection of cortisol's breakdown products in feces (Möstl and Palme 2002; Palme et al. 2005). Non-invasive stress markers (i.e., FGMs) can provide a mechanistic link between habitat change or disturbance and population health, and a powerful tool for tracking stress responses over extended time periods (Palme et al. 1999; Touma and Palme 2005). For example, extreme climate changes such as heat waves and/or severe winter weather were marked by increases in FGMs and high mortality rates in cattle in certain regions of the United States (Mader 2003). High predator abundances (Boonstra et al 1998; Scheuerlein et al 2001 Clinchy et al 2004; Mateo 2007) were reflected in increased FGMs in snowshoe hares, tropical stonechats, song sparrows, and Belgian's ground squirrels; increases in poaching activity elicited stress responses in African elephants (Gobush et al. 2008); and capture, handling, or transportation of animals (Bateson and Bradshaw 1997; Palme et al. 2000; Gentsch et al. 2018) are all positively correlated with FGMs. Stress responses to infectious diseases, specifically viruses have been associated with increased mortality risk. For example, in calves, under transportation stress, bovine herpesvirus 1 increased the risk of pneumonia or even mortality (Filion et al. 1984; Peterson et al. 1991), and in mice, herpes simplex and influenza A were associated with increased mortality under stressors such as restraint, and forced exercise (Ilbäck et al. 1984; Peterson et al. 1991). Functionally, stress responses aids in redistributing metabolic allocation to help the animal deal with emergencies - an adaptive response

essential to maintaining homeostasis (McEwen and Wingfield 2003). As such, stress responses can prompt upregulation of immune responses, especially cellular-mediated immunity during an acute stress response (Dhabhar and McEwen 1999; Sternberg 2006; Dhabhar 2009; Martin 2009). On the other hand, chronic stress can have negative health effects through down-regulation of immune responses (Reiche et al. 2004; Dhabhar 2009; Martin 2009), which may increase disease susceptibility (infections such as colds, herpes, human immunodeficiency virus, (Herbert and Cohen 1993; Leserman et al. 2000), and worsen disease progression of cancers (Glaser and Kiecolt-Glaser 2005; Dhabhar 2009). As such, physiological stress may respond to pathogenic challenges, but also modify the host's immune responses and susceptibility to infection - potentially modulating the host-pathogen interaction, and its outcomes for host health and disease dynamics. We investigated this putative cycle of stress and infections, using a longitudinal study of 200 individually marked, free-ranging African buffalo, in which we assessed FGMs and infections by viral and bacterial pathogens, and gastrointestinal parasites, every six months over a four-year period.

Buffalo are ecologically important, non-selective bulk grazers, which influence the function of savanna ecosystems by shaping plant communities and consequently affecting other herbivores (Mcnaughton 1978; Huntley 1991; Olff and Ritchie 1998). They are also an important prey species for lions (*Panthera leo*) and have economic value as a key species in the hunting and tourism industries. Buffalo are highly gregarious and carry many pathogens and parasites, some of which can cause important diseases in livestock (Michel and Bengis 2012). In livestock, cycles of stress and disease have been described. For example, the bovine respiratory disease complex (or "shipping fever") includes opportunistic infections by viral pathogens (e.g. bovine adenovirus-3, parainfluenza-3 virus, bovine respiratory syncytial virus, bovine viral diarrhea virus) when the host's immune responses are compromised due to stressors such as transportation (Earley et al. 2017). Primary viral infections are often succeeded by secondary infections by bacteria that are commonly present as commensals in the bovine upper respiratory tract (e.g. *Mannheimia haemolytica*, *Mycoplasma bovis*), (Timsit et al. 2013; Guzman and Taylor 2015), but gain access to

lower respiratory organs following viral infections. Under these circumstances, such bacteria act as pathogens, causing pneumonia in the host, and contributing to massive economic impacts on the US and global cattle industries (Griffin et al. 2010). By contrast, in natural populations in the absence of acute, highly stressful human interventions, it is less clear whether positive feedback cycles between stress responses and infections play a role in shaping disease dynamics. This question is of urgent importance in the context of the current surge in infectious disease emergence (Acevedo-Whitehouse and Duffus 2009; Altizer et al. 2013). Infectious diseases have been implicated in wildlife population declines and extinctions; yet in many cases, it is likely that multiple stressors acting in synergy are responsible for these declines. Here we use our longitudinal study design to investigate how stress responses (measured as FGMs) and infectious diseases interact in a natural mammalian population, asking (i) How do infections by a diverse suite of parasites affect stress responses (i.e., FGMs)?; (ii) How does stress responses affect the incidence of these infections?; and ultimately, (iii) Is there evidence for positive feedback-cycles between stress responses and disease in a relatively undisturbed population of wild buffalo?

2.3 Methods

Study site.—This study took place in the southern region of Kruger National Park (KNP), South Africa. KNP is approximately 19 485 km^2 in extent and lies between 22.5 and 25.5°S, and 31.0 and 31.57°E (Fig. 2.1). Rainfall occurs primarily during the summer (October-March) and varies from north to south from 400-700 mm, on average, per year. Gradients in rainfall and soil types (granite soils west and ecca shale, basalt and rhyolites succeeding east) contribute to strong regional variation in forage availability; and the seasonal and inter-annual variation in rainfall creates dramatic temporal variation in food quality and availability for herbivores. Landscape types within the main study area (southern region) varies from Lebombo mountain bushveld, Marula (*Sclerocarya birrea subsp. caffra*)/Knobthorn (*Vachellia nigrescens*) savannah, Delagoa thorn (*Vachellia welwitschii*) thickets, Sabie and Crocodile River thickets and Thornveld (Venter, Scholes & Eckhardt 2003). The

buffalo population has been estimated as approximately 30 000 throughout the park (Cross et al. 2009).

This study took advantage of a four-year field study investigating multi-parasitic infection patterns and immune profiles in female African buffalo in KNP (Ezenwa and Jolles 2015).

Mass Capture and Collaring.—Two hundred female African buffalo (2-5yrs. of age) were darted using a helicopter and a large ground team (South African National Parks' Veterinary Wildlife Services and Game Capture team) in the Lower Sabie section (during June/July 2008) and Crocodile Bridge section (during October 2008) of Kruger National Park (100 buffalo in each section). Each individual buffalo was fitted with either a radio (n=193) or satellite (n=7) collar. For identification purposes, each buffalo received a very high frequency (VHF) transmitter built into the collar accompanied with a unique letter and number combination that was branded on both sides of their rump. If an animal died it was replaced by a new individual from the herd from which the deceased animal originated (n=261).

Recaptures and Sample collection.—Each buffalo was recaptured every 6 months (biannually) for 4 years (June 2008 – August 2012), resulting in up to 9 captures per individual (fewer, if the animal died during the study period, or was added in later). Telemetry was used to locate specific buffalo and a vehicle to access them; occasionally, helicopter-assisted captures were utilized to complete regular captures of all individuals. All immobilizations were conducted by a veterinarian according to the South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. Each buffalo received a combination of etorphine hydrochloride (Novartis, Kempton Park, 1619, South Africa), azaperone (Janssen Pharmaceutical Ltd., Halfway House, 1685, South Africa) and in some cases hyaluronidase (Kyron Laboratories, Benrose, 2001, South Africa). This drug combination was delivered into the muscle of the rump remotely, using a dart propelled by a compressed air rifle (DAN-INJECT, International S.A., Skukuza, 1350, South Africa). The doses varied between 7-9 mg etorphine, 40-80 mg azaperone and 5000 i.u. hyaluronidase depending on the size of the animal. Following data collection, naltrexone (the antidote to etorphine

hydrochloride; 40 mg/ml, Kyron) was administered intravenously and the animal kept under observation until fully recovered. Each individual buffalo was sedated for approximately 10-15 minutes.

Environmental variables collected for each immobilized buffalo.—We divided seasons at the study site into early wet (October-December), late wet (January-March), early dry (April-June), and late dry season (July-September). Year was classified into rainfall years: October 2008-September 2009, October 2009-September, October 2010-September 2011, October 2011-August 2012. Fecal chlorophyll measures the green color signal recoverable in fecal material, and is an indicator of food quality (higher quality forage results in a stronger chlorophyll signal; Christianson and Creel 2009). Rectal fecal samples were used to determine chlorophyll optical density values at 415 wavelengths, which have shown peaks in the absorption spectra of fecal extracts of African herbivores (Christianson and Creel 2009).

Demographic variables collected from each immobilized buffalo.—Animals were aged according to body size and horn development in juveniles less than two years old (Jolles 2007), and incisor emergence patterns were used for individuals between 2 and 5 years old (Grimsdell 1973b). In older buffalo, the tooth wear of the first incisors was evaluated for age determination (Jolles 2007). For statistical analysis, age was recorded in months and presented as a continuous variable. Body condition was assessed by palpating the ribs, spine, hip, and base of the tail and scoring them on a scale of 1 (very poor) to 5 (excellent) and calculating the average of all four areas (Ezenwa et al. 2009). These manual body condition scores are correlated with host kidney fat index (Ezenwa et al. 2009). Herd membership was recorded as the herd each buffalo was captured with (Lower Sabie, Corner, Mountain, Powerline, Thicket, or Northwest). To account for the positive correlation between total horn length and age, the regression residuals of total horn width (cm) as a function of age (months) were calculated. A higher value was interpreted as above average horn size for their age, reflecting a measure of individual quality (Ezenwa and Jolles 2008). Rectal pregnancy tests were performed by a veterinarian and recorded as being pregnant or not. Lactation status (present or absent) was determined

by manual milking of all four teats (Jolles et al. 2005). Reproductive status was recorded as active if a buffalo were pregnant and/or lactating at the time of capture. Fifty percent of the buffalo (treatment group) received a long-acting antihelminthic bolus (Panacur® slow-release bolus, Hoechst Roussel), a de-wormer to examine the effects of gastro-intestinal parasite removal on bovine tuberculosis progression (Ezenwa and Jolles 2015). The control group received no bolus. Blood was collected from the jugular venipuncture into appropriate tubes for disease diagnostics. Blood and fecal samples were stored on ice and transported back to the lab within 8 hours. Aliquoted blood samples were stored at -20°C until analysis.

Stress response.—Fecal Glucocorticoid Metabolites (FGMs) were quantified using established methods validated for African buffalo (Spaan et al. 2017). Briefly, rectal fecal samples were collected from each immobilized buffalo. The concentration of steroid metabolites in the extracted fecal samples was measured with a double antibody RIA (^{125}I Corticosterone RIA kit; MP Biomedicals LLC, Santa Ana, CA 92707, USA). The FGM concentrations is not a reflection of the capture and handling effect, due to the 10-20hour lag time between the increase of circulating plasma cortisol and the appearance of its metabolites in the feces (Spaan et al. 2017).

Disease diagnostics

Each immobilized buffalo was tested for various parasites and pathogens. Methods for respiratory diseases have been previously described (Glidden et al. 2018). Briefly, separate competitive ELISA kits (Bio-X Diagnostics, Belgium) following the manufacturers' instructions were used to determine seropositivity and seronegativity for Bovine Respiratory Syncytial virus (BRSV) and Bovine Viral Diarrhea virus (BVDV). A direct ELISA test kit (Bio-X Diagnostics, Belgium) following manufacturer's instructions and provided formula was used to calculate the specific degree of seropositivity for Bovine Herpesvirus I (BHV), Bovine Parainfluenza-3 (PI3), Bovine Adenovirus-3 (AD3), *Mannheimia haemolytica* (MH) and Mycoplasma bovis (MB). All ELISA's were run on stored serum in November 2013.

To test for Mycobacterium bovis (BTB) a standardized interferon gamma (IFNy) assay, previously optimized for African buffalo (Michel et al. 2011) was used

with a BOVIGAM ELISA kit (BOVIGAM, Prionics, Switzerland) following manufacturer's instructions. An animal was considered BTB positive after two consecutive positive test results and remained infected thereafter (Ezenwa and Jolles 2015). To test for brucellosis an ELISA test kit (Brucellosis Serum Ab ELISA test, IDEXX #P04130) was used to determine seropositivity and seronegativity (Gorsich et al. 2015a).

The various parasites and pathogens were grouped into acute infections, including acute viruses (AD3, PI3, and BRSV) and acute bacteria (MH), as well as chronic infections, including chronic viruses (BVDV, BHV) and chronic bacteria (BTB, MB, Brucellosis, *Anaplasma centrale*, and *A. marginale*), see Box 2.1.

Diagnostic assays are available for these pathogens because they also occur in livestock. However, buffalo are likely to be exposed to many pathogens that do not occur in cattle, and that we therefore have no diagnostic tests for. To estimate the occurrence of exposure by unknown pathogens, we included an assay quantifying haptoglobin in our diagnostic panel. Haptoglobin, an acute phase protein has shown to be a reliable indicator of an acute infection caused by various infections in cattle (Horadagoda et al. 1999) and African buffalo (Glidden et al. 2018). To detect circulating plasma levels a commercially available ELISA kit for bovine haptoglobin (Life Diagnostics, product number HAPT 11) was run on all plasma samples in November of 2013 (Glidden et al. 2018).

Rectal fecal samples were used to determine gastrointestinal infections including, macroparasites such as strongyles and a microparasite protozoan such as coccidia, using a modified McMaster fecal egg counting technique (Ezenwa 2003). These fecal egg counts were used as a proxy of worm burdens (Ezenwa and Jolles 2015).

Schistosome infection burden was quantified using established methods validated for African buffalo (Beechler et al. 2017). Briefly, a lateral flow assay was used to measure circulating anodic antigen (antigen produced by schistosomes and released in the blood of host) concentrations (pg/ml) in serum (Beechler et al. 2017).

Diagnostics for haemoparasites are published elsewhere (Henrichs et al. 2016). Briefly, diagnostics for *Anaplasma centrale* and *A. marginale* were performed

at the Department of Veterinary Tropical Disease, University of Pretoria (Pretoria, South Africa) using Reverse Line Blot Hybridization from genomic DNA extractions of 200μL of whole blood (Henrichs et al. 2016).

All procedures conducted during this research were approved by South African National Parks Board (Reference No. CALJM728), and Oregon State University's Animal Care and Use Committee (OSU ACUP No. 3822).

Statistical Analysis

In order to look at FGMs as a personality trait (i.e., how repeatable is FGMs among individual buffalo?), the rptGaussian function of the rptR package (Stoffel et al. 2017) was used to calculate the repeatability, R to determine the variation of FGMs among group means (Animal ID) over the sum of group-level and residual (data-level) variance (Stoffel et al. 2017). The function was set at 1000 bootstraps and permutations.

Environmental and demographic drivers of stress.—To determine which environmental and demographic factors predict stress in buffalo, linear mixed-effect models (LMEMs) were used to account for the random effect of repeated measures (Bates et al. 2012). The dredge function, an automated model selection function of the multi-model inference (MuMIn) package (Bartoń 2016) was used to generate a set of models with combinations of all environmental and demographic variables in the global model and ranked based on the second-order Akaike information criteria (AICc). Only two-way interactions of the remaining explanatory variables in the best model were considered and a top-down strategy (Diggle et al. 2002) was followed. Briefly, starting with the best model including all possible two-way interactions, all interaction models were compared using the likelihood ratio test via ANOVA. Two-way interactions were only kept in the best model if statistically significant. In general, model comparisons were based on the maximum likelihood (ML) criterion and the residual maximum likelihood (REML) criterion was used to draw inferences on the final model selected (Bates et al. 2012).

P-values were obtained using the lmerTest package (Kuznetsova et al. 2016) and considered statistically significant if $P \leq 0.05$. Visual inspection of residual plots revealed the necessity to log transform the response variable (FGM). FGM results

were expressed as nanograms of glucocorticoid metabolites per gram of dry feces (ng/g). The dredge function was also used to determine the importance of each variable, where importance is the sum of the Akaike weights' of all possible model combinations (a value between 0-1).

Incidence measures.—Incidence was calculated for all acute and chronic disease diagnostics and defined whether a new infection occurred at that capture period (t_i), except for intestinal parasites (i.e., strongyles, coccidia, and schistosomes) for which we measured abundance. Incidence was determined from serological tests following manufacturers' instructions (Bio-X Diagnostics, Belgium) for: 1) BRSV and BVDV (competitive ELISA kits), using optical density readings (i.e., values ranged from 0-100%, and considered exposed if value > 50%) and 2) direct ELISA test kits for BHV, PI3, AD3, MB, MH serostatus (i.e., scored on a 0-5 scale and considered exposed if score increased by two or more points between two consecutive captures). Acknowledging the subtleties associated with acute infections that incidence cannot happen in consecutive time periods (i.e., if antibody titer is already high at t_0, then even if the animal is re-exposed, we cannot detect it, because the titer at t_1 would not be different). Other subtleties include BHV, *A. centrale*, and *A. marginale*, where we cannot distinguish new exposures from recrudescence of latent infections. Buffalo that tested positive for chronic infections such as BTB and brucellosis remained infected for the remaining study period and can be considered true incidence (Ezenwa and Jolles 2015; Gorsich et al. 2015b).

Acute infections were grouped into a binary variable based on whether a buffalo was infected by one or more acute infection (viral and/or bacterial). Similarly, chronic infections were also grouped by whether a buffalo was infected by one or more chronic infection (viral and/or bacterial). The average yearly incidence rate for the various acute (MH, PI3, AD3, BRSV) and chronic infections (MB, BHV, BTB, Brucella, BVDV, *Anaplasma centrale*, *A. marginale*) were calculated across the 4-year study period (October 2008-September 2009, October 2009-September2010, October 2010-September 2011, October 2011-August 2012). In the case of intestinal parasites, coccidia (an acute protozoan), strongyles and schistosomes (chronic infections), the average yearly fraction of the population infected were calculated

across the 4-year study period, due to results recorded as worm burden rather than incidence.

Do infections drive stress responses.—To test whether infections are associated with FGMs, each parasite and/or pathogen, was added individually or as a group (chronic/acute) to the best model (predicted by environmental and demographic variables), and only retained in the model if statistically significant. This is a multi-model selection, where each case represents a particular parasite and/or pathogen or group added to the best model (Burnham and Anderson 2004). The time point of stress at each capture interval (t_i) was compared to the incidence of each pathogen and/or parasite between the previous capture (t_{i-1}) and the current capture (t_i), to test whether disease incidence in the preceding 6 months affected cortisol levels. Importance and *P*-values were obtained as above. In addition, to account for variable samples sizes depending on the parasite/pathogen added to the best model, adjusted *P*-values were calculated to control for the false discovery rate (Benjamini and Hochberg 1995). The false discovery rate is the expected proportion of false discoveries amongst the rejected hypothesis (Benjamini and Hochberg 1995).

Does stress responses drive infections.—To test, whether FGMs is associated with infections, generalized linear mixed models (GLMMs) with the binomial family distribution and the logit link function (for binary variables) and LMEM (for continuous variables), were used to account for the random effect of repeated measures (Bates et al. 2012). All continuous explanatory variables within the global model were scaled by standardizing the coefficient estimates (i.e., centering by subtracting the mean of each variable and dividing it by its standard deviation) to ensure that all variables contribute evenly to the scale. Scaling was done by the scale function of the base package (R Core Team 2016).

Again, the dredge function was used to determine the best model for parasites and/or pathogens individually or as a group (chronic/acute) by generating a set of models with combinations of all environmental, demographic, and stress (i.e., FGMs) variables in the global model, and ranked based on the second-order Akaike information criteria (AICc). The incidence of each pathogen and/or parasite between time point (t_{i-1} and t_i) was compared to the time point of FGMs at the capture interval

(t_{i-1}) to test whether disease incidence is affected by previous cortisol levels. However, it is important to note that from previous analysis where acute infections are associated with elevated FGMs, we cannot test whether previous FGMs are associated with increased risk of acute infections. Because if increased antibodies at time point (t_{i-1} and t_i) caused elevated FGMs at t_i, then the antibodies cannot go up further between time point (t_i and t_{i+1}), even if there is exposure. This is a limitation of the serological diagnostic assay. Importance and *P*-values were obtained as above. The rsquared.glmm function of the MuMIn package (Bartoń 2016) was used to calculate the marginal and conditional R^2 for all the best models. The marginal R^2 represents the variance explained by the fixed effects, whereas the conditional R^2 represents the variance explained by both the fixed and random effects (Nakagawa and Schielzeth 2013).

The statistical program, R (R Core Team 2016) was used for all statistical analysis, including packages: lme4 (Bates et al. 2012), lmerTest (Kuznetsova et al. 2016), MuMln (Bartoń 2016), rptR (Stoffel et al. 2017) and ggplot2 (Wickham 2009).

2.4 Results

Overall, we obtained stress data from 269 free-ranging buffalo, sampled 2-9 times each, for a total of 536 buffalo-years of data on stress physiology in this natural population. Data coverage during this period was 74%, with 1320 fecal samples recovered from 1779 capture events. Cortisol levels, as evaluated from fecal glucocorticoid metabolites (FGMs), varied dramatically among individual buffalo, and within individuals over time (Fig. 2.3). Cortisol levels in individual buffalo were moderately repeatable ($R = 0.44$, $SE = 0.03$, $P = <0.0001$), suggesting that some buffalo tended towards consistently higher cortisol levels than others. In our models we controlled for this repeatability component of inter-individual variation by including Animal ID as a random effect. We then proceed to evaluate drivers of the remaining variation in FGMs representing plasticity in the level of FGMs expressed by buffalo.

Effects of reproductive status, individual quality, and body condition on stress responses.—Elevated FGMs in our study population of female buffalo was associated

with low body condition, poor individual quality (as indicated by small horns), but pregnancy in individuals with bigger horns (Supplementary Data SD2.1, Supplementary Data SD2.2, Supplementary Data SD2.3). In addition, strong seasonal and interannual variability in stress responses remained, indicating that temporally variable factors other than reproduction and nutrition played a role in stress physiology. In these basic models, which did not yet include any disease variables, year was the most important variable, followed by body condition, seasonal variability, horn residuals, and pregnant status (Fig. 2.4a).

Effects of infections on stress responses.—Infections were diverse and common in the buffalo (Fig. 2. 2 in Box 2.1), with incidence rates varying seasonally and among years (Beechler et al. 2017). The infections we measured included viral and bacterial pathogens, protozoal and helminth gastro-intestinal parasites; and both acute and chronically infecting parasites are represented (Box 2.1).

Acute infections, but not chronic infections, were associated with elevated FGM concentrations (Table 2.1). An effect of acute infections on stress responses was evident in models including either “acute infections”, “acute viruses” (see Box 2.1), bovine Adenovirus-3, or Haptoglobin (Table 2.1), an acute phase protein that is upregulated rapidly in buffalo during acute infections (Glidden et al. 2018). In addition, virus richness was associated with elevated FGMs in buffalo. Overall, the model including “acute infections” (whether an animal was infected by one or more acute bacteria or viruses) had the lowest AIC value (Table 2.1, AIC = 1125.8). Buffalos infected by one or more acute pathogen(s) during a given six-month capture period had 12% (8.4ng/g) higher FGM concentrations than buffalo that did not suffer any acute infections during the same time period (LMEM, $P = 0.0001$, Table 2.2, Fig. 2.5). Bovine adenovirus-3 (AD3) was the only single infection that had a significant effect on FGM (Table 2.1). AD3 explained a proportion of the acute infections variable, however, it did not drive it on its own and is still best represented by acute infections as a group (Supplementary Data SD2.4). In fact, acute infections were the most important variable driving FGM in our study population, followed by body condition, season, year, pregnant status, and horn residuals (Fig. 2.4b). Interestingly, once acute infections were included, year, season, horn residuals and pregnant status

all had reduced importance, suggesting that some of the variance in FGM due to these variables was actually driven by differences in acute infections associated with these variables.

