Long-term Physiological Trends and their Drivers: Linking Hair Hormone Concentrations with Telemetry Data in GPS-collared Serengeti Wildebeest

by

Callum George Buchanan

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Institute of Biodiversity, Animal Health, and Comparative Medicine

College of Medical, Veterinary, and Life Sciences

University of Glasgow

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Abstract

The environmental context in which animals live, such as their exposure to predation or availability of food, can strongly shape their physiological state and subsequent behaviour. Additionally, changes in reproductive state also affect animal behaviour. Temporal physiological data is therefore of great value in the study of free-living animals and how they interact with their environment. However, longitudinal measures of physiological state within individual animals are rarely studied, particularly in wild populations, because they are difficult to collect.

Some substances which circulate in the blood, including drugs and endogenous steroid hormones, are incorporated into hair as it grows, in concentrations that reflect their circulating concentrations at that time. Since most regions of hair grow throughout the life of an individual, by sectioning hair samples along their growth axis we can create sequential, distinct samples. Measuring hormone concentrations in each of these sections therefore allows for a temporal record of chronic hormone concentrations, covering the time the sample was grown.

This thesis is composed of a general introduction (Chapter 1) and discussion (Chapter 6), as well as four standalone data chapters (Chapters 2-5).

Chapter 2 used lengths of regrown hair collected from 6 wildebeest to calculate hair growth rate, showing no significant difference between individuals. This hair growth rate was used to calculate dates of growth for all sections of hair. Cortisol, the primary hormone responsible for the stress response, was measured in wildebeest tail hair, showing variation along the sample, and was stable in wildebeest hair when measured in *pg cortisol/cm hair*. Cortisol was also measured and varied along the length of a hair sample collected from a ~100-year-old taxidermied oryx, highlighting that cortisol appears to remain stable over longer periods of time, showing promise for the collection of physiological data from museum specimens.

Chapter 3 implemented this method to produce cortisol profiles for hair samples (sectioned longitudinally into 8mm segments) collected from GPS-collared wildebeest (*Connochaetes taurinus*) in the Serengeti-Mara ecosystem. Cortisol data was then paired with a variety of environmental and anthropogenic factors extracted from telemetry data to determine drivers of stress. Hair cortisol concentrations were found to be negatively related with *NDVI* (a remotely sensed metric of vegetative productivity) and *proximity to villages*. Cortisol was generally higher in samples taken from animals found dead than those that were live captured, suggesting chronic stress impacts negatively on survival. Interestingly, the effect of NDVI on cortisol differed between animals found dead and those captured. These results suggest that chronic stress increases when wildebeest are in locations where food availability is low and where villages are nearby and that chronically stressed animals (carcass samples) perceived stressors differently to the individuals who were alive at sampling.

Chapter 4 measured progesterone, one of the primary hormones responsible for the regulation of pregnancy, in wildebeest hair samples, showing variation between and within samples. It was not stable along the growth axis of the hair, but it was possible to correct for this post hoc, using relative metrics compared against a long-term mean. Corrected progesterone concentrations were significantly higher during the time between the rut and calving period. This corresponds to the time when the vast majority of migratory wildebeest are pregnant, showing promise for the use of this technique to determine reproductive trends in wild animals.

Chapter 5 focused on aldosterone, the primary hormone responsible for the regulation of sodium and water balance within the body. Sodium is an essential element which wildebeest obtain through forage; however, the sodium content of forage varies across the Serengeti-Mara ecosystem. Aldosterone was measured in tail hair taken from wildebeest resident to a high-sodium region of the ecosystem, and migrants, to determine whether spatial variation in sodium within the landscape could be detected in the physiological response of the animals. Aldosterone concentrations showed variation within and between hair samples, as well as a general increase along the growth axis of the hair; however, it was possible to detrend the data using the correction method

developed in Chapter 2. Overall, aldosterone was higher, and showed greater seasonal variation, in migratory wildebeest than in wildebeest resident to sodium-rich areas of the ecosystem, which suggests that migrants experience greater sodium stress. Aldosterone levels also correlated with season (i.e. proximity to time of maximum cumulative rainfall) and NDVI (i.e. vegetation greenness), which suggests that wildebeest are most sodium-deprived at the end of the dry season, when they occupy the most sodium-poor area of the ecosystem.

This thesis validates a relatively straight-forward and robust method for the collection of chronic physiological time series data relating to stress, reproduction, and sodium homeostasis. Additionally, samples collected from geo-tagged animals can be used to study the relationship between an animal's physiology and their environment. The ability to link spatial information with an individual animal's physiology over long time periods opens exciting new avenues for research relevant to movement ecology, conservation management and animal welfare across a wide range of taxa and settings.

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Author's declaration

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I declare that this dissertation has not been submitted for any other degree at the University of Glasgow or any other institution and does not exceed the required word limit.

Word count: 51,893

Callum Buchanan
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1 General Introduction

Terrestrial mammal migrations are under increasing threat from an unprecedented growth in human activity and land use change (Harris et al., 2009). Although the decline in migrations is not new, the rate of decline is accelerating (Wilcove and Wikelski, 2008). To understand the reasons behind this decline, we need to achieve a greater understanding of the factors that affect animal movement. However, the relatively large spatial range covered by migratory mammals makes them simultaneously vulnerable to a range of anthropogenic factors such as habitat destruction and fragmentation, overexploitation, and climate change, while also making them difficult to study (Vitousek et al., 1986, Pimm et al., 2001, Coppack and Both, 2002, Berger, 2004). Furthermore, population ecology research may often be limited in that it has to rely upon data gathered from point observations, due to the costs, both financially and in terms of time and resources, involved in the collection of time series data (Boitani and Fuller, 2000). This approach, however, only provides a narrow snapshot into the factors involved in the study area of interest. For example, one of the primary methods currently employed to study the wildebeest (*Connochaetes taurinus*) migration of the Serengeti involves the location of animal carcasses, collection of samples, and determination of their cause of death (Mduma et al., 1999). However, this only gives limited information on the physiological state of the animal at the time of death and gives no information about the animal's life leading up to that point. Many studies have now capitalised upon the ability to fit animals with GPS-collars, which allows an animal's movement to be tracked over time. In recent years, improvement in tracking technologies have made it possible to obtain high-resolution movement data that can be collected using increasingly-smaller and more affordable devices, which can be used in both marine and terrestrial systems (Kays et al., 2015). However, it is difficult to determine the underlying motivation behind movement decisions using telemetry data alone. Factors that may contribute to animal movement decisions include: habitat selection; landscape permeability; animal memory; evolved, innate responses; and the physiological state of the animal (