Effects of stress responses on infections.—FGMs were an important, but only marginally significant variable associated with the increased risk of acquiring one or more chronic bacteria (GLMM, $P = 0.0871$, Table 2.3). Interestingly, FGMs were an important variable associated with the reduced risk of acquiring acute infections (incidence), (Table 2.3). This was evident from significant effects of FGM on the incidence of AD3 and important, but not significantly different for the reduced number of "acute viruses" (LMEM, $P = 0.1191$, Table 2.3). However, this negative effect of FGMs on disease incidence (acute viruses, AD3) might be spurious, because previous exposure to these pathogens would both raise FGMs, and prevent (for a while) re-infection (Supplementary Data SD 2.5; Coon et al. *in review*).

2.5 Discussion

Taken together, our results show that acute infections, notably by viruses including bovine Adenovirus-3 (AD3), are associated with increased FGMs in free-ranging buffalo, and that, vice versa, FGMs can contribute to the risk of acquiring one or more chronic bacteria. These reciprocal effects of elevated FGMs and chronic infections imply the potential for positive feedback cycles between FGMs and chronic bacterial infections in a natural, relatively undisturbed, mammalian population (Fig. 2.6). This is to our knowledge the first study to demonstrate reciprocal effects between infectious diseases and FGMs in a wildlife population.

Our longitudinal study design enabled us to tease apart effects of multiple factors, including environmental variation, reproduction, nutrition, individual variation, as well as infectious diseases on stress physiology. As in many other studies (e.g, red deer, *Cervus elaphus*, (Corlatti et al. 2011); reviews (Boonstra 2005; Busch and Hayward 2009; Baker et al. 2013)), good body condition was associated with lower FGMs in the buffalo. Interestingly, however, buffalo body condition peaks during the late wet (January – March) season (Spaan 2015), when FGM concentrations were high. The seasonal peak in FGMs thus appears not to be driven

by variation in nutritional resource availability. Instead, it may relate to the mating season, which also peaks between January and March (Ryan et al. 2007). During this time, buffalo herds include a higher proportion of adult males (Turner et al. 2005), which engage in vigorous competition over receptive females, involving fights among males, escorting and chasing of females (Sinclair 1977; Mloszewski 2010). This pattern could be further explained by the increased risk of predation associated with the peak calving season, considering that predation is known factors to increase stress (e.g., snowshoe hares (Boonstra et al. 1998; Lima 1998; Clinchy et al. 2004)), but unfortunately, we lack data on the fluctuations in predator density to support this statement. Horn size in female buffalo has been shown to correlate with abundance and richness of gastrointestinal parasites and has therefore been interpreted as a honest signal of superior health / individual quality in buffalo (Ezenwa and Jolles 2008). While gastrointestinal parasites did not affect FGM in this study, it is possible that horn growth may depend on the overall infection burden of the buffalo, including the acute infections that are relevant to elevated FGMs in buffalo. Alternatively, horn size may indicate long-term variation in body condition: Buffalo in better condition, which also tend to have low FGMs, may be able to invest more in horn growth in the long run.

African buffalo are host for many parasites and pathogens (Michel and Bengis 2012). Animals in this study typically experienced an incidence of more than 2.14 pathogens/year and 39% of the population was infected with gastrointestinal parasites/year. Upper respiratory infections were common among our study animals with diverse, complex, dynamic patterns varying from sporadic outbreaks to annual cycles, and responding to diverse environmental drivers (Coon et al. *in review*). Many of the respiratory pathogens observed in the buffalo are part of the bovine respiratory disease complex (BRDC); and similar to the BRDC infection cycle in cattle (Earley et al. 2017), we show that in buffalo, these respiratory pathogens may circulate via a stress-mediated co-infection cycle, where primary viral infections are associated with a stress response, which may increase the host's susceptibility to subsequent infection by other chronic bacteria, including pneumonia-causing bacteria. Acute infections, especially by viruses, have a quick onset and last for only a short time, being cleared

quickly by the host through a vigorous inflammatory immune response (Elenkov and Chrousos 2006; Martin 2009). Once an infection is cleared, it is essential to suppress further inflammation to limit immunopathological effects, such as tissue damage (Dhabhar 2009), and excessive energy expenditure (McEwen 2004). In our study population, we observed elevated FGMs following acute respiratory infections (i.e., "acute infections", "acute viruses", haptoglobin, and individually, AD3). Once "acute infections" were included in the best model, year, season, horn residuals, and pregnant status all had reduced importance. Although this reduction of importance could be explained by the reduced samples size associated with the addition of acute infections (i.e., missing data for disease diagnostics) to the best model, the yearly variation that was seen previously might be captured within the importance of "acute infections" (Fig. 2.4). Considering that there were multiple disease incidences during the first year of the study (i.e., elevated FGMs during the first year of the study compared to the subsequent years).

Gastrointestinal parasitic infections (helminths and protozoan) did not elevate FGMs. These results are consistent with other studies including species such as zebra and springbok (Cizauskas et al. 2015), wild female black howler monkeys (*Alouatta pigra*), (Martínez-Mota et al. 2017), and Woylies (*Bettongia penicillata*), (Hing et al. 2017). Interestingly, other more chronic infections, including chronic viral and bacterial infections, also did not affect FGMs.

On the contrary, elevated FGMs was associated with an increased risk of acquiring one or more chronic bacteria (i.e., *Mycoplasma bovis*, *Anaplasma centrale*, *A. marginale*, BTB or Brucellosis). A potential explanation for these contradicting results is that bacteria are commonly accepted as a secondary infection following an acute viral infection (Wellenberg et al. 2002; Nandi et al. 2009; Earley et al. 2017). However, timing and duration of a stressor play an important role in stress responses (Martin 2009). For example, being chronically infected (i.e., BTB), desensitized the animals' response towards the particular disease, consequently allowing a shift in allostatic load (i.e. energy required and the energy available) that does not cause an emergency physiological response (McEwen and Wingfield 2003; McEwen 2004). The physiological mechanisms that underlie these observed linkages between

infections and FGMs are complicated. Admittedly, we don't have a good enough grasp on immune responses relevant to chronic incidence, but perhaps elevated FGMs increases inflammatory responses as a protective host response towards some pathogens with the tradeoff of suppressing other immune responses, increasing risk for other pathogens.

The reduced risk of another acute viral infection, specifically AD3 associated with previous elevated FGMs, can be explained by previous exposures that result in both higher FGMs and lower likelihood of subsequent infections (e.g., AD3 had a reduced risk of reinfection for at least 1year post-exposure, Coon et al. *in review*). An alternative explanation is that FGMs induces stronger pro-inflammatory immune responses which are (somewhat but detectably) protective against infection by acute pathogens (see chapter 3). Whether one of these two scenarios are true needs further investigation. Future analysis is needed to test whether the pattern between acute infections and stress is driven by the same individuals in successive times steps.

The longitudinal structure of our data allowed us to make progress towards understanding possible causal relationships between stress responses and infectious diseases in a wild mammalian population, because we were able to examine how prior, and subsequent infections were associated with variation in FGM. Nonetheless, experimental infections would be needed to demonstrate a causal relationship between acute infections and elevated FGM conclusively, and experimental manipulation of stress levels in buffalo could test whether susceptibility to chronic bacterial infections is indeed increased.

Environmental change often includes multiple stressors changing simultaneously - including exposure to pathogens and parasites. In this study, we showed that infections are a potential driver of FGMs in buffalo, along with long- and short-term indicators of body condition, demonstrating that FGMs may indeed function as an integrative measure that captures the effects of multiple potential stressors on the animals. Further, we showed that elevated FGMs may play a role in driving transmission cycles of acute infection, potentially leading to positive feedback cycles between stress responses and disease (acute and chronic), which could exacerbate effects of infections on vulnerable wildlife populations.

2.6 Tables and Figures

Table 2.1 Summary of diseases (parasites/pathogens) added to best model (log(FGM)~Year+Season+Body condition+Pregnant status+Horn residuals+Pregnant status:Horn residuals+(1|Animal ID). Bold font indicates statistical significance and sample sizes changed depending on the various diseases added to the best model. FGM = Fecal glucocorticoid metabolite, FDR = False discovery rate.

Parasite/pathogen added to best model	Variable	*P*-value	*P*-adjust FDR	Model AIC	*n*	Animals	Log FGM (β)
							Estimate ± *SE* (ng/g)
Acute Bacteria							
Mannheimia haemolytica (MH)	Binary	0.1763	0.4408	1246.8	919	219	-
Chronic Bacteria: yes	Binary	0.2577	0.4950	821.3	611	211	-
Mycobacterium bovis (BTB)	Binary	0.6116	0.7361	1277.1	980	201	-
Mycoplasma bovis (MB)	Binary	0.6183	0.7361	1248.4	919	219	-
Brucellosis	Binary	0.9655	0.9655	1687.4	1266	271	-
Anaplasma centrale	Binary	0.2673	0.4950	801.8	569	124	-
Anaplasma marginale	Binary	0.7516	0.7829	803.0	569	124	-
Acute Viruses: yes	Binary	**0.0008**	**0.0067**	**1297.8**	**1009**	**255**	**0.10 ± 0.02**
Bovine Adenovirus-3 (AD3)	Binary	**0.0135**	**0.0844**	**1289.4**	**1000**	**254**	**0.10 ± 0.04**
Bovine Parainfluenza-3 (PI3)	Binary	0.2772	0.4950	1421.2	1067	259	-
Bovine Respiratory Syncytial virus (BRSV)	Binary	0.1459	0.4053	1555.9	1186	274	-
Chronic Viruses: yes	Binary	0.3176	0.5293	1339.1	1012	257	-
Bovine Viral Diarrhea virus (BVDV)	Binary	0.6898	0.7498	1574.6	1198	274	-
Bovine Herpesvirus-1 (BHV)	Binary	0.4605	0.6772	1336.2	1011	257	-
Acute Infections: yes	Binary	**<0.0001**	**0.0025**	**1125.8**	**898**	**239**	**0.11 ± 0.03**
Chronic Infections: yes	Binary	0.1264	0.3950	896.1	657	223	-
Log (Haptoglobin)	Continuous	**0.0002**	**0.0025**	**1379.4**	**1074**	**261**	**0.04 ± 0.01**
Macroparasites:	Binary	0.3823	0.5973	1707.2	1271	271	-
Gastrointestinal helminths: yes	Binary	0.5829	0.7361	1739.5	1320	274	-
Schistosomes: yes	Binary	0.1007	0.3950	1704.8	1270	271	-
Microparasite - Protozoan							
Coccidia: yes	Binary	0.6613	0.7498	1739.6	1320	274	-
Virus richness	Continuous	**0.0299**	0.1495	**1215.5**	**934**	**247**	**0.04 ± 0.02**
Bacteria richness	Continuous	0.5162	0.7169	449.6	331	85	-
Parasite richness	Continuous	0.1214	0.3950	369.9	272	79	-

Table 2.2 Summary of the linear mixed-effects model predicting acute infectious diseases as drivers of FGMs while accounting for horn residuals, body condition, pregnant status, year, season, and the random effects of individual buffalo (n = 239) during the 4-year study period (n = 898). Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: pregnant = no; rainfall year = Oct08-Sept09; season = late wet; acute infections = no. FGM = fecal glucocorticoid metabolite. R^2m = 3.68% and R^2c = 49.62%.

Predictor	Effect[a]	Log FGM (β) Estimate ± *SE* (ng/g)	*t*-value	*P*-value
Fixed effects				
(Intercept)		**4.28 ± 0.11**	**37.84**	**<0.0001**
Horn residuals	↓	**-0.01 ± 0.004**	**-1.94**	**0.0526**
Body condition	↓	**-0.10 ± 0.03**	**-3.48**	**0.0005**
Pregnant: yes		0.04 ± 0.03	1.23	0.2204
Year: Oct09-Sept10	↓	**-0.11 ± 0.04**	**-2.48**	**0.0133**
Year: Oct10-Sept11		-0.07 ± 0.05	-1.56	0.1193
Year: Oct11-Aug12	↓	**-0.14 ± 0.06**	**-2.58**	**0.0101**
Season: early wet	↓	**-0.17 ± 0.05**	**-3.62**	**0.0003**
Season: early dry		-0.08 ± 0.04	-1.88	0.0603
Season: late dry		-0.08 ± 0.05	-1.84	0.0656
Acute infections: yes	↑	**0.11 ± 0.03**	**3.89**	**0.0001**
Interaction term				
Horn residuals:Pregnant (yes)		0.01 ± 0.005	1.11	0.2671
Random effects		Variance	*SD*	
Animal ID		0.13	0.36	
Residuals		0.14	0.37	

[a] Effect column indicate the direction of the slope predicted by the model.

Table 2.3 Summary of the best generalized linear mixed models predicting FGMs as drivers of infections (individually or as a group), accounting for the explanatory variables included in each best model, and random effects of individual buffalo. Bold font indicates statistical significance. Other explanatory variables are in order of importance. All continuous explanatory variables were scaled. Sample sizes are different for each dependent variable. FGM = Fecal glucocorticoid metabolite; R^2m = marginal and R^2c = conditional.

Parameter			FGM		Other explanatory variables accounted for:	R^2m (%)	R^2c (%)
Dependent variables:	*Animal ID*	*n*	(Intercept±*SE*) *β*±*SE*	*P*-value			
Acute Bacteria							
Mannheimia haemolytica (MH)	199	723	(-1.91±0.22) -	-	Age + **Season**	4.53	4.53
Chronic Bacteria: yes	195	483	(3.95±1.26) 0.31±0.18	0.0871	**Herd** + **Age** + **Season** + **Year** + **Horn residuals** + Body condition	40.13	41.34
Chronic Bacteria [a]	77	262	(0.74±0.12) -	-	**Season** + **Age** + Treatment	14.30	14.84
Mycoplasma bovis (MB)	199	723	(-1.75±0.20) -	-	**Season**	1.90	1.90
Anaplasma centrale	113	442	(-1.65±1.25) -	-	**Season** + **Year** + **Age** + Body condition	22.60	29.13
Anaplasma marginale	113	442	(-0.72±0.22) -	-	**Age** + **Season** + **Body condition**	11.28	18.35
Acute Viruses: yes	238	825	(-1.82±0.38) -	-	**Season** + **Year** + **Herd** + Age	7.78	7.78
Acute Viruses [a]	236	806	(0.13±0.10) -0.03±0.02	0.1191	**Season** + **Year** + **Age** + **Herd**	5.36	5.36
Bovine Adenovirus-3 (AD3)	**237**	**816**	**(-2.33±0.25) -0.37±0.16**	**0.0223**	**Age** + **Season** + Body condition	22.60	29.13
Bovine Parainfluenza-3 (PI3)	240	866	(-3.24±0.48) -	-	**Season** + **Herd** + **Year** + Reproductive status	14.72	14.72
Bovine Respiratory Syncytial virus (BRSV)	241	874	(-3.00±0.90) -	-	**Year** + **Season**	25.19	31.73

[a] Continuous response (dependent) variables used linear mixed effects models.

Table 2.3 *Continued…*

Parameter			FGM		Other explanatory variables accounted for:	R^2m (%)	R^2c (%)
Dependent variables:	*Animal ID*	*n*	(Intercept±*SE*) *β*±*SE*	*P*-value			
Chronic Viruses: Yes	232	812	(-3.46±0.78)	-	**Year** + **Body condition** + **Season**	11.81	11.81
Chronic Viruses [a]	232	811	(0.04±0.06)	-	**Body condition** + **Year** + **Season**	2.97	2.97
Bovine Viral Diarrhea virus (BVDV)	240	882	(-4.14±0.28)	-	-	0.00	28.78
Bovine Herpesvirus-1 (BHV)	233	813	(-3.74±0.79)	-	**Year** + **Body condition** + **Herd** + **Season**	12.98	12.98
Acute Infections: yes	220	714	(-1.28±0.36)	-	**Year** + **Season**	5.18	5.18
Chronic Infections: yes	204	520	(3.98±1.24)	-	**Herd** + **Year** + **Age** + **Season** + **Body condition** + **Horn residuals**	40.86	41.01
Log (Haptoglobin) [a]	242	880	(7.17±0.19)	-	**Year** + **Age** + **Treatment** + Horn residuals	4.60	31.16
Macroparasites	246	979	(0.71±0.10)	-	**Treatment** + **Body condition** + **Year** + **Season** + Reproductive status	7.41	36.89
Gastrointestinal helminths: yes	247	998	(-0.61±0.25)	-	**Treatment** + **Season** + **Body condition** + **Age** + Herd + Horn residuals	13.44	41.68
Schistosomes: yes	246	979	(-0.74±0.46)	-	**Season** + **Year** + **Horn residuals** + **Age** + **Herd** + Reproductive status	13.05	60.49
Microparasite - Protozoan Coccidia: yes	247	998	(0.73±0.48)	-	**Season** + **Herd** + **Year**	33.22	34.27
Virus richness[a]	225	756	(0.12±0.13)	-	**Year** + **Herd** + **Age** + **Season** + **Body condition**	5.48	5.48
Bacteria richness[a]	77	262	(0.85±0.13)	-	**Age** + **Season** + Treatment	12.10	13.19
Parasite richness[a]	70	214	(1.96±0.75)	-	**Year** + **Treatment** + **Reproductive status** + Season	16.55	17.40

[a] Continuous response (dependent) variables used linear mixed effects models.

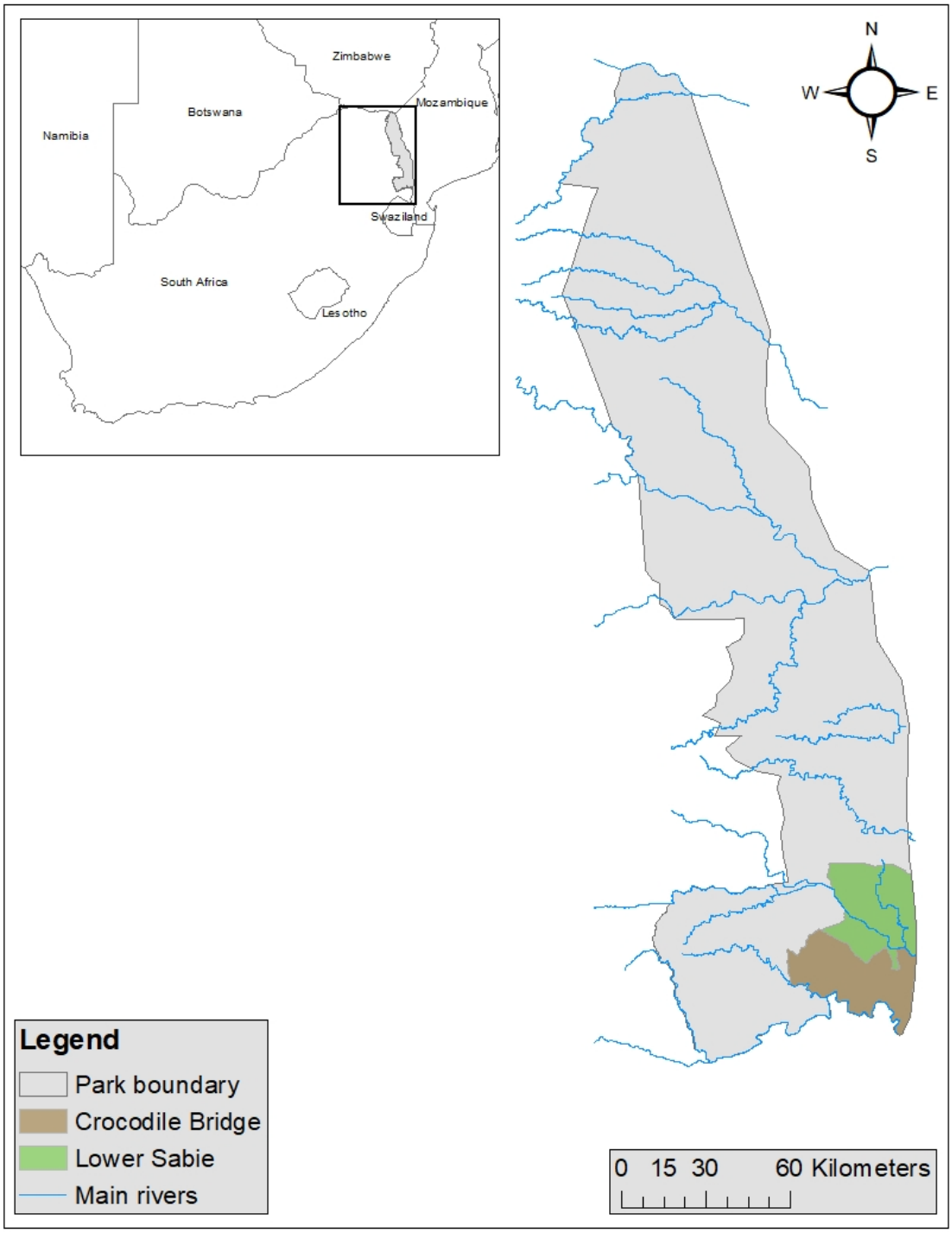

Figure 2.1 Location of the study site, Kruger National Park (KNP), South Africa. KNP is located on the north-eastern side of South Africa and data were collected at the south-eastern region of the park (indicated by green and brown).

Box 2.1: Includes Figure 2.2 that summarizes the average yearly incidence rate of various acute and chronic infections, as well as the average fraction of the population infected for micro- and macroparasites indicated by the shaded green box. The table gives a summary of the taxonomy, chronicity, and a brief biological description of each parasite/pathogen tested for in this study.

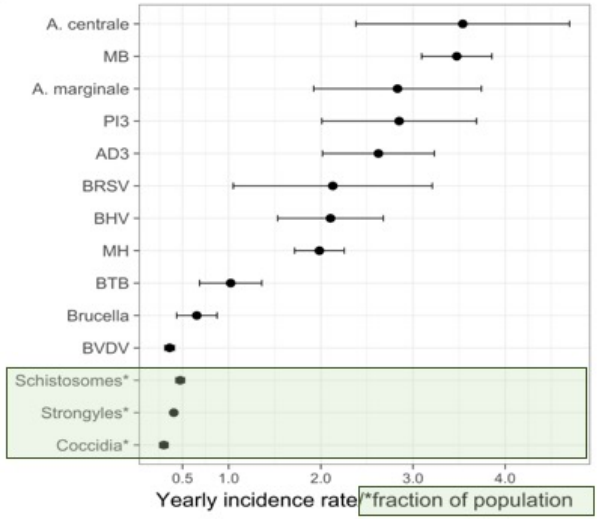

Figure 2.2 The average yearly incidence rate for the various acute (MH, PI3, AD3, BRSV) and chronic (MB, BHV, BTB, Brucella, BVDV, *A. centrale*, *A. marginale*) infections tested across the 4-year study period. Overall, viral infections (Acute: PI3, AD3, and BRSV; Chronic: BHV) showing highest variation of yearly incidence rates compared to chronic infections, except for MB. BVDV had the lowest, and the least variable yearly incidence rate. The asterisk indicate the average yearly fraction of the population infected with an acute infection (coccidia) and chronic infections (strongyles or schistosomes) tested across the 4-year study period (shaded, green box). A higher fraction of the population was infected with schistosomes and strongyles than coccidia . MB = *Mycoplasma bovis*; PI3 = Bovine parainfluenza-3; AD3 = Bovine adenovirus-3; BRSV = Bovine respiratory syncytial virus; BHV = Bovine herpesvirus-1; MH = *Mannheimia haemolytica*; BTB = Bovine tuberculosis; BVDV = Bovine viral diarrhea virus; *A* = *Anaplasma*.

Pathogen/ Parasite	Type	Brief biological description [a]
MH	Acute Bacteria	Primary bacterial agent leading to bovine respiratory disease (or commonly known as shipping fever) under stress conditions. Global distribution restricted to ruminants.
BTB	Chronic Bacteria	Mycobacterium bovis is the acting agent of this exotic emerging wildlife disease. Chronic infection that affects the lungs and the upper respiratory system of buffalo. Buffalo appear to be the primary maintenance hosts of this disease.
MB	Chronic Bacteria	Wide spread pathogen that causes respiratory disease, mastitis and arthritis in cattle, especially calves. Shedding occur via the respiratory tract of infected cattle for months or even years (act as reservoirs of infection).
Brucellosis	Chronic Bacteria	A zoonotic infection and the causative agent is Brucella abortus, resulting in abortions of wild and domestic ungulates. In African buffalo, reduced body condition followed after testing positive for brucellosis.
A. centrale	Chronic Bacteria	Tick borne, intracellular haemoparasite that inhabit red blood cells and replicate through binary fission.
A. marginale	Chronic Bacteria	Tick borne, intracellular haemoparasite that inhabit red blood cells and replicate through binary fission.
AD3	Acute Virus	Infections associated with respiratory and alimentary tract diseases, but causation remains uncertain. Widespread in cattle, especially calves and presumed to be transmitted via direct respiratory transmission or the fecal-oral route.
PI3	Acute Virus	Infection is commonly associated with respiratory diseases in cattle worldwide, especially under stress-induced conditions. Shedding occur through nasal and ocular secretions.
BRSV	Acute Virus	Part of the respiratory disease complex. Mostly affecting younger animals, especially calves of < 6 months old.
BVDV	Chronic Virus	Most prevalent infectious disease in cattle, causes huge economical losses. Risk of fetal infections can lead to embryonic death or teratogenic effects. Primary route for viral transmission is shedding via excretions and secretions.
BHV	Chronic Virus	Affects the respiratory and reproductive tract. Can be latent and reactivation is not unusual.
Schistosomes	Chronic	Trematode worms are worldwide causing inflammatory diseases in humans, livestock and wildlife by inhabiting mesenteric arteries of hosts after exposure to vector snails in infected aquatic habitats.
Strongyles	Chronic	Gastrointestinal parasite among the most widespread in ruminants, with a fecal-oral transmission route. Generalists in their host choice.
Coccidia	Acute	Protozoan, microparasite with a fecal-oral transmission route.

[a] Various peer-review journal articles and veterinary textbooks were used to compile descriptive information for each disease (parasite/pathogen) included in this study (Coetzer et al. 1994; Rock 1994; Jeyaseelan et al. 2002; Nicholas and Ayling 2003; Jolles et al. 2008; Nandi et al. 2009; Ellis 2010; Nettleton 2013; Gorsich et al. 2014; Lanyon et al. 2014; Gorsich et al. 2015b; Henrichs et al. 2016; Silvestro and Bratanich 2016; Beechler et al. 2017).

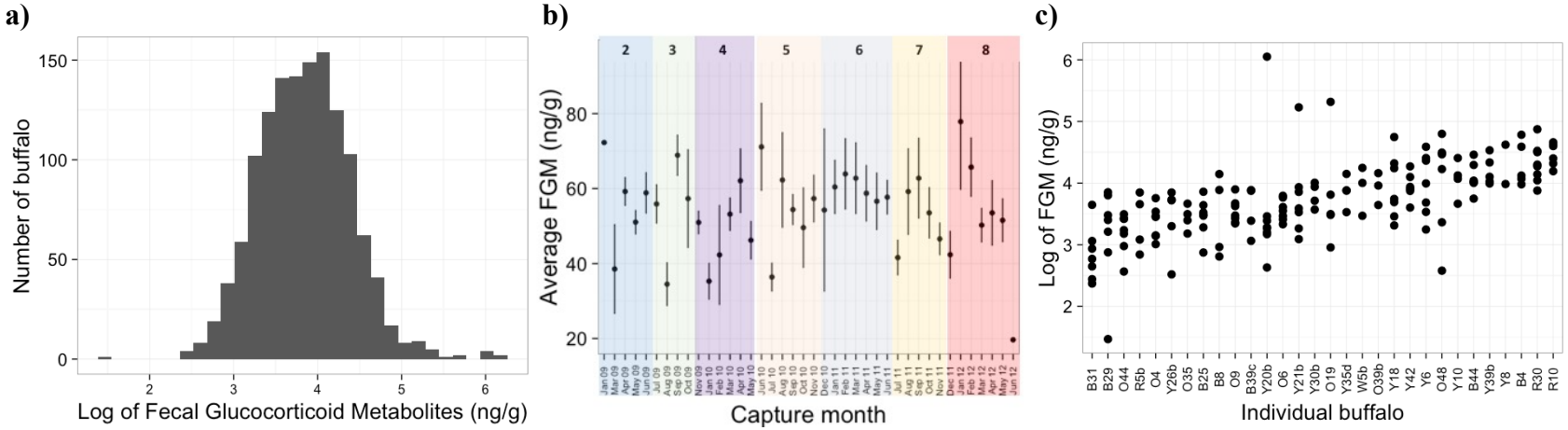

Figure 2.3 a) Fecal glucocorticoid metabolite (FGM) concentrations show majority of the study animals to be within an average FGM range with a few individuals above or below the norm. b) Summary of the temporal variation in the average FGMs over time (months) within the study population, emphasizes the seasonal variation associated with FGMs. Shaded colors represent different capture periods. c) Variation in FGMs among and within individuals taken from a randomly assigned subset of 30 buffalo, emphasizes the importance to control for these tendencies in FGMs by including animal ID in our models.

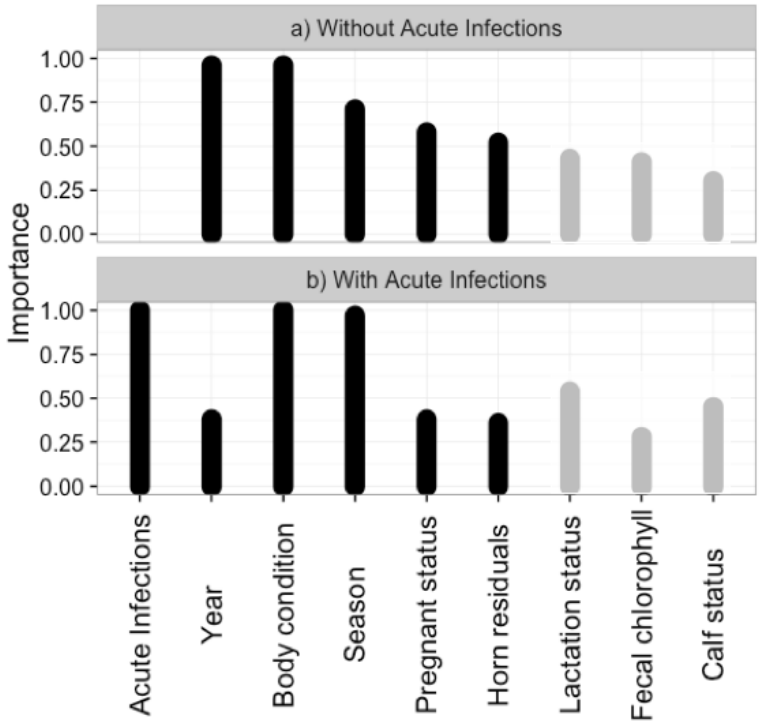

Figure 2.4 a) Summary of the importance values for each variable (environmental and demographic) included in the best model and b) includes acute infections. After addition of acute infections to the best model, year were no longer the most important variable and both pregnant status and horn residuals had reduced importance. Importance is the sum of the Akaike weights for all possible model combinations. The faded bars (lactation status, fecal chlorophyll, and calf status) are variables not included in the final best model. Sample size dropped from 1320 (274 buffalo) to 898 (239 buffalo) once acute infections were added to the best model.

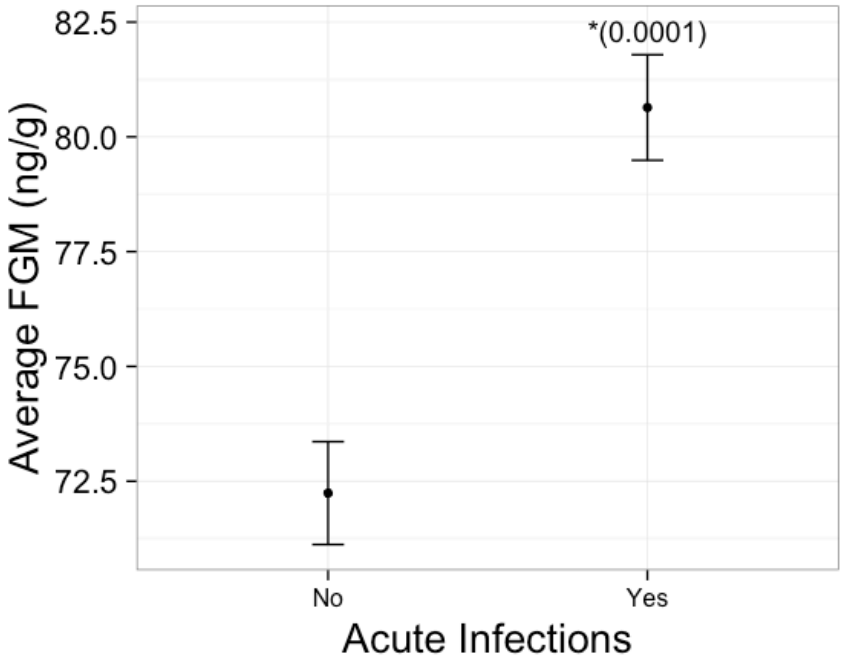

Figure 2.5 Summary of the average fecal glucocorticoid metabolite (FGM) concentrations whether infected by one or more acute infections. At the female population level, being infected by at least one acute infection is associated with higher stress (Table 2.2, LMEM, *P*-value =0.0001). Error bars indicate standard error.

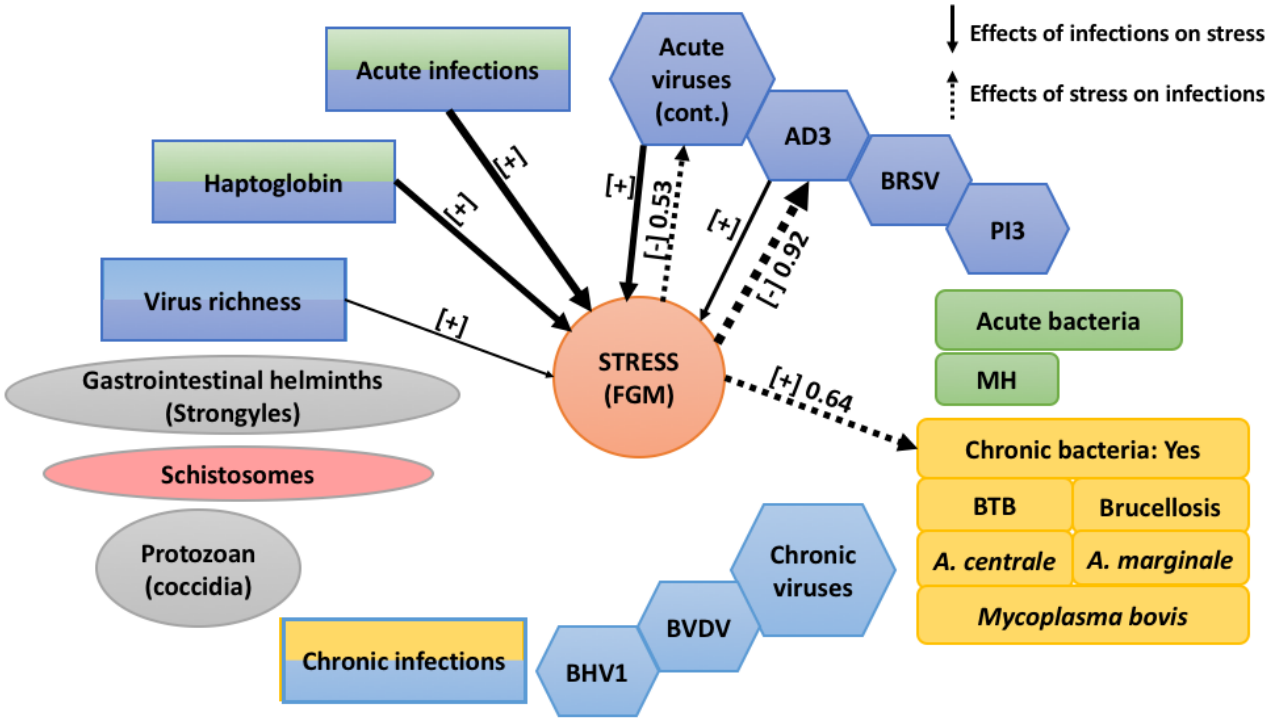

Figure 2.6 A schemathic diagram illustrating the reciprocal effects between stress responses and infections in female African buffalo. The solid arrows indicate the effect of infections on stress responses and the dashed arrows indicate the effect of stress responses on infections. The width of the arrows increases as the strength of that effect increases. The numbers associated with the dashed arrows are the importance value of the FGM variable for the specific parasite or pathogen (importance is the sum of the Akaike weights of all possible model combinations). Different shapes indicate viruses (hexagon) or bacteria (rectangular with rounded edges) and colours indicate acute (purple and green) or chronic (light blue and yellow). Gastrointestinal parasites are represented by the grey, oval circles (stronyles and coccidia), whereas Scistosomes are represented by the red oval circle. Rectangular blocks with mixed colours indicate the various groupings of parasites and pathogens. FGM = Fecal glucocorticoid metabolite; MH = *Mannheimia haemolytica*; *A.* = *Anaplasma*; BTB = Bovine tuberculosis; AD3 = Bovine adenovirus-3; PI3 = Bovine parainfluenza-3; BRSV = Bovine respiratory syncytial virus; BVDV = Bovine viral diarrhea virus; BHV = Bovine herpesvirus-1.

CHAPTER 3

Physiological consequences of stress responses in female African buffalo (*Syncerus caffer*)

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Currently *in preparation* for General and Comparative Endocrinology.

3.1 Abstract

The endocrine, nervous and immune systems form a complex integrated network that functions to maintain homeostasis. Stress in wildlife is often implicated in adverse health consequences–whether it is via immune suppression, altered leukocyte distribution, vascular damage, altered metabolic function, or weight loss, stress has the potential to increase disease susceptibility. On the other hand, the physiological stress response modulates immunity, which can include the upregulation of some immune responses. Although stress-immunity has been widely studied and concluded both immune enhancement and immune suppression, there are many factors that affect the enhanced or suppressed outcome of the stress-immune system. It is unclear, then, whether/when stress responses will increase vulnerability to infectious diseases, or decrease them, and therefore, at the population level, how stress responses are likely to affect disease dynamics. A shortcoming of many available datasets in wildlife is that they are cross-sectional, which can establish correlations between physiological stress responses and immune measures, but are limited in their ability to distinguish "fixed" animal traits from plastic physiological interactions.

We used a longitudinal dataset of 261 buffalo captured up to 9 times to assess associations between stress responses and a range of immune measures as well as physiological parameters. Our study design allows us to control for fixed animal effects, focusing on the plastic component in covariation between stress and immunity/physiology, as well as accounting for current pathogen exposures. We hypothesized that fecal glucocorticoid metabolites (FGMs) should enhance responses to acute infections, such as pro-inflammatory responses, and that it should suppress responses to chronic subclinical infections such as strongyles. Supporting our hypotheses, we found that buffalo with higher FGMs had elevated pro-inflammatory immune responses, as indicated by increased IFNγ (and trend: Il-12) responses, increased bactericidal activity and suggestively higher titers of neutrophils. However, limited to some immune suppression (i.e., proportion of eosinophils were negatively associated with FGMs), which are specifically an indicator of anti-macroparasite immunity. These findings are consistent with increased immune responses to acute

infections in buffalo with higher FGMs and decreased responses to parasitic infections (potentially strongyles: chronic, subclinical). In addition, our analysis revealed changes in red blood cell turnover rate, possibly indicative of subclinical anemia in stressed animals or could the increased hematopoiesis just be a part of the emergency phenotype that enables the animal to perform incredible physical demands (e.g., fight or flight responses). Liver enzymes in stressed animals were elevated, perhaps indicating toxic exposures to copper or poisonous plants. Unsurprisingly, season and year were important explanatory variables for almost all immunological and physiological measures. The associations between stress responses and physiological health parameters suggest that measuring FGMs can be used as a tool by wildlife managers to evaluate health within individuals.

3.2 Introduction

The immune system is a complex system that integrates with both the neural and endocrine systems. This complex system integration plays an important role in homeostasis (i.e., to maintain a constant internal condition). Glucocorticoids (GC), catecholamines, and cytokines are mediators of allostasis, the process of modifying behavioral and physiological responses in order to cope with predictable and unpredictable environmental changes (McEwen and Wingfield 2003). Rapidly changing environments (e.g., climate change, land use , and biodiversity loss), along with potential shifts in parasite distribution, have raised global concern (Patz et al. 2000b; Weiss and McMichael 2004a; Eisenberg et al. 2006; Parmesan 2006) and are leading to changes in stress physiology. Although, the emergency life history stage (ELHS) allows organisms to cope with unpredictable events that are indirect (e.g., loss of offspring, habitat deterioration) or direct (e.g., reduced resource availability, increased energy demands due to disease, adverse weather conditions or predator avoidance), (McEwen and Wingfield 2003; Demas and Nelson 2012; Wingfield 2013), this can have important effects on wildlife health, including altered immune profiles and changes in patterns of disease.

It is well-known that GC act as an immune suppressor and have been used as a treatment for autoimmune and inflammatory conditions (e.g., treatment of

Rheumatoid arthritis, Hench et al. 1949). Most stress-immunological hypotheses state stress to be an immune suppressor. For example, during a stress response, immunity is suppressed in order to redirect resources towards activities that increase survival, or immunity is of little significance sacrificing resources for more critical processes (Sapolsky et al. 2000), or immunity limits autoimmune damage by reducing self-damage (Raberg et al. 1998). However, GC can also be immune enhancers (Reichlin 1993; Chrousos 1995), especially if wounding occurred (Martin 2009). However, the complexity of the immune integration with the stress and the neural systems depends on various factors. For example, the context (i.e., the type), timing and duration of the stressor (Martin 2009).

This protective up-regulation of immune components as an initial response to GC, enhances pro-inflammatory immune responses followed by T lymphocytes, especially T helper 1 (Th-1) cells, and consequently coordinates with B lymphocytes (Martin 2009), which are responsible for the production of antibodies (Sompayrac 2015). However, under prolonged effects of GC, suppression of the same immune components (i.e., pro-inflammatory responses, Th-1 and B lymphocytes) can occur (Martin 2009). Certain immune components are more resistant to stress than others (Martin 2009), which changes with previous immune enhancements to particular stressors (e.g., GC insensitivity towards a previous stimulation), (Avitsur et al. 2001). The fact that the effects of stress on immunity can go in both directions (suppression/enhancement), thus, adds complexity to predicting stress-immune-disease outcomes.

During an acute stress response (i.e., restraining activity), elevated GC of mice enhanced immunity (Dhabhar 2009). The developmental immune stage of a rapid, first line of defense against disease infections is less costly; however, it has been found that the up-regulation of such a response has much higher immunopathological and energetic costs (Klasing 2003; Martin et al. 2008). Other types of immune responses such as Th-1 cytokines mediate inflammatory responses and cellular immunity, whereas Th-2 cytokines are anti-inflammatory cytokines, and induces humoral immunity and antibody production of lymphocyte B cells (i.e., mature lymphocytes in the bone marrow; Sompayrac 2015). Physiological and

ecological drivers may alter defense strategies in buffalo and such changes in the immune response are thought to be associated with variability in parasite abundance and incidence (Jolles et al. 2008; Beechler et al. 2012; Anderson et al. 2013).

Previous studies are mostly cross-sectional and therefore an important limitation, given that (i) stress responses and their effects depend on circumstances (e.g., type and duration of a stressor, and nutrition), and (ii) limited in their ability to distinguish "fixed" animal traits from plastic physiological interactions. This is central to understanding how stress will alter disease dynamics in natural populations.

Here we investigated links between physiological stress and immune/physiological responses in a well-studied model system for studying disease processes in natural populations, African buffalo (Sinclair 1977; Prins 1996; Winterbach 1998; Jolles 2003; Cross et al. 2005; Michel and Bengis 2012; Melletti and Burton 2014). Not only are they carriers of many pathogens and parasites, with the potential of some to spillover to livestock (Michel and Bengis 2012), but a great system to study immune-mediated interactions between host and parasite/pathogen existence (Jolles et al. 2008; Beechler et al. 2015; Ezenwa and Jolles 2015). Buffalo's size and fierce nature require chemical immobilization during any handling for management or research purposes. Immobilization is necessary for the collection of blood for all physiological health (e.g., immune, hematology, and biochemical) measures, but measuring FGMs as a stress marker, avoids measuring capture stress and instead measure the animals pre-capture FGMs (i.e., 10-20 hours pre-capture). Measuring FGMs has become a widely used and acceptable non-invasive tool to monitor responses to stressful situations (Möstl and Palme 2002; Palme et al. 2005) and has been validated for the use in African buffalo (Spaan et al. 2017).

We used a longitudinal study design including 261 buffalo across 4 years, allowing us to capture both "fixed"/repeatable and plastic components of the stress responses, asking: How does stress (i.e., measured as FGMs) affect immune responses that mediate acute and chronic infections? If the adaptive function of stress responses is to help animal cope with immediately harmful situations, then one might expect stress to upregulate immune responses to acute infections (especially microparasites, which can multiply quickly in the host), but suppress immune

responses to less imminently risky infections, such as macroparasites (which don't reproduce within the host) and thus don't suddenly increase in severity and harmfulness. Lastly, to gain a more holistic insight into health effects, we looked at physiological outcomes of variation in FGM, in concert with the immunological outcomes.

3.3 Methods

Methods regarding buffalo capture, sample collection, and handling methods have been published elsewhere (Beechler et al. 2012; Anderson et al. 2013; Ezenwa and Jolles 2015).

Study site.—This study took place in the southern region of Kruger National Park (KNP), South Africa. KNP is approximately 19 485 km^2 in extent and lies between 22.5 and 25.5°S, and 31.0 and 31.57°E (Fig. 2.1). The average rainfall occurs during the summer (October-March) and varies north to south from 400-700 mm per year. The buffalo population has been estimated as approximately 30 000 throughout the park (Cross et al. 2009). This study took advantage of a four-year field study investigating multi-parasitic infection patterns and immune profiles in female African buffalo in KNP (Ezenwa and Jolles 2015).

Mass Capture and Collaring.—Two hundred female African buffalo (2-5yrs. of age) were darted using a helicopter and a large ground team (South African National Parks' Veterinary Wildlife Services and Game Capture team) in the Lower Sabie section (during June/July 2008) and Crocodile Bridge section (during October 2008) of Kruger National Park (100 buffalo in each section). Each individual buffalo was fitted with either a radio (n=193) or satellite (n=7) collar. For identification purposes, each buffalo received a very high frequency (VHF) transmitter built into the collar accompanied with a unique color and number combination that was branded on both sides of their rump. If an animal died it was replaced by a new individual from the herd from which the dead animal originated (n=261).

Recaptures and Sample collection.—Each buffalo was recaptured on a 6-month period (biannually) for 4 years (June 2008 – August 2012), resulting in each individual buffalo being captured 2-9 times. Telemetry was used to locate specific

buffalo and a vehicle to access them, however occasionally helicopter captures were needed to recapture buffalo within the specific time period. All immobilizations were conducted by a veterinarian according to the South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. Each buffalo received a combination of etorphine hydrochloride (Novartis, Kempton Park, 1619, South Africa), azaperone (Janssen Pharmaceutical Ltd., Halfway House, 1685, South Africa) and in some cases hyaluronidase (Kyron Laboratories, Benrose, 2001, South Africa). This drug combination was delivered into the muscle of the rump remotely using a dart propelled by a compressed air rifle (DAN-INJECT, International S.A., Skukuza, 1350, South Africa). The doses varied between 7-9 mg etorphine, 40-80 mg azaperone and 5000 i.u. hyaluronidase depending on the size of the animal.

Environmental variables collected for each immobilized buffalo.—Seasonal variability of the study site allowed for the division into early wet (October-December), late wet (January-March), early dry (April-June), and late dry (July-September). Year was classified into rainfall years (October 2008-September 2009, October 2009-September, October 2010-September 2011, October 2011-August 2012) to allow for biological relevance (Ryan et al. 2006).

Demographic variables collected from each immobilized buffalo.—Animals were aged according to body size and horn development in juveniles less than two years old (Jolles 2007), and incisor emergence patterns were used for individuals between 2 and 5 years old (Grimsdell 1973). In older buffalo, the tooth wear of the first incisor was evaluated for age determination (Jolles 2007). For statistical analysis, age was recorded in months and presented as a continuous variable. To account for potential non-linearities in the relationships of some dependent variable (monocytes, erythrocytes, mean corpuscular volume, albumin, globulins, creatinine kinase) with age, we included a curvature by squaring age. Body condition was assessed by palpating the ribs, spine, hip and base of tail and scoring them on a scale of 1 (very poor) to 5 (excellent) and calculating the average of all four areas (Ezenwa et al. 2009). These manual body condition scores are correlated with host kidney fat index (Ezenwa et al. 2009). Herd membership was recorded as the herd each buffalo was

captured with (Lower Sabie, Crocodile Bridge, or Northwest). To account for the positive correlation between total horn length and age, the regression residuals of total horn width (cm) as a functions of age (months) were calculated. A higher value was interpreted as above average horn size for their age, reflecting a measure of individual quality (Ezenwa and Jolles 2008). Reproductive status was categorized depending on pregnancy and lactation status. Rectal pregnancy tests were performed by a veterinarian and recorded as being pregnant or not. Lactation status (present or absent) were determined by manual milking of all four teats (Jolles et al. 2005) of each female buffalo. Each female buffalo was classified as reproductively active when pregnant and or lactating at the time of capture. Fifty percent of the buffalo (treatment group) received a long-acting antihelminthic bolus (Panacur® slow-release bolus, Hoechst Roussel), a de-wormer to examine the effects of gastrointestinal parasite removal on bovine tuberculosis progression (Ezenwa and Jolles 2015). The control group received no bolus. Blood was collected through jugular venipuncture into 9ml ethylenediaminetetraacetic acid (EDTA), lithium-heparinized and serum tubes. Blood and fecal samples was stored on ice within 5 minutes post immobilization, and transported back to the lab within 8 hours. EDTA collected blood was used for hematological analysis and blood smears. Lithium-heparinized collected whole blood was used for bacterial killing assays (BKA). Blood plasma was aliquoted from centrifuged lithium heparinized tubes for 10 minutes at 5 000 g. Aliquoted blood samples were stored at -20°C until analysis.

Following data collection, naltrexone (the antidote to etorphine hydrochloride; 40 mg/ml, Kyron) was administered intravenously and the animal kept under observation until fully recovered. Each individual buffalo was only sedated for 10-15 minutes.

Stress response.—Fecal glucocorticoid metabolites (FGMs) were quantified using established methods for African buffalo (Spaan et al. 2017). The FGM concentrations is not a reflection of the capture and handling effect, due to the 10-20hour lag time between the increase of circulating plasma cortisol and the appearance of its metabolites in the feces (Spaan et al. 2017).

Immunological measures.—We used stained blood smears (Giemsa stain) to perform differential white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). These counts represent a baseline measure of immune resources available to the host (Beechler et al. 2009). A bacterial killing assay (BKA) was used to assess innate immunity for extracellular pathogens (the ability of immune factors in the blood plasma to kill the bacteria, *Escherichia coli*). The method used was modified from (French and Neuman-Lee 2012) and adapted for the use in African buffalo.

We have also assessed each buffalo's acute immune response by stimulating blood *in vitro* with mitogens (i.e., pokeweed stimulant, Sigma-Aldrich, product #L9379) to measure the following cytokines (immunological active proteins) using enzyme-linked immunosorbent assays (ELISAs): Interferon gamma (IFNγ) and Interleukin12 (Il-12) that are associated with an immune response to microparasites, and Interleukin 4 (Il-4) with an immune response to macroparasites. IFNγ and Il-12 are pro-inflammatory cytokines, secreted by T helper 1 (Th-1) lymphocytes (Sompayrac 2015), and regulates cellular mediated immunity (Elenkov et al. 2006). IFNγ is critical for immunity against viral, intracellular bacterial infections, tumor control, and an important activator for macrophages, neutrophils, and natural killer cells (Sompayrac 2015). IFNγ is also an important messenger molecule of the Th-1 response to tuberculosis (Flynn 1993; Welsh et al. 2005). Whereas, Il-4 are anti-inflammatory cytokines, secreted by Th-2 lymphocytes and regulates humoral immunity that provides protection against multicellular parasites and extracellular bacteria (Elenkov et al. 2006). Il-12 enhances IFNγ and inhibit Il-4 synthesis by T cells (Elenkov and Chrousos 1999).

Haptoglobin, an acute phase protein has shown to be a reliable indicator of an acute infection caused by various infections in cattle (Horadagoda et al. 1999) and buffalo (Glidden et al. 2018) was included in the global model to avoid confounding results for FGMs being associated with an immune response to a current acute infection. A commercially available ELISA kit for bovine haptoglobin (Life Diagnostics, product number HAPT 11) was used (Glidden et al. 2018).

Physiological parameters.—For hematological analysis, blood collected in EDTA vacutainers was used to generate hematology results with an ABC™ Hematology Analyzer for each buffalo captured (see Table 3.1 for more details). To further investigate immunoglobulins (as response to an adaptive immune response), we used serum biochemical measures (which are commonly used as a health measure), especially globulins (GLOB) which measures proteins that function as antibodies, enzymes, complement and carriers (Couch et al. 2017). A chemistry analyzer, Abaxis Vetscan VS2 (Abaxis Inc., Union City, CA, USA) and methods outlined by (Couch et al. 2017) was used for the serum biochemistry measures (see Table 3.1 for more details).

All procedures conducted during this research were approved by South African National Parks Board (Reference No. CALJM728), and Oregon State University's Animal Care and Use Committee (OSU ACUP No. 3822).

Statistical Analysis

Immunological measures.—The differential white blood cell counts were expressed as a proportion of each particular leukocyte counted. The neutrophil-lymphocyte ratio (NLR) was calculated because it is a common use in veterinary as an indication of an acute stress (i.e., replicating an acute capture stress) and expressed as milliliters (ml).

The absolute value of bacterial colonies killed (*Escherichia coli*) was calculated as the average absorbance in control wells (only bacteria and broth) subtracting the average absorbance in experimental wells (with plasma, bacteria, and broth).

Does stress drive immunity.—To test whether stress is associated with immune responses, linear mixed-effect models (LMEMs) were used to account for the random effect (i.e., animal ID) of repeated measures (Bates et al. 2012). The best model for each immune measure was determined using dredge, an automated model selection function of the multi-model inference (MuMIn) package (Bartoń 2016) to generate a set of models with combinations of all environmental (i.e., year and season), demographic (i.e., body condition, age, age squared, reproductive status, horn residuals, herd, and treatment) variables, FGMs (i.e., the stress variable of

interest), and haptoglobin in the global model, and ranked based on the second-order Akaike information criteria (AICc). All continuous explanatory variables within the global model were scaled by standardizing the coefficient estimates (i.e., centering by subtracting the mean of each variable and dividing it by its standard deviation) to ensure that all variables contribute evenly to the scale. Scaling was done by the scale function of the base package (R Core Team 2016). Haptoglobin was forced into the best models of immune measures if dropped by the dredge function in order to avoid the possibility that we may see spurious effects of FGMs on immune measures based on an animals' acute infections. Visual inspections of residual plots did not reveal any obvious deviations of normality or homoscedasticity.

P-values were obtained using the lmerTest package (Kuznetsova et al. 2016) and considered statistically significant if $P \leq 0.05$. FGM results were expressed as nanograms glucocorticoid metabolites per gram of dry feces (ng/g). To account for variable growth on the control plates of the BKA, an offset term for control plates were included in the global model with the absolute value of bacterial colonies killed as the response variable. Similarly, a control offset term was also included for the Il-12 pokeweed stimulated response. The dredge function of the MuMIn package (Bartoń 2016) was also used to determine the importance of each variable, where importance is the sum of the Akaike weights' of all possible model combinations (a value between 0-1). The rsquared.glmm function of the MuMIn package (Bartoń 2016) was used to calculate the marginal and conditional R^2 for all the best models. The marginal R^2 represents the variance explained by the fixed effects, whereas the conditional R^2 represents the variance explained by both the fixed and random effects (Nakagawa and Schielzeth 2013).

Physiological effects of FGMs.—To gain a more holistic insight into health effects of stress along with the immunological outcomes, we test whether stress is associated with hematological or biochemical responses. The same procedure was followed as above (i.e., does stress drive immunity), except for the use of separate models for each hematological or biochemistry response variable as the dependent variable.

The statistical program, R (R Core Team 2016) were used for all statistical analysis, including packages: lme4 (Bates et al. 2012), lmerTest (Kuznetsova et al. 2016), MuMln (Bartoń 2016), and ggplot2 (Wickham 2009).

3.4 Results

Overall, we obtained stress data from 261 free-ranging buffalo (2-9 samples/animal), and data coverage was 75%, with 1326 fecal samples recovered from 1779 capture events. Fecal glucocorticoid metabolites (FGMs) were moderately repeatable among individual buffalo, and within individuals over time ($R = 0.44$, $SE = 0.03$, $P = <0.0001$, Fig. 2.3 from Chapter 2). FGMs in individual buffalo were moderately repeatable, suggesting that some buffalo tended towards consistently higher stress levels than others. FGMs reflects plasma cortisol concentrations 10-20 hours prior, so that here, the stress response is preceding immune responses.

Hypothesis 1: FGMs should enhance responses to acute infections, such as pro-inflammatory response.—Elevated FGMs enhanced innate immune responses, such as the bactericidal competency of female African buffalo and proportion of neutrophils (marginally significant). A one standard deviation rise in FGMs was associated with a 0.02 standard deviation increase in the bactericidal competence ($SE = 0.008$, $t_{815} = 2.81$, $P < 0.0050$, Table 3.2), after accounting for year, control as an offset, herd, body condition, season, haptoglobin, and the repeated measures of individual buffalo (Table 3.2, Fig. 3.1). Increased FMGs were associated with an increase in the proportion of neutrophils available and an important explanatory variable (Supplementary Data SD3.3), but not significantly different in the best model (Table 3.2, Fig. 3.1).

Increased FGMs were associated with increased immune responses, especially Th-1 cytokines (IFNγ and Il-12), whereas Th-2 responses (Il-4, antagonists for Th-1) were not associated with FGMs. A one standard deviation rise in FGMs were associated with a 0.04 standard deviation increase in an interferon gamma response, after accounting for body condition, year, season, age, age squared, haptoglobin, and the repeated measures of individual buffalo ($SE = 0.02$, $t_{1044} = 2.19$, $P < 0.0287$, Table 3.2, Fig. 3.1). Increased FGMs were associated with an increased Il-12 response and

an important explanatory variable (Supplementary Data SD3.3), but not significantly different in the final best model (Table 3.2).

Hypothesis 2: FGMs should suppress responses to chronic subclinical infections, such as strongyles.—Limited by only two immune measures towards parasite responses (i.e., eosinophils and Il-4) there is some evidence that anti-parasitic exposures (i.e., proportion of eosinophils) were suppressed in animals with high FGMs. A one standard deviation rise in FGMs was associated with a 0.004 standard deviation decrease in the proportion of eosinophils available, after accounting for season, haptoglobin, reproductive status, age, year, herd, horn residuals, body condition, and the repeated measures of individual buffalo (SE = 0.002, t_{1039}= -1.96, P < 0.0507, Tables 3.2, Fig. 3.1). Whereas, no association between FGMs and Il-4 were detected (Table 3.2).

Physiological effects of FGMs.—Physiological outcomes of variation in FGMs, revealed that variation in FGMs was associated with changes in red blood cell profiles, specifically, an increase in mean corpuscular volume (MCV) in more stressed animals, which is indicative of a larger proportion of immature erythrocytes (β = 0.22, SE = 0.08, t_{783} = 2.98, P < 0.0029, Table 3.2, Fig. 3.2). Although, increased FGMs were associated with a decrease in platelets and an important explanatory variable (Supplementary Data SD3.3), but not significantly different in the final best model (Table 3.2, Fig. 3.2).

FGMs were an important explanatory variable associated with gamma-glutamyl transferase (GGT, enzymes produced primarily by the liver), BUN (a byproduct of protein metabolism) and the mineral, Calcium (Fig. 3.2). FGMs were not associated with globulins, albumin, or total protein as potential indicators of immunoglobulins. However, the sample size was significantly reduced for the serum biochemistry health measures, that could have potentially hidden important effects.

A one standard deviation rise in FGMs was associated with a 0.43 standard deviation increase in the enzyme GGT, after accounting for body condition, year, herd and the repeated measures of individual buffalo (SE = 0.15, t_{353}= 2.87, P < 0.0044, Tables 3.2 and Supplementary Data SD3.1). Increased FGMs were associated with an increase in BUN and calcium, and both were important explanatory variables

(Supplementary Data SD3.3), but not significantly different in the final best model (Table 3.2, Fig. 3.2).

3.5 Discussion

Results from this study support our hypotheses that elevated fecal glucocorticoid metabolites (FGMs) in African female buffalo is associated with 1) immune enhancement through the upregulation of pro-inflammatory immune responses to acute infections, and 2) limited to some immune suppression (i.e. proportion of eosinophils) which are specifically an indicator of anti-macroparasite immunity. Interestingly, the elevation of gamma-glutamyl transferase (GGT) shown in this study, could be indicative of subclinical liver damage, as GGT is an inducible enzyme with elevations typically linked to the damage in the liver – in this case perhaps due to copper or plant toxicities (Gummow et al. 1991; Grobler 1999; Grobler and Swan 1999; Thompson 2018). Whereas, the changes in erythrocyte turnover rate could be indicative of subclinical anemia in stressed animals.

Dependent on the immune component, FGMs enhanced or suppressed immunity of female African buffalo. For example, increased FGMs is associated with higher bactericidal competency, and to a lesser extent, the proportion of neutrophils (immune enhancement). This coincides with a previous study which found neutrophil counts and bactericidal competence to be positively correlated (Beechler et al. 2012) in whole blood, whereas we show that this extends to plasma competence. However, the proportion of eosinophils decreased with increased FGMs. Interestingly, stress had no effect on any of the other baseline measures of immunity (e.g., total leukocyte count, lymphocytes, monocytes, or basophils). The first line of defense of immunity is quick to respond, developmentally inexpensive, but energetically costly in upregulation, and plays an important role in directing subsequent immune responses (Klasing 2003). Therefore, increased glucocorticoids, increased glucose availability, enhance individuals ability to fight a bacterial infection. Neutrophils are associated with phagocytosis and inflammation (Voigt and Swist 2013), and in cattle, increased numbers of neutrophils are indicative of a response to localized, acute bacterial infections (Schalm 1962). Here we controlled for potential effects of current acute

infections on immune responses of interest (by including haptoglobin as an explanatory variable in all models). The fact that we nonetheless see increases in various pro-inflammatory responses in animals with higher FGMs, suggests that it is indeed the stress response readying the animal for responses to acute infections, and not vice versa.

On the other hand, eosinophils function against most parasites, are attracted to foreign or degraded proteins associated with inflammation and allergic conditions, have some form of phagocytic ability, it can detoxify some chemicals, and it can kill organisms in the presence of antibodies (Voigt and Swist 2013). The down-regulation of eosinophils with increased FGMs, is not uncommon and has been reported as eosinopenia under stress conditions (Voigt and Swist 2013), but could also be a defense mechanism of the body, considering the energetically expensive upregulation of one immune component (in this case neutrophils). For example, vertebrates increased plasma cortisol concentrations were associated with increased neutrophils but decreased lymphocytes (Davis et al. 2008).

As in this study, it is common in ruminants to have a higher percentage of lymphocytes compared to neutrophils (Voigt and Swist 2013; Roland et al. 2014). Although there was no detectable effect of FGMs on lymphocytes, increased FGMs were associated with increased interferon gamma (IFNγ) response to pokeweed stimulation. IFNγ is a cytokine (i.e., glycoproteins that regulate cell growth and development) secreted by T helper 1 (Th-1) lymphocytes (Sompayrac 2015). IFNγ is critical for immunity against viral, intracellular bacterial infections and for tumor control. IFNγ is also an important activator for macrophages, neutrophils, and natural killer cells (Sompayrac 2015). Similarly, increased FGMs were associated with an increase in Il-12 (also produced by a Th-1 cell), and even though FGMs was an important explanatory variable for Il-12 this response was not significantly different. On the contrary, FGMs were not associated with Il-4 stimulation, an antagonistic response towards IFNγ and Il-12. As seen here, one would expect cytokine production from Th cells to be suppress by one or the other, because Th-1 cells suppress Th-2 cells and vice versa (Abbas et al. 1996; Yazdanbakhsh et al. 2002). The opposite results were suggested in animals and humans where glucocorticoids

affected immunity by suppressing Th-1 cytokine production such as Il-12 and IFNγ *in vivo* and *in vitro* (Elenkov et al. 2006). However, the Th-1/Th-2 balance can be skewed one way or the other depending on the pathological condition (Elenkov and Chrousos 1999; Elenkov et al. 2006). Perhaps, the consequence of this stress-related change in immune responses may help animals defend themselves against acute (short-term) pathogen invasions (immune-enhancement) and self-damage (suppression). On the other hand, perhaps it allows parasites (hemo- or gastrointestinal parasites) to reproduce more. Therefore, stress could change disease dynamics and parasite communities within a host.

Increased FGMs were associated with elevated gamma-glutamyl transferase (GGT), perhaps indicating toxic exposure to copper or poisonous plants. Hepatotoxicity, including copper toxicity (i.e., exceeding the copper storage capacity of the liver result in elevated copper concentrations in blood plasma) is commonly associated with elevated GGTs in blood plasma. Toxins relevant to ruminants in this context include minerals (e.g., copper and iron), as well as plant toxins that are processed in the liver (e.g., *Lantana camara*, *Panicum coloratum*, pyrrolizidine alkaloid). In our study area, copper toxicity has been demonstrated in cattle and impala (Grobler and Swan 1999) and traced to mining activities just outside of the park boundaries (Gummow et al. 1991; Grobler 1999). In addition, some of the toxic plants in the study area produce toxins that can cause increases in GGT, such as *Lantana camara* (Kellerman and Coetzer 1985; Barr and Anderson 2009; Vardien et al. 2012), which is not uncommon for cattle to feed on, especially in highly infested areas (Kellerman and Coetzer 1985). In Red kangeroos (*Megaleira rufus*) exposure to *L. camara* resulted in elevated GGT's and supported the diagnosis of hepatotoxicity (Johnson and Jensen 1998). The stress-associated increase in the GGT that we observed in the buffalo could thus signify an increased vulnerability of stressed animals to toxins that are present in their environment. However, further investigation is needed to determine the direction of this effect (i.e., whether stressed animals are more susceptible or if liver damage itself is causing the stress response?). Interestingly, a current analysis of predictors for survival in buffalo also shows that

elevated GGT is predictive of mortality (Glidden et al. *in prep.*), underscoring the relevance of these findings for buffalo health and survival.

The observed higher mean corpuscular volume (MCV) in buffalo with elevated FGMs, indicating a shift in the red blood cell population towards younger erythrocytes (Voigt and Swist 2013). This could be due to a reduction in erythrocyte life span, and/or an increase in erythropoiesis, which is typically indicative of a subclinical anemia. Stress might thus encourage a vigorous hematopoietic response to erythrocyte loss due to blood-borne parasites or toxicosis. For example, hepatocellular death due to excessive copper release into the bloodstream, resulting into vascular damage such as premature erythrocyte death that leads to an acute hemolytic crisis (Thompson 2018) and increased hematocrit levels (Gummow et al. 1991). Alternatively, perhaps these stressed animals are not controlling their hemoparasitic and blood-sucking worm populations as well, and therefore need to upregulate their erythrocyte production.

This is one of the first studies using longitudinal data on a wild mammalian species to characterize the effects of stress (i.e., FGM as stress marker) on immune responses and physiology. Our findings are consistent with the idea that stress responses modulate immune responses to prioritize defenses against acute, microbial infections, while potentially deprioritize defenses against parasitic infections. Moreover, we discovered evidence linking stress responses to changes in liver function and hematopoiesis, which may indicate alterations in the animal's ability to tolerate toxic and parasitic exposures. As expected both year and season were important explanatory variables of the majority of immunological/physiological measures, as expected in a highly variable environment with seasonal (e.g., hot, wet summers compared to cold, dry winters) and yearly variation.

In conclusion, our results reinforce the idea of the physiological stress response as a double-edged sword: immune defenses against some pathogens are enhanced, at the cost of defenses against parasites; vulnerability to toxic exposures may be exacerbated, while the capacity for replenishment of erythrocytes is enhanced. The physiological effects of stress responses likely reflect trade-offs, honed by evolution to provide the most critical emergency functions the animal is likely to

require. To what extent these responses are plastic, allowing them to remain adaptive in the face of rapid environmental change, is a critical question in ecological physiology.

3.6 Tables and Figures

Table 3.1: Biological description and relevance of the various immunological and physiological (i.e., hematological and biochemical) measures.

Type	Category	Description [a]
Bacterial Killing Assays	Immune	Measures bacterial competency of blood plasma in the presence of an extracellular pathogen, *Escherichia coli*.
Leukocytes	Immune/ hematologic	Measures the relative number of immune cells in a cubic mm of blood (10^3/mm^3). Various causes dependent on the leukocyte type.
Neutrophils	Immune/ hematologic	Associated with phagocytosis and inflammation. In cattle, elevated neutrophils are indicative of a response to localized acute bacterial infections. Increases are associated with stress, inflammation, or infections.
Lymphocytes	Immune/ hematologic	Associated with antibody production and cell-mediated immunity. Generally associated with viral infections. Most common leukocyte in ruminants. Lymphocytes that migrate to the thymus are called T-lymphocytes and functions as T helper 1 or T helper 2 cells.
Eosinophils	Immune/ hematologic	Associated with parasitic worm infections. Eosinopenia (i.e., reduced numbers) can result under stress conditions.
Monocytes	Immune/ hematologic	Develop into macrophages. Non-specific defense against pathogen infected cells via phagocytosis. Activate B and T lymphocytes.
Basophils	Immune/ hematologic	Associated with allergic reactions.
Interferon gamma (IFNy)	Immune/ hematologic	T helper 1 cytokine (immunological active protein) that is associated with an immune response to microparasites.
Interleukin 4 (Il-4)	Immune/ hematologic	T helper 2 cytokine (immunological active protein) that is associated with an immune response to macroparasites.
Interleukin 12 (Il-12)	Immune/ hematologic	T helper 1 cytokine (immunological active protein) that is associated with an immune response to microparasites.
Haptoglobin	Immune/ hematologic	Acute phase protein that is a reliable indicator of an acute infection by various infections in cattle.
Erythrocytes	Hematologic	Measures the relative number of erythrocytes in a cubic mm of blood (10^6/mm^3). Increases associated with polycythemia, decreases associated with blood loss or hemolysis.
Hemoglobin (HGB)	Hematologic	Measured as grams of hemoglobin per deciliter of blood(g/dl). The protein, hemoglobin is the molecule responsible for carrying oxygen. Therefore, HGB is an indication of the oxygen carrying ability of blood.

[a] Various peer-review journal articles and veterinary textbooks were used to compile descriptive information for each immunological, hematological, and biochemical measure used in this study (Schalm 1962; Voigt and Swist 2013; Couch et al. 2017; Whitbread n.d.; Sompayrac 2015; Horadagoda et al. 1999).

Table 3.1 *Continued…*

Type	Category	Description [a]
Hematocrit (HCT)	Hematologic	Measures the percentage of blood volume comprised of erythrocytes (MCV*Erythrocytes/10). Increases are associated with polycythemia, whereas decreases are associated with blood loss, anemia, hemolysis, or bone marrow diseases.
Platelets (PLT)	Hematologic	Measures the relative number of platelets in a cubic mm of blood ($10^3/mm^3$). Plays an important role during blood clotting (prevent blood loss) during injury, including internal tissue damage of blood vessels.
Mean Corpuscular Volume (MCV)	Hematologic	MCV measures the average volume of the individual erythrocyte, expressed in femtoliters (fl) and an increased size in MCV indicates immature erythrocytes.
Mean Corpuscular Hemoglobin (MCH)	Hematologic	Measured as a function of HGB and erythrocytes and expressed as pictogram (pg).
Mean Corpuscular Hemoglobin Concentration (MCHC)	Hematologic	Measures the average concentration of hemoglobin in erythrocytes (HGB*100/HCT) and expressed as grams of hemoglobin per deciliter of blood (g/dl). Another measure to indicate oxygen carrying ability of blood.
Albumin (ALB)	Biochemical	Liver proteins measured as grams per deciliters (g/dL). Increases due to dehydration. Decreases due to liver failure, protein deficiency, hemorrhage, or some viral conditions.
Globulins (GLOB)	Biochemical	Measures all proteins that functions as antibodies, enzymes, complement and carriers. Measured as grams per deciliter (g/dL).
Total protein (TP)	Biochemical	All proteins (summation of albumin and globulins). Increases due to dehydration or chronic inflammation. Decreases associated with protein deficiency, hemorrhage, or some viral conditions. Measured as grams per deciliters (g/dL).
Gamma glutamyl transferase (GGT)	Biochemical	Mostly liver enzymes. Indicate potential liver damage due to variable factors that can lead to increased hepatocyte necrosis. Increases with long-term liver damage and useful metric in ruminants. Measured as units per liter (U/L).
Creatinine kinase (CK)	Biochemical	Muscle enzyme that is indicative of muscle damage due to increased muscle activity. Measured as units per liter (U/L).
Alkaline phosphatase (ALP)	Biochemical	Liver and bone enzymes. Measured as units per liter (U/L). Elevated levels are associated with liver damage, biliary disease, bone formation or raised levels of circulating steroids.

[a] Various peer-review journal articles and veterinary textbooks were used to compile descriptive information for each immunological, hematological, and biochemical measure used in this study (Schalm 1962; Voigt and Swist 2013; Couch et al. 2017; Whitbread n.d.; Sompayrac 2015; Horadagoda et al. 1999).

Table 3.1 *Continue…*

Type	Category	Description [a]
Aspartate aminotransferase (AST)	Biochemical	Liver and muscle enzymes. Measured as units per liter (U/L).
Calcium (Ca)	Biochemical	Most common mineral in the body, essential for muscle contraction and bone formation. Can increase due to dehydration. Measured as milligram per deciliter (mg/dL).
Blood Urea Nitrogen (BUN)	Biochemical	Byproduct of protein metabolism. Decreases indicative of liver damage and protein deficiency. Increases associated with dehydration, or alterations related with protein metabolism. Measured as grams per deciliter (g/dL).
Phosphorous (P)	Biochemical	Play a role in muscle and nerve function. Increases associated with renal failure. Decreases associated with downer cows and stress. Measured as milligram per deciliter (mg/dL).
Magnesium (Mg)	Biochemical	Play a role in muscle and nerve function. Decreases in ruminants is associated with acute and chronic dietary deficiencies. Measured as milligram per deciliter (mg/dL).

[a] Various peer-review journal articles and veterinary textbooks was used to compile descriptive information for each immunological, hematological, and biochemical measure used in this study (Schalm 1962; Voigt and Swist 2013; Couch et al. 2017; Whitbread n.d.; Sompayrac 2015; Horadagoda et al. 1999).

Table 3.2: Summary of the best fit linear mixed-effects model, where FGMs is associated with immune, hematological and/or biochemical health measures. Bold font indicates statistical significance. Other explanatory variables are listed in order of importance. Sample sizes changed depending on the immune, hematologic, or biochemical measure of interest. FGM = Fecal glucocorticoid metabolite; R^2m = marginal; R^2c = conditional.

Parameter			FGM		Other explanatory variables accounted for:	R^2m (%)	R^2c (%)
Dependent variables	*Animal ID*	*n*	(Intercept±*SE*) *β*±*SE*	*P*-value			
Immune measures							
Bacterial killing assay (BKA)	**258**	**1024**	**(-0.69±0.03) 0.02±0.008**	**0.0050**	**Year** + **offset(control)** + **Herd** + **Body condition** + **Season** + Haptoglobin	26.72	28.51
Total leukocyte count (log)	253	946	(1.84±0.05) -	-	**Year** + **Season** + **Body condition** + **Reproductive status** + **Age** + Haptoglobin	37.25	48.36
Neutrophils	261	1071	(0.26±0.009) 0.004±0.002	0.0812	**Body condition** + **Haptoglobin** + **Season** + **Age** + **Year**	16.13	36.10
Lymphocytes	261	1071	(0.34±0.02) -	-	**Season + Year + Body condition + Herd** + Haptoglobin	15.93	21.65
Monocytes	261	1071	(0.25±0.02) -	-	**Year** + **Season** + **Age** + **Herd** + **Age²** + Haptoglobin	10.53	10.53
Eosinophils	**261**	**1071**	**(0.15±0.008) -0.004±0.002**	**0.0507**	**Season** + **Haptoglobin** + **Reproductive status** + **Age** + **Year** + Herd + **Horn residuals** + Body condition	25.43	38.79
Basophils	261	1071	(0.003±0.0004) -	-	**Year** + **Herd** + **Season +** Horn residuals + Body condition + Haptoglobin	7.13	8.68
Neutrophil Lymphocyte Ratio (NLR)	252	922	(2.65±0.24) -	-	**Season** + **Reproductive status** + Age² + Age + Haptoglobin	3.27	6.09
Interferon gamma (IFN*γ*) stimulation	**259**	**1057**	**(1.14±0.08) 0.04±0.02**	**0.0287**	**Year** + **Season** + **Herd** + Treatment + Haptoglobin	5.51	38.89
Interleukin 4 (Il-4) stimulation	250	887	(1175.55±146.26)	-	**Year** + **Herd** + **Body condition** + Season + Haptoglobin	11.88	22.11

Table 3.2 *Continue…*

Parameter			FGM		Other explanatory variables accounted for:	R^2m (%)	R^2c (%)
Dependent variables	*Animal ID*	*n*	(Intercept±*SE*) *β*±*SE*	*P*-value			
Immune measures							
IFNγ:Il-4 ratio stimulation	249	881	(0.002±0.0002) -	-	**Year** + **Season** + Body condition + Haptoglobin	5.30	21.70
Interleukin 12 stimulation	231	483	(5.19±0.24) -	-	**Year** + **Herd** + **Body condition** + Haptoglobin	29.16	29.16
Interleukin 12 response	231	483	(-0.68±0.41) 0.19±0.10	0.0616	**Year** + **Season** + Haptoglobin	2.95	2.95
Hematologic measures							
Total erythrocyte count	248	922	(7.64±0.29) -	-	**Year** + **Season** + **Body condition** + **Age** + **Age^2** + Haptoglobin	30.26	41.88
Hemoglobin (Hgb)	248	922	(9.43±0.36) -	-	**Season** + **Year** + **Herd** + **Body condition** + Haptoglobin	15.10	23.28
Hematocrit (HCT)	248	922	(28.06±1.03)	-	**Year** + **Season** + **Body condition** + Haptoglobin	20.67	31.42
Mean corpuscular hemoglobin (MCH)	248	922	(11.87±0.24) -	-	**Year** + **Season** + **Body condition** + **Herd** + **Reproductive status** + **Age** + Haptoglobin	41.60	49.20
Mean corpuscular hemoglobin concentration (MCHC)	248	922	(32.21±0.52) -	-	**Year** + **Season** + **Body condition** + **Herd** + **Age** + Haptoglobin	28.20	28.64
Mean corpuscular volume (MCV)	**248**	**922**	**(37.01±0.40)** **0.22±0.08**	**0.0029**	**Year** + **Season** + **Reproductive status** + **Age** + **Age^2** + **Herd** + Haptoglobin	43.40	80.94
Platelets (PLT)	248	922	(491.79±23.72) -7.98±5.49	0.1465	**Year** + **Season** + **Treatment** + **Haptoglobin** + Body condition	8.89	22.84

Table 3.2 *Continue…*

Parameter			FGM		Other explanatory variables accounted for:	R^2m (%)	R^2c (%)
Dependent variables	*Animal ID*	*n*	(Intercept±*SE*) *β*±*SE*	*P*-value			
Biochemical panel measures							
Albumin (ALB)	151	407	(4.46±0.07) -	-	**Year** + **Season** + **Reproductive status** + **Haptoglobin** + **Body condition** + **Age²** + Herd	12.85	60.20
Globulins (GLOB)	151	407	(4.10±0.13) -	-	**Season** + **Year** + **Haptoglobin** + **Body condition** + **Age²** + **Age**	31.98	65.38
Albumin Globulin Ratio (ALB:GLOB)	151	407	(0.95±0.04) -	-	**Season** + **Haptoglobin** + **Body condition** + **Age²** + **Year** + **Age** + **Reproductive status**	24.47	62.07
Total protein (TP)	151	407	(9.19±0.50) -	-	**Season** + **Year** + **Horn residuals** + **Body condition** + Haptoglobin	0.86	99.30
Gamma glutamyl transferase (GGT)	**151**	**407**	**(12.47±0.47)** **0.43±0.15**	**0.0044**	**Body condition** + **Year** + **Herd** + Haptoglobin	9.93	49.76
Creatinine kinase (CK)	151	407	(143.39±43.73) -	-	**Season** + **Year** + **Age²** + Reproductive status + Treatment + Age + Haptoglobin	12.28	64.14
Alkaline phosphatase (ALP)	151	407	(103.92±7.26) -	-	**Season** + Horn residuals + Haptoglobin	4.76	71.21
Aspartate aminotransferase (AST)	151	407	(99.50±7.73)	-	**Year** + **Body condition** + **Season** + Horn residuals + Haptoglobin	5.02	91.18
Calcium (Ca)	151	407	(7.87±0.16) -0.08±0.05	0.1525	**Year** + Treatment + Herd + Haptoglobin	14.93	14.93
Blood Urea Nitrogen (BUN)	151	407	(10.66±0.68) 0.29±0.17	0.0878	**Season** + **Year** + **Body condition** + Haptoglobin	28.40	28.47
Phosphorous (P)	151	407	(5.95±0.15)	-	**Year** + **Body condition** + **Herd** + Horn residuals + Reproductive status + Haptoglobin	13.82	26.14
Magnesium (Mg)	151	407	(2.70±0.08) -	-	**Season** + **Reproductive status** + **Haptoglobin** + **Year** + **Age** + Body condition	23.54	45.20

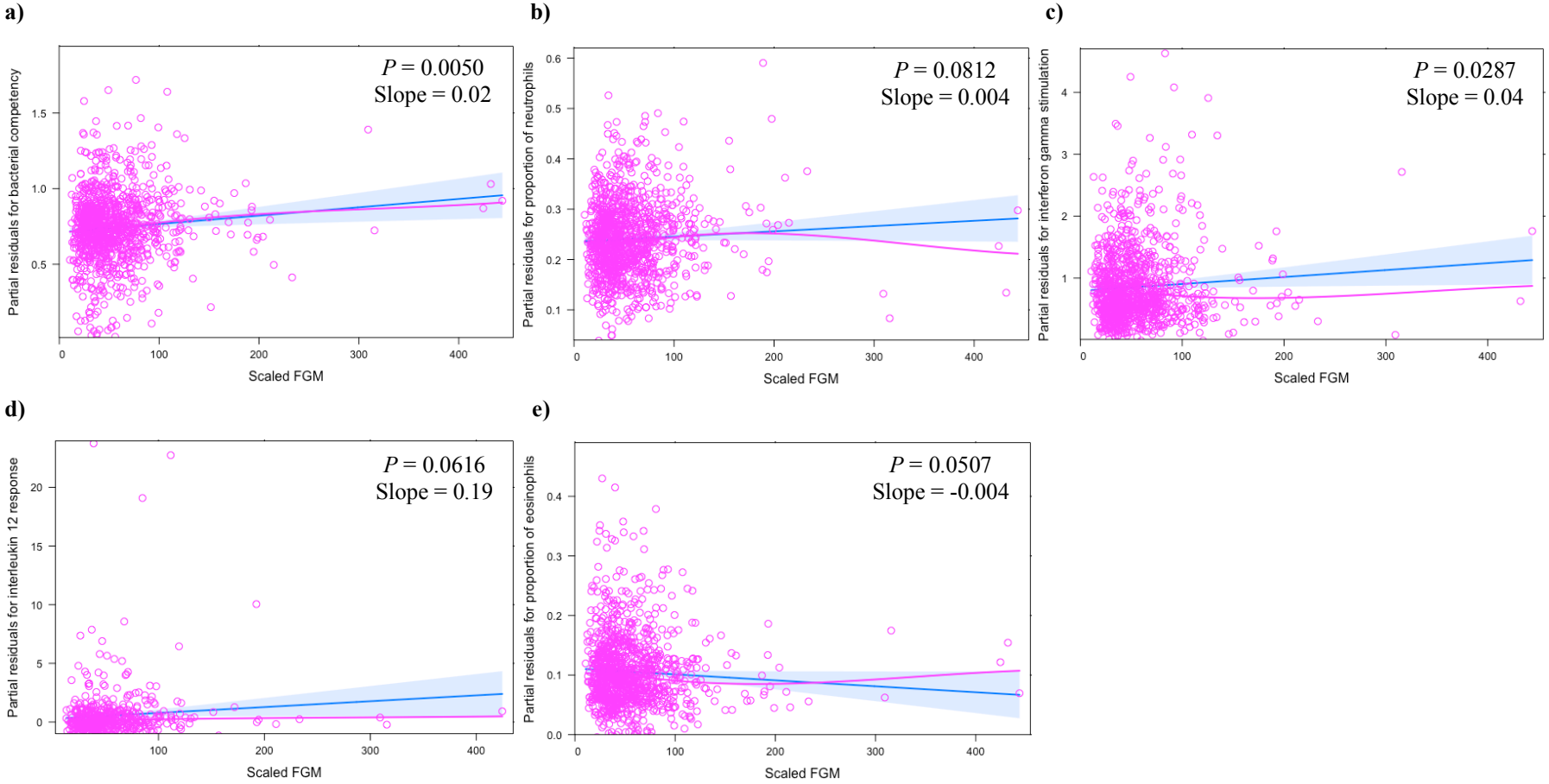

Figure 3.1 Effect displays with partial residuals for fecal glucocorticoid metabolites (FGM) on immunological measures, a) bacterial competency, b) proportion of neutrophils, c) interferon gamma stimulation, d) interleukin 12 response, and e) proportion of eosinophils. The blue line indicates the slope predicted by the best model for each immunological measure that were associated with FGMs (see Supplementary Data SD3.1 for the effects of other independent variables accounted for in each best model). The shaded blue indicates confidence intervals and the pink line indicates the slope with a "loess" function showing a non-linear trend.

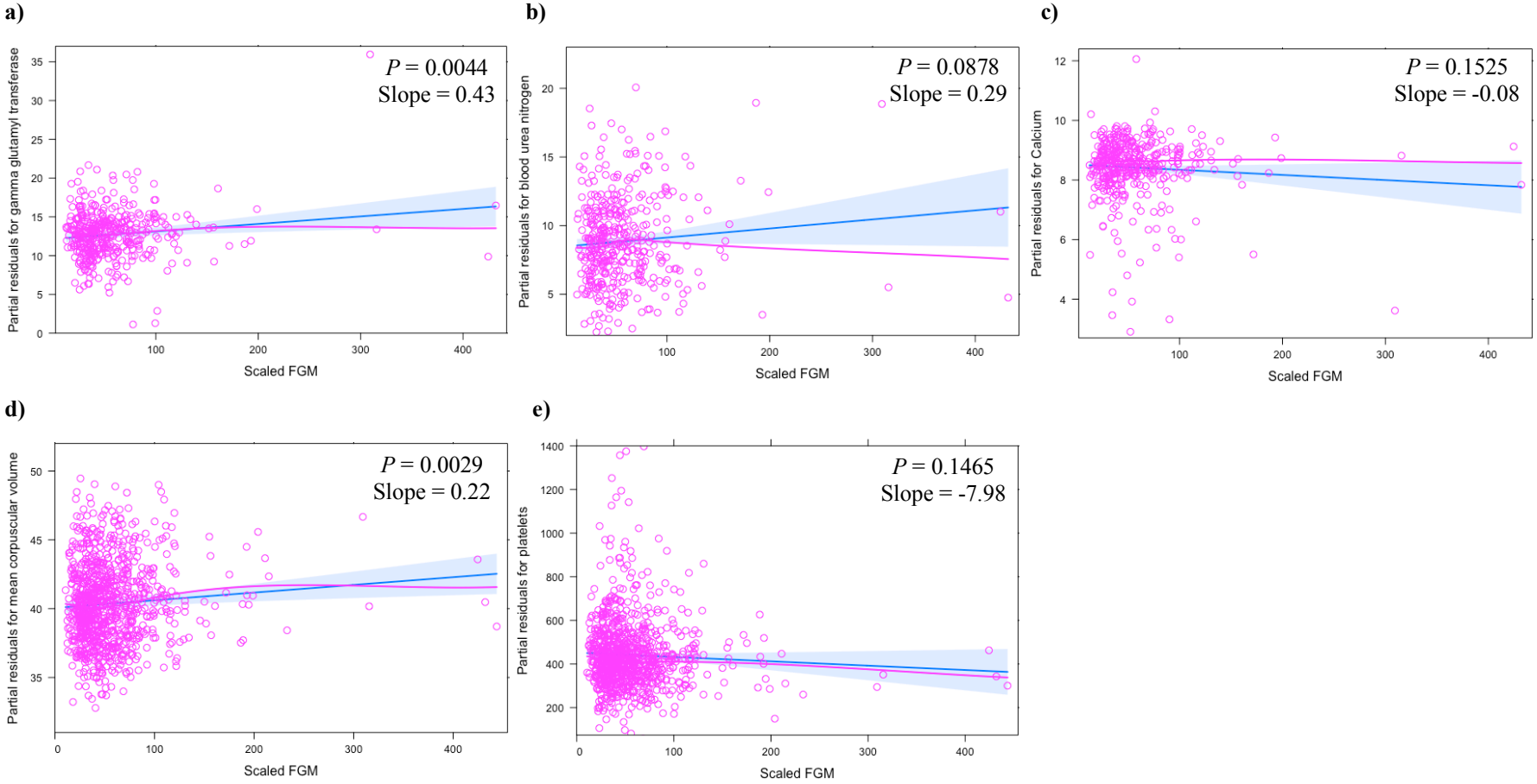

Figure 3.1 Effect displays with partial residuals for fecal glucocorticoid metabolites (FGM) on physiological health measures, a) gamma glutamyl transferase, b) blood urea nitrogen, c) calcium, d) mean corpuscular volume, and e) platelets. The blue line indicates the slope predicted by the best model for each physiological health measure that were associated with FGMs (see Supplementary Data SD3.1 for the effects of other independent variables accounted for in each best model). The shaded blue indicates confidence intervals and the pink line indicates the slope with a "loess" function showing a non-linear trend.

CONCLUSION

Rapidly changing environments in the face of anthropogenic stressors and increased risk of pathogen/parasite distributions have raised global concern (Patz et al. 2000a; Weiss and McMichael 2004b; Eisenberg et al. 2006; Parmesan 2006). Therefore, the ability to predict and manage changes in infectious disease prevalence and severity depends critically on a mechanistic understanding linking anthropogenic environmental modifications to disease dynamic outcomes (Daszak et al. 2001). Animals respond to exposure to unpredictable events by mounting a physiological stress response, which activates the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky 2002), followed by a cascade of effects, orchestrated by stress hormones (e.g., predominantly cortisol in African buffalo), (Siegel 1980; Sapolsky et al. 2000; Tsigos and Chrousos 2002; Palme et al. 2005). The endocrine, nervous and immune systems form a complex integrated network that functions to maintain homeostasis. Cortisol levels can become elevated in response to a broad range of disturbances and as such, a non-invasive measure of stress may serve as an integrative measure of animal health (McEwen 2004; Mormède et al. 2007; Palme 2012) at the interface of environmental drivers and immunological and infection consequences.

Functionally, stress responses aids in redistributing metabolic allocation to help the animal deal with emergencies - an adaptive response essential to maintaining homeostasis (McEwen and Wingfield 2003). As such, physiological stress may respond to pathogenic challenges, but also modify the host's immune responses and susceptibility to infection - potentially modulating the host-pathogen interaction, and its outcomes for host health and disease dynamics. However, these are complex integrated systems that are dependent on many factors.

I evaluated the use of two commercially available assays (a radioimmunoassay and enzyme immunoassay), to measure fecal glucocorticoid metabolites (FGMs), as a non-invasive measure of stress responses in African buffalo. There is a lag time of 10 – 20 hours post an ACTH-induced plasma cortisol concentration peak and adult buffalo mounted a stress response to immobilization and handling compared to subadults, suggesting an age-related difference. Therefore,

FGMs providing a non-invasive and efficient means of assessing adrenocortical activity in buffalo.

Using a longitudinal study of 200 individually marked, free-ranging African buffalo, in which FGMs, infections by viral and bacterial pathogens, gastrointestinal parasites, immunological, and physiological (hematological and biochemical) measures were collected, every six months over a four-year period, allowed me to investigate stress responses (measured as FGMs) as an integrative physiological link between relevant measures of animal health at the interface of environmental drivers and immunological and infection consequences.

Environmental change often includes multiple stressors changing simultaneously - including exposure to pathogens and parasites. Results from this dissertation showed that infections are a potential driver of FGMs in buffalo, along with long- and short-term indicators of body condition, demonstrating that FGMs may indeed function as an integrative measure that captures the effects of multiple potential stressors on the animals. Further, stress responses may play a role in driving transmission cycles of acute infections, potentially leading to feedback cycles between stress responses and disease (i.e., acute infections elevate FGMs, followed by the increased risk of obtaining some chronic bacteria), which could exacerbate effects of infections on vulnerable wildlife populations.

This is one of the first studies using longitudinal data on a wild mammalian species to characterize the effects of stress (i.e., FGM as stress marker) on immune responses and physiology. Our findings are consistent with the idea that stress responses modulate immune responses to prioritize defenses against acute, microbial infections, while deprioritizing defenses against chronic subclinical parasitic infections (i.e., acknowledging weak evidence for the suppressed effect).

Ultimately, this study evaluated the reliability of a non-invasive tool for future use in African buffalo, and it provides some mechanistic, animal physiology-based link between current accelerated environmental changes and immunological and infection consequences at the population level. It allows us to control for fixed animal effects, focusing on the plastic component in covariation between stress responses, immunity/physiology, and infectious consequences. The longitudinal design of the

data allowed me to make progress towards understanding possible causal relationships between stress and infectious diseases in a wild mammalian population, because I was able to examine how prior, and subsequent infections were associated with variation in FGMs. Although, this longitudinal study design did enable me to tease apart effects of multiple factors, including environmental variation, reproduction, nutrition, individual variation, as well as infectious diseases on stress physiology. Experimental infections would be needed to demonstrate a causal relationship between acute infections and elevated stress responses conclusively, and experimental manipulation of stress levels in buffalo could test whether susceptibility to acute infections is indeed increased. Future analysis could also investigate whether elevated FGMs is associated with greater pro-inflammatory responses if animals experienced acute infections. Also, FGMs in individual buffalo were moderately repeatable (i.e., 44%), suggesting that some buffalo tended towards consistently higher cortisol levels than others, and approximately, 46% of the variance in FGMs attributed to individual animals. Therefore, further investigation is needed at the individual level, in order to address stress physiology as a personality trait.

Further investigation is needed to determine the direction of the stress-associated increase in the GGT that we observed in the buffalo, in order to determine whether stressed animals are more susceptible or if liver damage itself causing the stress response. Follow up work could potentially test for copper levels in buffalo blood.

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APPENDIX I:

Chapter 1 Supplementary Data

Supplementary Data SD1.1 Summary of linear mixed effects model for plasma cortisol concentration (nmol/L) of African buffalo (*Syncerus caffer*), accounting for time, age, sex, and random effects of individual buffalo (n = 8) during both trials (weeks) of the validation experiment (n = 197). Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: treatment = saline; week = week 2; age = adult; sex = female; time = 0 minutes (initial plasma cortisol level). Sample size for age category = 2 adults and 6 subadults. Sample size for sex category = 3 males and 5 females.

Predictor	Effect[a]	Plasma cortisol (ß) Estimate ± *SE* (nmol/L)	*t*-value	*P*-value
Fixed effects				
(Intercept)		-41.91 ± 49.92	-0.84	0.4397
Treatment: ACTH		77.58 ± 50.01	1.55	0.1661
Time: 5 minutes		4.10 ± 21.92	0.19	0.8519
Time: 10 minutes		6.51 ± 21.92	0.30	0.7668
Time: 15 minutes		20.05 ± 21.92	0.92	0.3618
Time: 20 minutes		18.83 ± 21.92	0.86	0.3918
Time: 25 minutes		23.93 ± 21.92	1.09	0.2767
Time: 30 minutes		19.89 ± 21.92	0.91	0.3657
Time: 35 minutes		22.08 ± 22.73	0.97	0.3328
Time: 40 minutes		18.51 ± 21.92	0.84	0.3997
Time: 45 minutes		21.63 ± 21.92	0.99	0.3254
Time: 50 minutes		19.44 ± 21.92	0.89	0.3766
Time: 55 minutes		23.04 ± 21.92	1.05	0.2949
Time: 60 minutes		26.54 ± 21.92	1.21	0.2278
Week: 1	↑	**142.06 ± 44.11**	**3.22**	**0.0309**
Age: subadult		100.92 ± 55.03	1.83	0.1384
Sex: male		-85.79 ± 50.94	-1.68	0.1653
Interaction terms				
Treatment (ACTH):Time (5 minutes)		13.46 ± 31.00	0.43	0.6647
Treatment (ACTH):Time (10 minutes)	↑	**59.19 ± 31.00**	**1.91**	**0.0580**
Treatment (ACTH):Time (15 minutes)		56.46 ± 31.00	1.82	0.0704
Treatment (ACTH):Time (20 minutes)	↑	**66.07 ± 31.59**	**2.09**	**0.0380**
Treatment (ACTH):Time (25 minutes)	↑	**66.54 ± 31.60**	**2.11**	**0.0368**
Treatment (ACTH):Time (30 minutes)	↑	**63.56 ± 31.60**	**2.01**	**0.0459**
Treatment (ACTH):Time (35 minutes)	↑	**78.73 ± 32.93**	**2.39**	**0.0180**
Treatment (ACTH):Time (40 minutes)	↑	**64.11 ± 31.60**	**2.03**	**0.0441**
Treatment (ACTH):Time (45 minutes)	↑	**69.91 ± 31.60**	**2.21**	**0.0283**
Treatment (ACTH):Time (50 minutes)	↑	**79.44 ± 31.60**	**2.51**	**0.0129**
Treatment (ACTH):Time (55 minutes)	↑	**69.78 ± 31.60**	**2.21**	**0.0286**
Treatment (ACTH):Time (60 minutes)	↑	**73.48 ± 31.60**	**2.33**	**0.0213**
Treatment (ACTH):Week (1)		21.14 ± 87.34	0.24	0.8208
Treatment (ACTH):Age (subadult)	↓	**-88.90 ± 15.98**	**-5.56**	**<0.0001**
Treatment (ACTH):Sex (Male)		14.94 ± 15.20	0.98	0.3271
Random effects				
		Variance	*SD*	
Animal ID (intercept)		3419	58.47	
Residuals		1922	43.84	

[a] Effect column indicate the direction of the slope predicted by the model.

Supplementary Data SD1.2 Summary of linear mixed effects model for the FGMs (for both assays) comparing ACTH and saline treated African buffalo (*Syncerus caffer*), accounting for time, age, sex, and random effects of individual buffalo ($n = 8$) during both trials (weeks) of the validation experiment. Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: treatment = saline; week = week 2; age = adult; sex = female and time = initial FGM level. Sample size for age category = 2 adults and 6 subadults. Sample size for sex category = 3 males and 5 females. FGM = fecal glucocorticoid metabolite; EIA = Enzyme immunoassay; RIA = Radioimmunoassay.

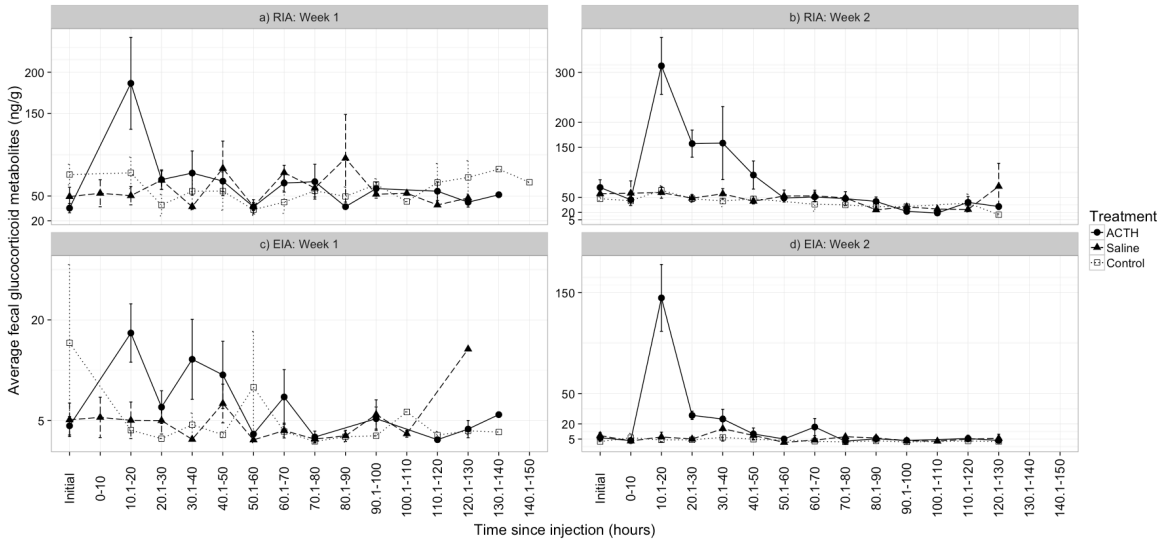
Predictor	Effect[a]	RIA ($n = 164$)			EIA ($n = 155$)		
		Log FGM (ß) Estimate ± *SE* (ng/g)	*t*-value	*P*-value	Log FGM (ß) Estimate ± *SE* (ng/g)	*t*-value	*P*-value
Fixed effects							
(Intercept)		**3.92 ± 0.22**	**17.76**	**< 0.0001**	**2.51 ± 0.35**	**7.15**	**<0.0001**
Treatment: ACTH		0.13 ± 0.28	0.45	0.6526	-0.42 ± 0.45	-0.92	0.3597
Time: 0 – 10 hours		0.00 ± 0.27	0.01	0.9919	-0.02 ± 0.42	-0.06	0.9566
Time: 10.1 – 20 hours		0.10 ± 0.21	0.48	0.6320	0.05 ± 0.34	0.15	0.8852
Time: 20.1 – 30 hours		0.07 ± 0.22	0.31	0.7540	-0.24 ± 0.35	-0.69	0.4896
Time: 30.1 – 40 hours		-0.07 ± 0.22	-0.30	0.7639	-0.10 ± 0.37	-0.27	0.7898
Time: 40.1 – 50 hours		0.04 ± 0.21	0.17	0.8619	0.22 ± 0.34	0.65	0.5172
Time: 50.1 – 60 hours		-0.22 ± 0.25	-0.90	0.3710	-0.65 ± 0.42	-1.55	0.1241
Time: 60.1 – 70 hours		0.20 ± 0.22	0.91	0.3660	-0.23 ± 0.37	-0.63	0.5273
Time: 70.1 – 80 hours		0.02 ± 0.21	0.11	0.9123	0.09 ± 0.37	0.25	0.8008
Time: 80.1 – 90 hours		-0.15 ± 0.26	-0.57	0.5670	0.12 ± 0.42	0.29	0.7730
Time: 90.1 – 100 hours		-0.24 ± 0.22	-1.07	0.2867	-0.27 ± 0.35	-0.77	0.4428
Time: 100.1 – 110 hours		-0.12 ± 0.26	-0.46	0.6440	-0.59 ± 0.42	-1.39	0.1674
Time: 110.1 – 120 hours	🡻	**-0.58 ± 0.25**	**-2.35**	**0.0205**	-0.03 ± 0.42	-0.07	0.9422
Time: 120.1 – 130 hours		0.03 ± 0.30	0.11	0.9152	-0.26 ± 0.47	-0.56	0.5758
Time: 130.1 – 140 hours		0.18 ± 0.47	0.38	0.7073	0.40 ± 0.75	0.54	0.5913
Week: 1		0.17 ± 0.15	1.14	0.2957	-0.03 ± 0.25	-0.10	0.9219
Age: Subadult	🡻	-0.13 ± 0.19	-0.69	0.5137	**-1.19 ± 0.31**	**-3.82**	**0.0064**
Sex: Male		0.07 ± 0.18	0.38	0.7155	-0.05 ± 0.28	-0.19	0.8563

[a] Effect column indicate the direction of the slope predicted by

Supplementary Data SD1.2 *Continued*…

Predictor	Effect[a]	RIA (*n* = 164)			EIA (*n* = 155)		
		Log FGM (ß) Estimate ± *SE* (ng/g)	*t*-value	*P*-value	Log FGM (ß) Estimate ± *SE* (ng/g)	*t*-value	*P*-value
Interaction terms							
Treatment (ACTH):Time (0 – 10 hours)		-0.24 ± 0.43	-0.55	0.5843	-0.80 ± 0.69	-1.15	0.2527
Treatment (ACTH):Time (10.1 – 20 hours)	↑	**1.50 ± 0.31**	**4.85**	**<0.0001**	**2.59** ± 0.49	**5.29**	**<0.0001**
Treatment (ACTH):Time (20.1 – 30 hours)	↑	**0.74 ± 0.31**	**2.35**	**0.0204**	**1.54** ± 0.50	**3.09**	**0.0025**
Treatment (ACTH):Time (30.1 – 40 hours)	↑	**0.80 ± 0.31**	**2.55**	**0.0118**	**1.42** ± 0.51	**2.79**	**0.0062**
Treatment (ACTH):Time (40.1 – 50 hours)		0.39 ± 0.30	1.30	0.1971	0.48 ± 0.48	0.99	0.3226
Treatment (ACTH):Time (50.1 – 60 hours)		0.13 ± 0.36	0.37	0.7151	0.85 ± 0.63	1.35	0.1797
Treatment (ACTH):Time (60.1 – 70 hours)		-0.05 ± 0.31	-0.17	0.8681	0.85 ± 0.50	1.69	0.0937
Treatment (ACTH):Time (70.1 – 80 hours)		0.02 ± 0.31	0.06	0.9503	-0.62 ± 0.51	-1.22	0.2263
Treatment (ACTH):Time (80.1 – 90 hours)		-0.10 ± 0.39	-0.25	0.8067	-0.43 ± 0.69	-0.62	0.5357
Treatment (ACTH):Time (90.1 – 100 hours)		0.04 ± 0.31	0.14	0.8906	0.35 ± 0.50	0.70	0.4848
Treatment (ACTH):Time (100.1 – 110 hours)		-0.67 ± 0.53	-1.27	0.2074			
Treatment (ACTH):Time (110.1 – 120 hours)		0.49 ± 0.35	1.41	0.1599	-0.17 ± 0.58	-0.30	0.7651
Treatment (ACTH):Time (120.1 – 130 hours)		-0.10 ± 0.40	-0.25	0.8020	0.34 ± 0.63	0.54	0.5924
Treatment (ACTH):Week (1)		-0.25 ± 0.28	-0.92	0.4066	-0.33 ± 0.44	-0.76	0.4898
Treatment (ACTH):Age (subadult)		-0.13 ± 0.18	-0.69	0.4910	0.30 ± 0.31	0.96	0.3401
Treatment (ACTH):Sex (male)		0.02 ± 0.17	0.14	0.8888	0.38 ± 0.28	1.36	0.1773
Random effects							
		Variance	*SD*		Variance	*SD*	
Animal ID (intercept)		0.025	0.157		0.059	0.243	
Residuals		0.183	0.428		0.462	0.680	

[a] Effect column indicate the direction of the slope predicted by model.



Supplementary Data SD1.3 a) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups, as well as for the control (open squares, dotted line) group (*n* = 4 per group) during week 1 and b) week 2 from the RIA results. c) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups, as well as for the control (open squares, dotted line) group (*n* = 4 per group) during week 1 and d) week 2 from the EIA results. Error bars represent *SE*. Note variable scales on the y-axes.

APPENDIX II:

Chapter 2 Supplementary Data

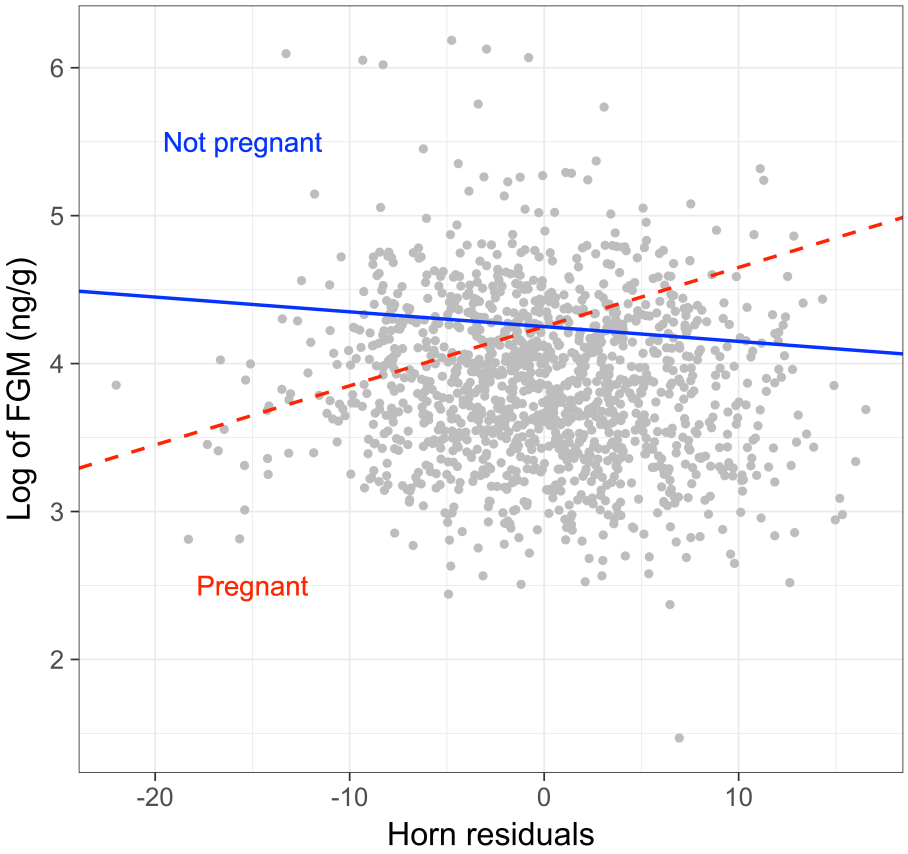
Supplementary Data SD2.1 Summary of the 5 best models during model selection to determine which environmental and demographic factors predict stress in female African buffalo.

Model:	Explanatory variables:	df	AICc	delta	weight
1	Year + Body condition + Season + Pregnant status + Horn residuals + (1|Animal ID)	12	1122.4	0	0.026
2	Year + Body condition + Season + Pregnant status + (1|Animal ID)	11	1122.8	0.47	0.021
3	Year + Body condition + Season + Pregnant status + Horn residuals + Lactation status + (1|Animal ID)	13	1123.2	0.78	0.018
4	Year + Body condition + Season + Pregnant status + Horn residuals + Chlorophyll 415 + (1|Animal ID)	13	1123.3	0.89	0.017
5	Year + Body condition + Season + Pregnant status + Lactation status + (1|Animal ID)	12	1123.7	1.30	0.014

Supplementary Data SD2.2 Summary output of the best fit linear mixed-effects model predicting environmental and demographic drivers of stress, while accounting for random effects of individual buffalo (n = 274) during the 4year study period (n = 1320). Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: pregnant = no; rainfall year = Oct08-Sept09; season = late wet. FGM = fecal glucocorticoid metabolite.

Predictor		Log FGM (ß)		
	Effect [a]	Estimate ± *SE* (ng/g)	*t*-value	*P*-value
Fixed effects				
Intercept ($ß_0$)		**4.25 ± 0.10**	**42.50**	**<0.0001**
Horn residuals	↓	**-0.01± 0.004**	**-2.06**	**0.0399**
Body condition	↓	**-0.06 ± 0.03**	**-2.33**	**0.0200**
Pregnant: Yes		0.04 ± 0.03	1.38	0.1681
Year: Oct09-Sept10	↓	**-0.15 ± 0.04**	**-3.95**	**<0.0001**
Year: Oct10-Sept11		**-0.14 ± 0.04**	**-3.34**	**0.0009**
Year: Oct11-Aug12	↓	**-0.22 ± 0.04**	**-4.99**	**<0.0001**
Season: Early wet	↓	**-0.11 ± 0.04**	**-2.51**	**0.0121**
Season: Early dry		**-0.08 ± 0.04**	**-2.29**	**0.0224**
Season: Late dry		**-0.09 ± 0.04**	**-2.35**	**0.0189**
Interaction term				
Horn residuals:Pregnant (Yes)	↑	**0.01 ±0.004**	**1.98**	**0.0481**
Random effect		Variance	*SD*	
Animal ID		0.12	0.35	
Residuals:		0.16	0.40	

[a] Effect column indicate the direction of the slope predicted by the model.

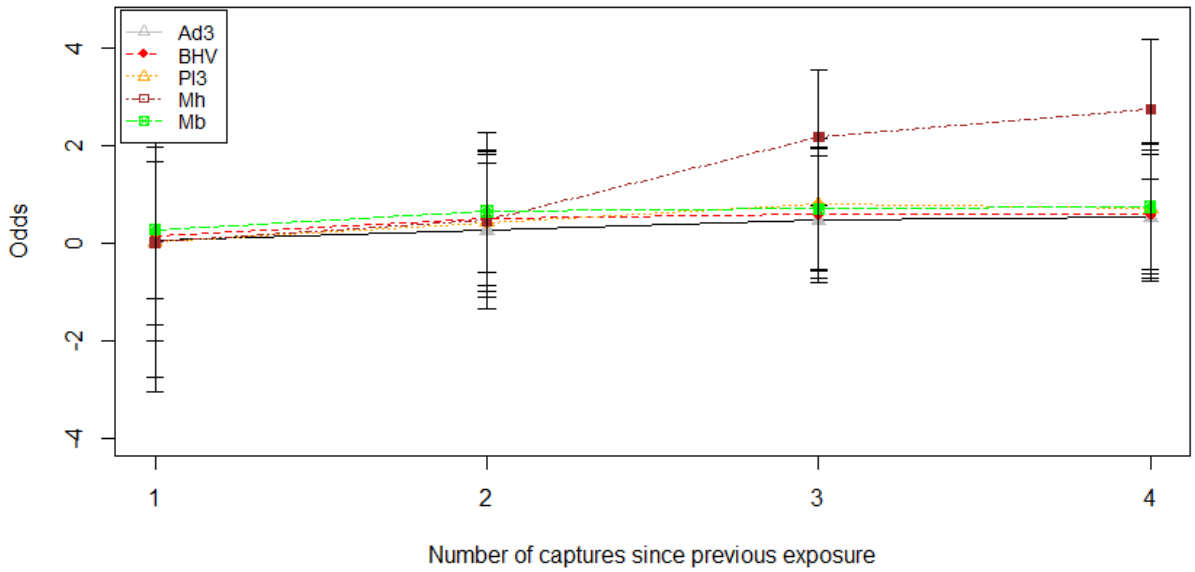


Supplementary Data SD2.3 The change in the log of fecal glucocorticoid metabolites (FGMs) and horn residuals (low value is indicative of below average horn size) is dependent on whether female African buffalo were pregnant (dashed, red line) or not (solid, blue line). Increased FGMs is associated with a decrease (intercept = 4.25, slope = -0.01) in individual quality (indicated by small horns), unless buffalo were pregnant (slope = 0.04), while accounting for body condition, year, season and repeated measures.

Supplementary Data SD2.4 Summary of the best linear mixed-effects model predicting acute infectious diseases (with or without AD3) as drivers of FGMs, while accounting for horn residuals, body condition, pregnant status, year, season, and the random effects of individual buffalo (n = 239) during the 4-year study period (n = 898). Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: pregnant = no; rainfall year = Oct08-Sept09; season = late wet; acute infections = no. FGM = fecal glucocorticoid metabolite, AD3 = bovine adenovirus-3.

		Acute infections include AD3			Acute infections exclude AD3		
Predictor	Effect [a]	Log FGM (ß) Estimate ± *SE* (ng/g)	*t*-value	*P*-value	Log FGM (ß) Estimate ± *SE* (ng/g)	*t*-value	*P*-value
Fixed effects							
Intercept ($ß_0$)		**4.28 ± 0.11**	**37.84**	**<0.0001**	**4.29 ± 0.11**	**37.55**	**<0.0001**
Horn residuals	↓	**-0.01 ± 0.004**	**-1.94**	**0.0526**	**-0.01 ± 0.004**	**-2.02**	**0.0437**
Body condition	↓	**-0.10 ± 0.03**	**-3.48**	**0.0005**	**-0.10 ± 0.03**	**-3.33**	**0.0009**
Pregnant: Yes		0.04 ± 0.03	1.23	0.2204	0.04 ± 0.03	1.26	0.2066
Year: Oct09-Sept10	↓	**-0.11 ± 0.04**	**-2.48**	**0.0133**	**-0.10 ± 0.04**	**-2.29**	**0.0225**
Year: Oct10-Sept11		-0.07 ± 0.05	-1.56	0.1193	-0.06 ± 0.05	-1.35	0.1728
Year: Oct11-Aug12	↓	**-0.14 ± 0.06**	**-2.58**	**0.0101**	**-0.13 ± 0.06**	**-2.26**	**0.0243**
Season: Early wet	↓	**-0.17 ± 0.05**	**-3.62**	**0.0003**	**-0.17 ± 0.05**	**-3.47**	**0.0005**
Season: Early dry		-0.08 ± 0.04	-1.88	0.0603	**-0.08 ± 0.04**	**-1.95**	**0.0517**
Season: Late dry		-0.08 ± 0.05	-1.84	0.0656	-0.08 ± 0.05	-1.80	0.0728
Acute infections: Yes	↑	**0.11 ± 0.03**	**3.89**	**0.0001**	**0.06 ± 0.03**	**1.97**	**0.0492**
Interaction term							
Horn residuals:Pregnant (Yes)		0.01 ± 0.005	1.11	0.2671	0.01 ± 0.006	1.063	0.2879
Random effect		Variance *SD*			Variance *SD*		
Animal ID		0.13 0.36			0.13 0.36		
Residuals:		0.14 0.37			0.14 0.38		

[a] Effect column indicate the direction of the slope predicted by the model.



Supplementary Data SD2.5 The odds of exposure at time t_i given exposure since previous captures. For all pathogens, log odds of exposure at time t_i are low if the animal was exposed approximately 6month previously (1 capture previously); odds of exposure at time t_i are highest if an animal had not been exposed in the previous 24months (within the previous 4 captures) (Coon et al. *in review*.).

APPENDIX III:

Chapter 3 Supplementary Data

Supplementary Data SD3.1: Summary of the effect (positive or negative or both for categorical variables with more than 2 levels) of each independent variable on the dependent variables best model (linear mixed-effects model with Animal ID as the random effect). Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.'. FGM = fecal glucocorticoid metabolite.

	Year	Season	Herd	FGM	Body condition	Age	Age^2	Horn residuals	Reproductive status	Haptoglobin	Treatment
Innate immunity											
Bacterial killing assay (BKA)	+***	-*	+***	+**	-***					+	
Total leukocyte count (log)	+***	-***			+***	-*			-*		
Neutrophils	-**	-/+*		+	-***	+***				+***	
Lymphocytes	-***	+***	-*		+**						
Monocytes	+***	-***	+**			-***	+*			-	
Eosinophils	+**	-***	-/+	-.	+	+***		+	-***	-***	
Basophils	-***	-/+*	-**		+			+			
Neutrophil Lymphocyte Ratio (NLR)		-***				-	+		-*		
Adaptive immunity											
Interferon gamma (IFNy) stimulation	-***	-/+***	-/+*	+*						+	-.
Interleukin 4 (Il-4) stimulation	-/+***	-/+.	-/+*		-**						
IFNy:Il-4 ratio stimulation	-/+***	-**			+						
Interleukin 12 (Il-12) stimulation	-**		-**		+***						
Interleukin 12 (Il-12) response	+**	+**		+						+	

Supplementary Data SD3.1 *Continue…*

	Year	Season	Herd	FGM	Body condition	Age	Age^2	Horn residuals	Reproductive status	Haptoglobin	Treatment
Hematology measures											
Total erythrocyte count	+***	-/+***			+***	-**	+*				
Hemoglobin (Hgb)	+***	+***	-/+*		+**						
Hematocrit (HCT)	+***	+***			+***						
Mean corpuscular hemoglobin (MCH)	-/+***	+***	-**		-***	+***			+**		
Mean corpuscular hemoglobin concentration (MCHC)	-***	+***	-/+*		-***	-*					
Mean corpuscular volume (MCV)	-/+***	+***	-*	+**		+***	-*		+***	+	
Platelets (PLT)	-***	-/+**		-	-					+*	

Supplementary Data SD3.1 *Continue…*

	Year	Season	Herd	FGM	Body condition	Age	Age^2	Horn residuals	Reproductive status	Haptoglobin	Treatment
Biochemical panel measures											
Albumin (ALB)	-/+*	-***	-*		+*		+.		+**	-**	
Globulins (GLOB)	+*	-***			-**	+**	-**			+***	
Albumin Globulin Ratio (ALB:GLOB)	+*	-***			-**	+**	-**		-*	+***	
Total protein (TP)	-/+*	-***			-*			+**			
Gamma glutamyl transferase (GGT)	-/+		+*	+**	-***					-	
Creatinine kinase (CK)	+**	+***				+	-*		-.		+
Alkaline phosphatase (ALP)		-/+***						-			
Aspartate aminotransferase (AST)	+***	-/+**			-***			-			
Calcium (Ca)	-/+***		+*	-						+	+.
Blood Urea Nitrogen (BUN)	-***	-/+***		+	-*					+	
Phosphorous (P)	+***		-/+*		+***			-	-		
Magnesium (Mg)	-**	+***			-	+**			+***	-**	

Supplementary Data SD3.2: Summary of the 10 best linear mixed effects models (LMEMs) for each dependent variable (various immunological, hematology, and biochemical measures) to determine which demographic and environmental factors are associated with immune responses in female African buffalo (*Syncerus caffer*). All models included Animal ID as a random effect and all continuous explanatory variables were scaled. Explanatory variables are in order of most to least important based on the importance value. FGM = Fecal glucocorticoid metabolites; R^2m = marginal and R^2c = conditional

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Bacterial killing assay (BKA) *Escherichia coli*							
2660	Year + Offset (control) + Herd + Body condition + FGM + Season + Haptoglobin	13	-11.52	0.00	0.05	26.71	28.53
2684	Year + Offset (control) + Herd + Body condition + FGM + Age + Season + Age^2	15	-11.52	0.00	0.05	26.92	28.43
2172	Year + Offset (control) + Herd + Body condition + FGM + Age + Age^2	12	-11.51	0.01	0.05	26.52	27.89
2148	Year + Offset (control) + Herd + Body condition + FGM	10	-10.74	0.78	0.03	26.25	27.96
2176	Year + Offset (control) + Herd + Body condition + FGM + Age + Age^2 + Reproductive status	13	-10.24	1.28	0.02	26.55	27.91
2688	Year + Offset (control) + Herd + Body condition + FGM + Age +Season + Age^2 + Reproductive status	16	-10.09	1.43	0.02	26.94	28.43
2300	Year + Offset (control) + Herd + Body condition + FGM + Age + Age^2 + Haptoglobin	13	-10.05	1.47	0.02	26.53	27.86
2668	Year + Offset (control) + Herd + Body condition + FGM + Age + Season	14	-9.95	1.57	0.02	26.73	28.57
2788	Year + Offset (control) + Herd + Body condition + FGM + Season + Haptoglobin	14	-9.95	1.57	0.02	26.72	28.51
2812	Year + Offset (control) + Herd + Body condition + FGM + Age + Season + Age^2 + Haptoglobin	16	-9.93	1.59	0.02	26.93	28.40

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: log Total leukocyte count							
559	Year + Season + Body condition + Reproductive status + Age	12	898.42	0.00	0.04	37.25	48.36
560	Year + Season + Body condition + Reproductive status + Age + Herd	14	899.19	0.77	0.03	37.49	48.31
687	Year + Season + Body condition + Reproductive status + Age + Haptoglobin	13	899.34	0.92	0.03	37.31	48.35
567	Year + Season + Body condition + Reproductive status + Age^2	12	899.59	1.17	0.02	37.13	48.31
623	Year + Season + Body condition + Reproductive status + Age + FGM	13	899.75	1.33	0.02	37.28	48.41
815	Year + Season + Body condition + Reproductive status + Age + Horn residuals	13	899.87	1.45	0.02	37.29	48.34
575	Year + Season + Body condition + Reproductive status + Age + Age^2	13	900.03	1.61	0.02	37.24	48.43
816	Year + Season + Body condition + Reproductive status + Age + Herd + Horn residuals	15	900.15	1.72	0.02	37.58	48.25
1583	Year + Season + Body condition + Reproductive status + Age + Treatment	13	900.21	1.79	0.02	37.26	48.45
688	Year + Season + Body condition + Reproductive status + Age + Herd + Haptoglobin	15	900.24	1.82	0.02	37.54	48.30
568	Year + Season + Body condition + Reproductive status + Age^2 + Herd	14	900.30	1.88	0.02		

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Neutrophils							
747	Body condition + Haptoglobin + Season + Age + Year + FGM	13	-2604.37	0.00	0.06	16.13	36.10
751	Body condition + Haptoglobin + Season + Age + Year + FGM + Reproductive status	14	-2604.06	0.31	0.05	16.24	36.15
683	Body condition + Haptoglobin + Season + Age + Year	12	-2603.35	1.03	0.04	15.87	35.98
687	Body condition + Haptoglobin + Season + Age + Year + Reproductive status	13	-2603.16	1.22	0.03	15.98	36.02
1003	Body condition + Haptoglobin + Season + Age + Year + FGM + Horn residuals	14	-2602.97	1.40	0.03	16.15	36.01
763	Body condition + Haptoglobin + Season + Age + Year + FGM + Age^2	14	-2602.52	1.85	0.03	16.07	36.14
1771	Body condition + Haptoglobin + Season + Age + Year + FGM + Treatment	14	-2602.42	1.95	0.02	16.14	36.23
1007	Body condition + Haptoglobin + Season + Age + Year + FGM + Reproductive status + Horn residuals	15	-2602.42	1.95	0.02	16.24	36.09
745	Body condition + Haptoglobin + Season + Age + FGM	10	-2602.13	2.24	0.02	14.81	34.86
1775	Body condition + Haptoglobin + Season + Age + Year + FGM + Reproductive status + Treatment	15	-2602.09	2.28	0.02	16.24	36.27

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Lymphocytes							
548	Season + Year + Body condition + Herd + Haptoglobin	12	-727.48	0.00	0.03	15.93	21.65
676	Season + Year + Body condition + Herd + Haptoglobin	13	-727.43	0.05	0.03	16.09	21.99
552	Season + Year + Body condition + Herd + Reproductive status	13	-727.14	0.34	0.02	16.04	21.74
680	Season + Year + Body condition + Herd + Haptoglobin + Reproductive status	14	-727.07	0.42	0.02	16.20	22.09
700	Season + Year + Body condition + Herd + Haptoglobin + Age^2 + Age	15	-726.48	1.00	0.02	16.33	22.09
572	Season + Year + Body condition + Herd + Age^2 + Age	14	-726.38	1.11	0.01	16.16	21.77
696	Season + Year + Body condition + Herd + Haptoglobin + Reproductive status + Age^2	15	-726.26	1.23	0.01	16.28	22.10
692	Season + Year + Body condition + Herd + Haptoglobin + Age^2	14	-726.14	1.34	0.01	16.14	22.03
568	Season + Year + Body condition + Herd + Reproductive status + Age^2	14	-726.11	1.37	0.01	16.10	21.75
1700	Season + Year + Body condition + Herd + Haptoglobin + Treatment	14	-726.03	1.45	0.01	16.15	22.24

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Monocytes							
668	Year + Season + Age + Herd + Age^2 + Haptoglobin	14	-852.86	0.00	0.06	10.53	10.53
700	Year + Season + Age + Herd + Age^2 + Haptoglobin + Body condition	15	-852.17	0.69	0.05	10.63	10.63
540	Year + Season + Age + Herd + Age^2	13	-852.03	0.83	0.04	10.30	10.30
1692	Year + Season + Age + Herd + Age^2 + Haptoglobin + Treatment	15	-851.89	0.96	0.04	10.61	10.61
1724	Year + Season + Age + Herd + Age^2 + Haptoglobin + Body condition + Treatment	16	-851.30	1.55	0.03	10.73	10.86
572	Year + Season + Age + Herd + Age^2 + Body condition	14	-851.18	1.68	0.03	10.39	10.39
672	Year + Season + Age + Herd + Age^2 + Haptoglobin + Reproductive status	15	-851.10	1.75	0.03	10.54	10.54
732	Year + Season + Age + Herd + Age^2 + Haptoglobin + FGM	15	-851.06	1.79	0.03	10.54	10.54
1564	Year + Season + Age + Herd + Age^2 + Treatment	14	-850.89	1.97	0.02	10.36	10.36
924	Year + Season + Age + Herd + Age^2 + Haptoglobin + Horn residuals	15	-850.83	2.02	0.02	10.52	10.52

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Eosinophils							
1008	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Horn residuals + Body condition	17	-2943.66	0.00	0.05	25.67	39.02
1007	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Horn residuals + Body condition	15	-2943.57	0.09	0.05	25.35	38.68
976	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Horn residuals	16	-2943.44	0.22	0.04	25.50	38.78
768	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Body condition + Age^2	17	-2942.89	0.77	0.03	25.58	38.80
736	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Age^2	16	-2942.75	0.92	0.03	25.41	38.54
752	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Body condition	16	-2942.63	1.03	0.03	25.43	38.79
1024	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Horn residuals + Body condition + Age^2	18	-2942.52	1.15	0.03	25.70	38.98
720	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd	15	-2942.51	1.16	0.03	25.27	38.55
992	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Herd + Horn residuals + Age^2	17	-2942.30	1.36	0.03	25.53	38.73
1023	Season + Haptoglobin + Reproductive status + Age + Year + FGM + Horn residuals + Body condition + Age^2	16	-2942.15	1.51	0.02	25.36	38.68

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Basophils							
804	Year + Herd + Season + Horn residuals + Body condition	13	-9019.25	0.00	0.03	7.13	8.68
820	Year + Herd + Season + Horn residuals + Body condition + Age^2	14	-9018.64	0.61	0.02	7.25	8.76
772	Year + Herd + Season + Horn residuals	12	-9018.63	0.62	0.02	6.89	8.03
812	Year + Herd + Season + Horn residuals + Body condition + Age	14	-9018.48	0.76	0.02	7.24	8.72
548	Year + Herd + Season + Body condition	12	-9017.92	1.33	0.02	6.84	8.50
932	Year + Herd + Season + Horn residuals + Body condition + Haptoglobin	14	-9017.52	1.72	0.01	7.16	8.72
788	Year + Herd + Season + Horn residuals + Age^2	13	-9017.35	1.90	0.01	6.95	8.08
868	Year + Herd + Season + Horn residuals + Body condition + FGM	14	-9017.33	1.92	0.01	7.14	8.75
808	Year + Herd + Season + Horn residuals + Body condition + Reproductive status	14	-9017.31	1.94	0.01	7.14	8.72
780	Year + Herd + Season + Horn residuals + Age	13	-9017.25	2.00	0.01	6.94	8.06

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Innate immunity: Neutrophil Lymphocyte Ratio (NLR)							
533	Season + Reproductive status + Age^2	8	4597.39	0.00	0.02	3.27	6.09
789	Season + Reproductive status + Age^2 + Horn residuals	9	4597.46	0.07	0.02	3.44	6.42
781	Season + Reproductive status + Horn residuals + Age	9	4597.89	0.51	0.01	3.38	6.40
534	Season + Reproductive status + Age^2 + Herd	10	4598.30	0.92	0.01	3.65	6.20
535	Season + Reproductive status + Age^2 + Year	11	4598.32	0.94	0.01	3.65	6.20
525	Season + Reproductive status + Age	8	4598.36	0.97	0.01	3.76	6.43
597	Season + Reproductive status + Age^2 + FGM	9	4598.66	1.27	0.01	3.10	5.98
773	Season + Reproductive status + Horn residuals	8	4598.73	1.34	0.01	3.36	6.05
790	Season + Reproductive status + Age^2 + Horn residuals + Herd	11	4598.81	1.43	0.01	3.08	6.61
541	Season + Reproductive status + Age^2 + Age	9	4598.87	1.48	0.01	3.76	6.47
853	Season + Reproductive status + Age^2 + Horn residuals + FGM	10	4598.87	1.48	0.01	3.27	6.09

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Adaptive immunity: Interferon gamma (IFNy) stimulation							
1732	Year + Season + Herd + FGM + Treatment + Haptoglobin	14	1836.36	0.79	0.03	5.51	38.89
1612	Year + Season + Herd + FGM + Treatment + Age	14	1836.60	1.03	0.03	5.60	39.09
1620	Year + Season + Herd + FGM + Treatment + Age^2	14	1836.83	1.25	0.02	5.56	39.05
1636	Year + Season + Herd + FGM + Treatment + Body condition	14	1837.08	1.51	0.02	5.51	38.92
580	Year + Season + Herd + FGM	12	1837.24	1.67	0.02	4.68	38.74
1740	Year + Season + Herd + FGM + Treatment + Haptoglobin + Age	15	1837.27	1.69	0.02	5.67	39.08
1860	Year + Season + Herd + FGM + Treatment + Horn residuals	14	1837.39	1.82	0.02	5.49	38.96
1748	Year + Season + Herd + FGM + Treatment + Haptoglobin + Age^2	15	1837.52	1.94	0.02	5.63	39.04
1608	Year + Season + Herd + FGM + Treatment + Reproductive status	14	1837.61	2.04	0.02	5.44	38.87
Adaptive immunity: Interleukin 4 (Il-4) stimulation							
548	Year + Herd + Body condition + Season	12	14769.21	0.00	0.04	11.88	22.11
556	Year + Herd + Body condition + Season + Age	13	14769.52	0.31	0.03	12.05	22.08
36	Year + Herd + Body condition	9	14769.60	0.39	0.03	11.34	21.33
564	Year + Herd + Body condition + Season + Age^2	13	14769.64	0.43	0.03	12.03	22.02
612	Year + Herd + Body condition + Season + FGM	13	14770.77	1.56	0.02	11.91	22.20
620	Year + Herd + Body condition + Season + Age + FGM	14	14770.93	1.72	0.02	12.10	22.16
804	Year + Herd + Body condition + Season + Horn residuals	13	14770.98	1.77	0.02	11.90	22.16
52	Year + Herd + Body condition + Age^2	10	14770.99	1.78	0.02	11.40	21.27
44	Year + Herd + Body condition + Age	10	14771.01	1.80	0.01	11.40	21.29
676	Year + Herd + Body condition + Season + Haptoglobin	13	14771.07	1.86	0.01	11.88	22.27

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Adaptive immunity: Interferon gamma Interleukin 4 Ratio (IFNy:Il-4)							
547	Year + Season + Body condition	10	-9266.22	0.00	0.02	5.30	21.70
803	Year + Season + Body condition + Horn residuals	11	-9266.05	0.17	0.02	5.54	21.96
1827	Year + Season + Body condition + Horn residuals + Treatment	12	-9265.57	0.65	0.02	5.79	21.97
1571	Year + Season + Body condition + Treatment	11	-9265.51	0.71	0.02	5.51	21.71
515	Year + Season	9	-9265.43	0.79	0.01	4.99	21.26
771	Year + Season + Horn residuals	10	-9265.04	1.18	0.01	5.21	21.51
611	Year + Season + Body condition + FGM	11	-9264.86	1.36	0.01	5.38	21.66
1539	Year + Season + Treatment	10	-9264.83	1.39	0.01	5.21	21.27
675	Year + Season + Body condition + Haptoglobin	11	-9264.72	1.50	0.01	5.35	21.54
1795	Year + Season + Horn residuals + Treatment	11	-9264.67	1.55	0.01	5.47	21.53
Adaptive immunity: Interleukin 12 (Il-12) stimulation							
164	Year + Herd + Body condition + Haptoglobin	9	2187.33	0.87	0.03	29.29	29.29
292	Year + Herd + Body condition + Horn residuals	9	2187.39	0.93	0.03	29.29	29.29
52	Year + Herd + Body condition + Age^2	9	2187.52	1.06	0.03	29.27	29.27
44	Year + Herd + Body condition + Age	9	2187.61	1.15	0.03	29.25	29.25
1060	Year + Herd + Body condition + Treatment	9	2188.12	1.66	0.02	29.18	29.18
420	Year + Herd + Body condition + Horn residuals + Haptoglobin	10	2188.21	1.75	0.02	29.42	29.42
40	Year + Herd + Body condition + Reproductive status	9	2188.42	1.96	0.02	29.13	29.13
308	Year + Herd + Body condition + Horn residuals + Age^2	10	2188.49	2.03	0.02	29.38	29.38
100	Year + Herd + Body condition + FGM	9	2188.50	2.04	0.02	29.12	29.12

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Adaptive immunity: Interleukin 12 (Il-12) response							
579	Year + FGM + Season	9	2141.78	0.00	0.01	2.95	2.95
1603	Year + FGM + Season + Treatment	10	2141.99	0.22	0.01	3.31	3.31
835	Year + FGM + Season + Horn residuals	10	2142.80	1.02	0.01	3.16	3.16
611	Year + FGM + Season + Body condition	10	2143.19	1.42	0.01	3.08	3.08
515	Year + Season	8	2143.25	1.48	0.01	2.25	2.25
1859	Year + FGM + Season + Treatment + Horn residuals	11	2143.28	1.50	0.01	3.47	3.47
1635	Year + FGM + Season + Treatment + Body condition	11	2143.32	1.54	0.01	3.46	3.46
595	Year + FGM + Season + Age^2	10	2143.39	1.61	0.01	3.04	3.04
1539	Year + Season + Treatment	9	2143.40	1.63	0.01	2.63	2.63
587	Year + FGM + Season + Age	10	2143.45	1.68	0.01	3.03	3.03
Hematology measures: Total erythrocyte count							
571	Year + Season + Body condition + Age + Age^2	12	3802.57	0.00	0.07	30.26	41.88
575	Year + Season + Body condition + Age + Age^2 + Reproductive status	13	3803.01	0.44	0.05	30.39	41.78
827	Year + Season + Body condition + Age + Age^2 + Horn residuals	13	3803.76	1.19	0.04	30.31	41.90
831	Year + Season + Body condition + Age + Age^2 + Reproductive status + Horn residuals	14	3804.05	1.48	0.03	30.46	41.80
635	Year + Season + Body condition + Age + Age^2 + FGM	13	3804.06	1.48	0.03	30.26	41.87
1595	Year + Season + Body condition + Age + Age^2 + Treatment	13	3804.26	1.68	0.03	30.27	41.95
639	Year + Season + Body condition + Age + Age^2 + Reproductive status + FGM	14	3804.45	1.88	0.03	30.40	41.77
699	Year + Season + Body condition + Age + Age^2 + Haptoglobin	13	3804.60	2.02	0.02	30.24	41.87
559	Year + Season + Body condition + Age + Reproductive status	12	3804.64	2.06	0.02	30.08	41.91
1599	Year + Season + Body condition + Age + Age^2 + Treatment	14	3804.72	2.15	0.02	30.40	41.86

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Hematology measures: Hemoglobin							
548	Season + Year + Herd + Body condition	12	4318.07	0.00	0.09	15.10	23.28
612	Season + Year + Herd + Body condition + FGM	13	4319.74	1.67	0.04	15.12	23.31
676	Season + Year + Herd + Body condition + Haptoglobin	13	4319.79	1.72	0.04	15.10	23.25
804	Season + Year + Herd + Body condition + Horn residuals	13	4319.87	1.81	0.04	15.11	23.29
564	Season + Year + Herd + Body condition + Age^2	13	4320.05	1.98	0.03	15.10	23.36
552	Season + Year + Herd + Body condition + Reproductive status	13	4320.07	2.01	0.03	15.09	23.30
556	Season + Year + Herd + Body condition + Age	13	4320.11	2.05	0.03	15.09	23.36
1572	Season + Year + Herd + Body condition + Treatment	13	4320.12	2.05	0.03	15.09	23.38
740	Season + Year + Herd + Body condition + FGM + Haptoglobin	14	4321.42	3.36	0.02	15.12	23.28
868	Season + Year + Herd + Body condition + FGM + Horn residuals	14	4321.53	3.46	0.02	15.13	23.31
Hematology measures: Hematocrit							
547	Year + Season + Body condition	10	6285.33	0.00	0.02	20.67	31.42
563	Year + Season + Body condition + Age^2	11	6285.48	0.15	0.02	20.91	31.48
555	Year + Season + Body condition +Age	11	6285.61	0.29	0.02	20.90	31.53
611	Year + Season + Body condition + FGM	11	6285.62	0.30	0.02	20.78	31.52
551	Year + Season + Body condition + Reproductive status	11	6285.64	0.32	0.02	20.78	31.59
627	Year + Season + Body condition + FGM + Age^2	12	6285.97	0.64	0.02	21.01	31.60
615	Year + Season + Body condition + FGM + Reproductive status	12	6286.09	0.76	0.02	20.88	31.69
619	Year + Season + Body condition + FGM + Age	12	6286.13	0.80	0.02	20.99	31.64
819	Year + Season + Body condition + Age^2 + Horn residuals	12	6286.14	0.81	0.02	21.02	31.57
567	Year + Season + Body condition + Age^2 + Reproductive status	12	6286.23	0.90	0.01	20.98	31.66

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Hematology measures: Platelets (PLT)							
1763	Year + Season + Treatment + Haptoglobin + Body condition + FGM	13	11969.93	0.00	0.04	8.89	22.84
1699	Year + Season + Treatment + Haptoglobin + Body condition	12	11970.01	0.08	0.04	8.68	22.59
1703	Year + Season + Treatment + Haptoglobin + Body condition + Reproductive status	13	11970.59	0.65	0.03	8.82	22.40
1767	Year + Season + Treatment + Haptoglobin + Body condition + FGM + Reproductive status	14	11970.66	0.72	0.03	9.01	22.66
1667	Year + Season + Treatment + Haptoglobin	11	11970.91	0.98	0.03	8.38	22.41
1731	Year + Season + Treatment + Haptoglobin + FGM	12	11971.33	1.40	0.02	8.54	22.64
1671	Year + Season + Treatment + Haptoglobin + Reproductive status	12	11971.53	1.59	0.02	8.51	22.22
2019	Year + Season + Treatment + Haptoglobin + Body condition + FGM + Horn residuals	14	11971.67	1.73	0.02	8.91	23.03
1955	Year + Season + Treatment + Haptoglobin + Body condition + Horn residuals	13	11971.80	1.87	0.02	8.70	22.79
1771	Year + Season + Treatment + Haptoglobin + Body condition + FGM + Age	14	11971.93	2.00	0.02	8.86	22.93

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Hematology measures: Mean corpuscular volume (MCV)							
608	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd	15	4180.17	0.00	0.11	43.40	80.93
640	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Body condition	16	4180.90	0.73	0.07	43.36	80.96
607	Year + Season + Reproductive status + Age + FGM + Age^2	13	4181.44	1.27	0.06	43.01	81.00
1632	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Treatment	16	4181.87	1.70	0.05	43.44	80.98
736	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Haptoglobin	16	4181.87	1.70	0.05	43.38	80.94
864	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Horn residuals	16	4181.98	1.81	0.04	43.36	80.99
639	Year + Season + Reproductive status + Age + FGM + Age^2 + Body condition	14	4182.09	1.92	0.04	42.96	81.05
1664	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Body condition + Treatment	17	4182.58	2.41	0.03	43.40	81.01
768	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Body condition + Haptoglobin	17	4182.62	2.45	0.03	43.34	80.96
896	Year + Season + Reproductive status + Age + FGM + Age^2 + Herd + Body condition + Horn residuals	17	4182.73	2.56	0.03	43.32	81.00

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Hematology measures: Mean corpuscular hemoglobin (MCH)							
560	Year + Season + Body condition + Herd + Reproductive status + Age	14	3466.18	0.00	0.07	41.60	49.20
688	Year + Season + Body condition + Herd + Reproductive status + Age + Haptoglobin	15	3466.44	0.26	0.06	41.68	49.18
1584	Year + Season + Body condition + Herd + Reproductive status + Age + Treatment	15	3466.57	0.39	0.06	41.69	49.26
1712	Year + Season + Body condition + Herd + Reproductive status + Age + Haptoglobin + Treatment	16	3466.62	0.44	0.05	41.78	49.23
576	Year + Season + Body condition + Herd + Reproductive status + Age + Age^2	15	3466.75	0.57	0.05	41.68	48.89
704	Year + Season + Body condition + Herd + Reproductive status + Age + Age^2 + Haptoglobin	16	3466.99	0.82	0.05	41.76	48.88
1600	Year + Season + Body condition + Herd + Reproductive status + Age + Age^2 + Treatment	16	3467.42	1.24	0.04	41.75	48.99
1728	Year + Season + Body condition + Herd + Reproductive status + Age + Age^2 + Haptoglobin + Treatment	17	3467.48	1.30	0.04	41.84	48.97
624	Year + Season + Body condition + Herd + Reproductive status + Age + FGM	15	3467.86	1.69	0.03	41.60	49.26
752	Year + Season + Body condition + Herd + Reproductive status + Age + FGM + Haptoglobin	16	3468.04	1.87	0.03	41.68	49.25

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Hematology measures: Mean corpuscular hemoglobin concentration (MCHC)							
556	Year + Season + Body condition + Herd + Age	13	4956.22	0.00	0.06	28.20	28.64
684	Year + Season + Body condition + Herd + Age + Haptoglobin	14	4956.84	0.62	0.04	28.30	28.81
564	Year + Season + Body condition + Herd + Age^2	13	4957.38	1.16	0.03	28.11	28.49
560	Year + Season + Body condition + Herd + Age + Reproductive status	14	4957.47	1.25	0.03	28.24	28.68
572	Year + Season + Body condition + Herd + Age + Age^2	14	4957.76	1.54	0.03	28.24	28.83
812	Year + Season + Body condition + Herd + Age + Horn residuals	14	4957.82	1.60	0.03	28.23	28.80
1580	Year + Season + Body condition + Herd + Age + Treatment	14	4957.95	1.73	0.02	28.21	28.72
692	Year + Season + Body condition + Herd + Age^2 + Haptoglobin	14	4957.95	1.73	0.02	28.21	28.66
688	Year + Season + Body condition + Herd + Age + Haptoglobin + Reproductive status	15	4958.08	1.86	0.02	28.34	28.84
620	Year + Season + Body condition + Herd + Age + FGM	14	4958.19	1.97	0.02	28.19	28.62

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Globulins (GLOB)							
699	Season + Year + Haptoglobin + Body condition + Age^2 + Age	13	763.93	0.00	0.10	31.98	65.38
703	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Reproductive status	14	764.23	0.29	0.08	32.29	65.29
700	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Herd	15	765.03	1.10	0.06	32.43	65.66
704	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Reproductive status + Herd	16	765.62	1.69	0.04	32.67	65.65
955	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Horn residuals	14	765.71	1.78	0.04	31.82	65.61
959	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Reproductive status + Horn residuals	15	765.92	1.98	0.04	32.14	65.53
763	Season + Year + Haptoglobin + Body condition + Age^2 + Age + FGM	14	765.95	2.02	0.03	31.97	65.30
1723	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Treatment	14	766.06	2.12	0.03	31.84	65.53
767	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Reproductive status + FGM	15	766.27	2.33	0.03	32.28	65.22
1727	Season + Year + Haptoglobin + Body condition + Age^2 + Age + Reproductive status + Treatment	15	766.33	2.40	0.03	32.16	65.44

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Albumin (ALB)							
696	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Herd	15	231.35	0.00	0.08	12.85	60.20
704	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Herd + Age	16	231.66	0.31	0.07	13.36	59.32
695	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2	13	232.80	1.46	0.04	11.65	59.09
688	Year + Season + Reproductive status + Haptoglobin + Body condition + Herd + Age	15	232.82	1.47	0.04	12.25	60.31
680	Year + Season + Reproductive status + Haptoglobin + Body condition + Herd	14	233.16	1.81	0.03	11.26	59.79
703	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Age	14	233.21	1.87	0.03	12.09	58.14
1720	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Herd + Treatment	16	233.43	2.08	0.03	12.80	60.38
952	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Herd + Horn residuals	16	233.51	2.16	0.03	12.81	60.36
760	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Herd + FGM	16	233.51	2.17	0.03	12.83	60.12
960	Year + Season + Reproductive status + Haptoglobin + Body condition + Age^2 + Herd + Age + Horn residuals	17	233.67	2.32	0.02	13.42	59.67

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Albumin Globulin Ratio (ALB:GLOB)							
703	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status	14	-258.20	0.00	0.14	24.47	62.07
704	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + Herd	16	-257.07	1.13	0.08	24.84	62.71
959	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + Horn residuals	15	-256.38	1.82	0.06	24.33	62.22
1727	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + Treatment	15	-256.07	2.13	0.05	24.35	62.24
767	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + FGM	15	-256.05	2.15	0.05	24.45	61.99
960	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + Herd + Horn residuals	17	-255.18	3.02	0.03	24.72	62.88
1728	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + Herd + Treatment	17	-254.99	3.21	0.03	24.74	62.87
699	Season + Haptoglobin + Body condition + Age^2 + Year + Age	13	-254.95	3.25	0.03	23.70	61.63
768	Season + Haptoglobin + Body condition + Age^2 + Year + Age + Reproductive status + Herd + FGM	17	-254.92	3.28	0.03	24.83	62.63
701	Season + Haptoglobin + Body condition + Age^2 + Age + Reproductive status	11	-254.79	3.41	0.03	23.57	61.73

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Total protein (TP)							
803	Season + Year + Horn residuals + Body condition	11	1467.16	0.00	0.06	0.86	99.30
804	Season + Year + Horn residuals + Body condition + Herd	13	1467.95	0.79	0.04	1.14	99.31
1827	Season + Year + Horn residuals + Body condition + Treatment	12	1468.04	0.88	0.04	1.61	99.31
931	Season + Year + Horn residuals + Body condition + Haptoglobin	12	1468.73	1.57	0.03	0.86	99.30
807	Season + Year + Horn residuals + Body condition + Reproductive status	12	1468.75	1.59	0.03	0.86	99.30
1828	Season + Year + Horn residuals + Body condition + Treatment + Herd	14	1468.79	1.63	0.03	1.94	99.31
867	Season + Year + Horn residuals + Body condition + FGM	12	1468.94	1.78	0.02	0.87	99.30
811	Season + Year + Horn residuals + Body condition + Age	12	1469.01	1.85	0.02	0.99	99.30
819	Season + Year + Horn residuals + Body condition + Age^2	12	1469.18	2.02	0.02	0.90	99.30
1955	Season + Year + Horn residuals + Body condition + Treatment + Haptoglobin	13	1469.62	2.47	0.02	1.61	99.31
Biochemical panel measure: Alkaline phosphatase (ALP)							
769	Season + Horn residuals	7	4446.83	0.00	0.03	4.76	71.21
513	Season	6	4446.99	0.16	0.03	4.04	70.65
517	Season + Reproductive status	7	4448.07	1.24	0.02	4.12	70.78
773	Season + Horn residuals + Reproductive status	8	4448.36	1.53	0.01	4.74	71.23
793	Season + Horn residuals + Age^2 + Age	9	4448.47	1.64	0.01	6.15	71.70
833	Season + Horn residuals + FGM	8	4448.51	1.68	0.01	4.79	71.36
785	Season + Horn residuals + Age^2	8	4448.52	1.69	0.01	4.94	71.12
529	Season + Age^2	7	4448.58	1.75	0.01	4.26	70.58
521	Season + Age	7	4448.83	1.99	0.01	4.14	70.59
801	Season + Horn residuals + Body condition	8	4448.83	1.99	0.01	4.76	71.23

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Aspartate aminotransferase (AST)							
803	Year + Body condition + Season + Horn residuals	11	4140.78	0.00	0.05	5.02	91.18
819	Year + Body condition + Season + Horn residuals + Age^2	12	4141.57	0.80	0.03	5.56	91.26
867	Year + Body condition + Season + Horn residuals + FGM	12	4141.91	1.14	0.03	5.11	90.97
547	Year + Body condition + Season	10	4141.93	1.15	0.03	4.73	90.94
811	Year + Body condition + Season + Horn residuals + Age	12	4142.17	1.39	0.02	5.23	91.24
931	Year + Body condition + Season + Horn residuals + Haptoglobin	12	4142.53	1.76	0.02	5.03	91.14
1827	Year + Body condition + Season + Horn residuals + Treatment	12	4142.65	1.88	0.02	5.17	91.25
883	Year + Body condition + Season + Horn residuals + FGM + Age^2	13	4142.67	1.89	0.02	5.67	91.05
611	Year + Body condition + Season + FGM	11	4142.68	1.90	0.02	4.87	90.71
807	Year + Body condition + Season + Horn residuals + Reproductive status	12	4142.83	2.05	0.02	5.02	91.16
Biochemical panel measure: Gamma glutamyl transferase (GGT)							
100	Body condition + FGM + Year + Herd	10	2046.02	0.00	0.06	9.95	49.54
1124	Body condition + FGM + Year + Herd + Treatment	11	2047.53	1.51	0.03	10.25	49.86
104	Body condition + FGM + Year + Herd + Reproductive status	11	2047.54	1.52	0.03	9.98	49.71
356	Body condition + FGM + Year + Herd + Horn residuals	11	2047.84	1.82	0.02	10.07	49.62
228	Body condition + FGM + Year + Herd + Haptoglobin	11	2047.90	1.88	0.02	9.93	49.76
116	Body condition + FGM + Year + Herd + Age^2	11	2048.07	2.05	0.02	9.97	49.83
108	Body condition + FGM + Year + Herd + Age	11	2048.13	2.11	0.02	9.90	49.79
99	Body condition + FGM + Year	8	2048.51	2.49	0.02	8.12	49.36
1128	Body condition + FGM + Year + Herd + reproductive status + Treatment	12	2048.94	2.92	0.01	10.31	50.02
98	Body condition + FGM + Herd	7	2049.16	3.14	0.01	8.57	47.66

Supplementary Data SD3.2 *Continue…*

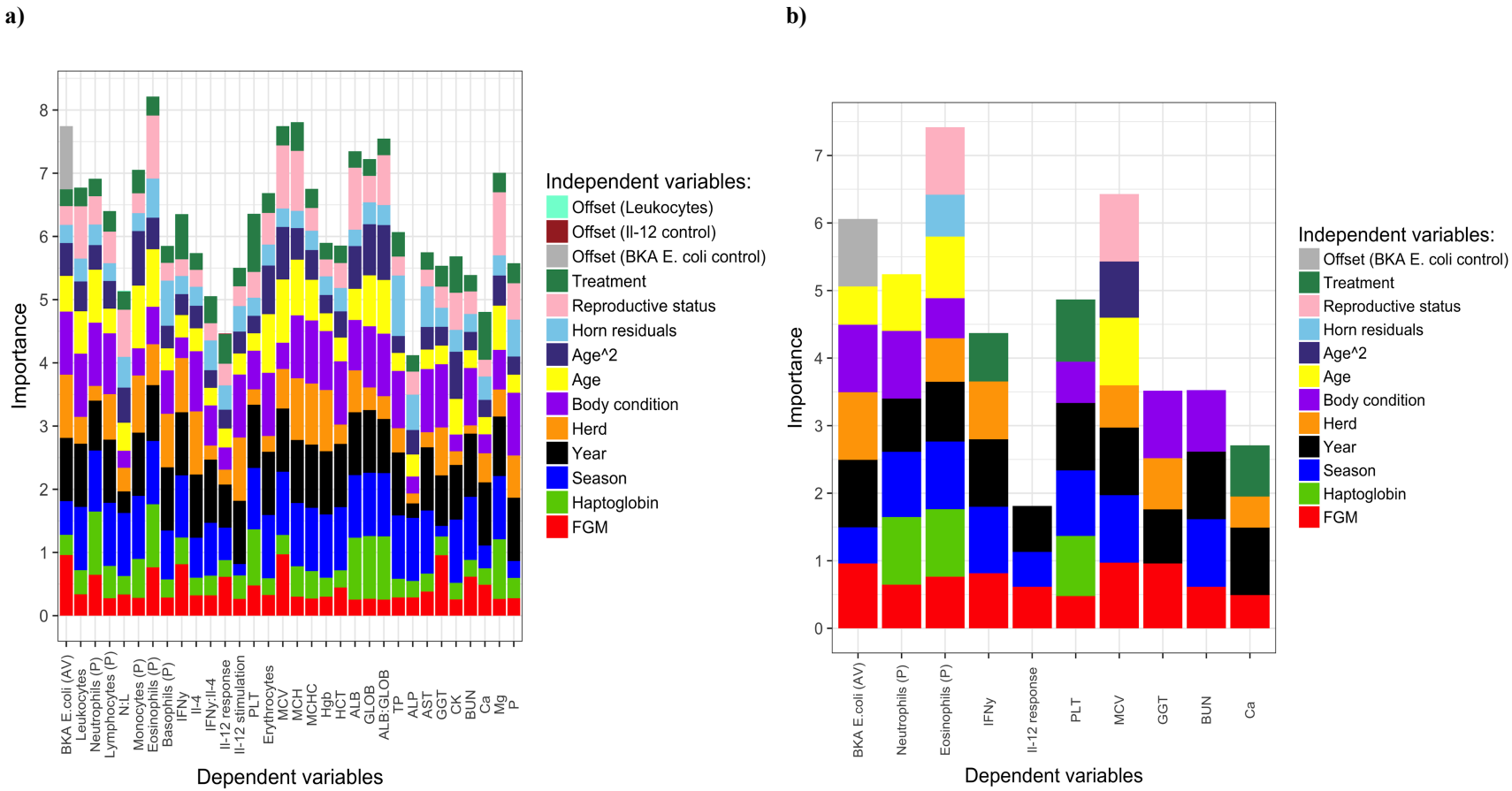
Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Creatine kinase (CK)							
1567	Season + Year + Age^2 + Reproductive status + Treatment + Age	13	5466.37	0.00	0.02	12.28	64.14
1559	Season + Year + Age^2 + Reproductive status + Treatment	12	5466.56	0.20	0.02	11.88	64.30
535	Season + Year + Age^2 + Reproductive status	11	5466.79	0.43	0.02	11.10	64.15
1823	Season + Year + Age^2 + Reproductive status + Treatment + Age + Horn residuals	14	5466.98	0.61	0.02	13.09	64.09
1555	Season + Year + Age^2 + Treatment	11	5466.99	0.62	0.02	11.45	63.69
543	Season + Year + Age^2 + Reproductive status + Treatment + Age	12	5467.04	0.68	0.02	11.42	64.06
531	Season + Year + Age^2	10	5467.61	1.25	0.01	10.64	63.59
1563	Season + Year + Age^2 + Treatment + Age	12	5467.88	1.52	0.01	11.66	63.50
799	Season + Year + Age^2 + Reproductive status + Age + Horn residuals	13	5468.00	1.64	0.01	12.08	64.05
1815	Season + Year + Age^2 + Reproductive status + Treatment + Horn residuals	13	5468.11	1.75	0.01	12.17	64.22
Biochemical panel measure: Calcium (Ca)							
1092	Year + Treatment + FGM + Herd	10	1197.28	0.00	0.02	14.95	14.95
1028	Year + Treatment + Herd	9	1197.29	0.01	0.02	14.55	14.55
1027	Year + Treatment	7	1197.43	0.15	0.01	13.71	13.71
1539	Year + Treatment + Season	10	1197.50	0.22	0.01	14.91	14.91
1091	Year + Treatment + FGM	8	1197.69	0.41	0.01	14.06	14.06
1603	Year + Treatment + FGM + Season	11	1197.73	0.45	0.01	15.26	15.26
1859	Year + Treatment + FGM + Horn residuals + Season	12	1198.25	0.97	0.01	15.55	15.55
1795	Year + Treatment + Horn residuals + Season	11	1198.33	1.05	0.01	15.14	15.14
1348	Year + Treatment + FGM + Herd + Horn residuals	11	1198.37	1.09	0.01	15.13	15.13
1060	Year + Treatment + Herd + Body condition	10	1198.53	1.25	0.01	14.69	14.69

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Blood Urea Nitrogen (BUN)							
611	Season + Year + Body condition + FGM	11	2139.81	0.00	0.06	28.44	28.47
547	Season + Year + Body condition	10	2140.68	0.86	0.04	28.01	28.55
615	Season + Year + Body condition + FGM + Reproductive status	12	2141.08	1.26	0.03	28.54	28.54
867	Season + Year + Body condition + FGM + Horn residuals	12	2141.61	1.80	0.03	28.44	28.48
627	Season + Year + Body condition + FGM + Age^2	12	2141.74	1.93	0.02	28.45	28.89
551	Season + Year + Body condition + Reproductive status	11	2141.81	1.99	0.02	28.10	28.25
619	Season + Year + Body condition + FGM + Age	12	2141.86	2.04	0.02	28.42	28.79
739	Season + Year + Body condition + FGM + Haptoglobin	12	2141.89	2.08	0.02	28.40	28.47
1635	Season + Year + Body condition + FGM + Treatment	12	2141.89	2.08	0.02	28.41	28.64
563	Season + Year + Body condition + Age^2	11	2142.55	2.74	0.02	28.03	29.01
Biochemical panel measure: Phosphorous (P)							
296	Year + Body condition + Herd + Horn residuals + Reproductive status	11	1160.92	0.00	0.03	13.82	26.14
40	Year + Body condition + Herd + Reproductive status	10	1161.29	0.37	0.02	13.14	25.98
292	Year + Body condition + Herd + Horn residuals	10	1161.66	0.73	0.02	13.26	24.92
808	Year + Body condition + Herd + Horn residuals + Reproductive status + Season	14	1162.17	1.25	0.02	14.78	26.85
424	Year + Body condition + Herd + Horn residuals + Reproductive status + Haptoglobin	12	1162.41	1.49	0.01	13.95	25.79
1320	Year + Body condition + Herd + Horn residuals + Reproductive status + Treatment	12	1162.60	1.68	0.01	13.89	26.61
420	Year + Body condition + Herd + Horn residuals + Haptoglobin	11	1162.71	1.79	0.01	13.50	24.55
36	Year + Body condition + Herd	9	1162.72	1.80	0.01	12.41	24.62
39	Year + Body condition + Reproductive status	8	1162.73	1.81	0.01	11.42	24.95
360	Year + Body condition + Herd + Horn residuals + Reproductive status + FGM	12	1162.86	1.94	0.01	13.82	26.05

Supplementary Data SD3.2 *Continue…*

Model:	Explanatory variables:	df	AICc	delta	weight	R^2m (%)	R^2c (%)
Biochemical panel measure: Magnesium (Mg)							
687	Season + Reproductive status + Haptoglobin + Year + Age + Body condition	13	440.57	0.00	0.05	23.54	45.20
688	Season + Reproductive status + Haptoglobin + Year + Age + Body condition + Herd	15	440.71	0.14	0.05	24.40	46.11
655	Season + Reproductive status + Haptoglobin + Year + Age	12	441.08	0.51	0.04	23.13	43.96
695	Season + Reproductive status + Haptoglobin + Year + Body condition + Age ^2	13	441.75	1.18	0.03	23.19	44.85
943	Season + Reproductive status + Haptoglobin + Year + Age + Body condition + Horn residuals	14	442.01	1.44	0.02	23.77	46.00
696	Season + Reproductive status + Haptoglobin + Year + Body condition + Age^2 + Herd	15	442.01	1.44	0.02	24.02	45.71
1712	Season + Reproductive status + Haptoglobin + Year + Age + Body condition + Herd + Treatment	16	442.19	1.62	0.02	24.57	46.31
1711	Season + Reproductive status + Haptoglobin + Year + Age + Body condition + Treatment	14	442.30	1.73	0.02	23.62	45.38
703	Season + Reproductive status + Haptoglobin + Year + Age + Body condition + Age^2	14	442.30	1.73	0.02	23.53	45.48
704	Season + Reproductive status + Haptoglobin + Year + Age + Body condition + Age^2 + Herd	16	442.44	1.87	0.02	24.38	46.41



Supplementary Data SD3.3 Stacked bar summarizing the importance value (i.e., value between 0-1) of each independent variable: a) for each dependent variable in the global model (linear mixed effects model with Animal ID as the random effect), and b) from the best model (linear mixed effects model with Animal ID as the random effect) for each dependent variable that included FGMs as an important predictor. The importance value can be calculated as the sum of the Akaike weights for each independent variable of all possible model combinations. FGM was an important predictor of innate immunity (measured as BKA *E. coli*, Neutrophils, and

Eosinophils), adaptive immunity (measured as response to pokeweed stimulation for INFy, and Il-12), hematology measures (measured as MCV, and PLT), and biochemical panel measures (including GGT, BUN, and Ca). Season and year were important explanatory variables for majority of the immunological and health measures (excluding GGT, Ca, P for season, and ALP, N:L for year). FGM = fecal glucocorticoid metabolite; BKA = bacterial killing assay; AV = absolute value; N:L = neutrophil lymphocyte ratio; IFNy = interferon gamma; Il = interleukin; PLT = platelets; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; Hgb = hemoglobin; HCT = hematocrit; ALB = albumin; GLOB = globulin; TP = total protein; ALP = alkaline phosphotase; AST = asparatate aminotransferase; GGT = gamma-glutamyltransferase; CK = creatine kinase; BUN = blood urea nitrogen; Ca = calcium; Mg = magnesium; P = phosphorous.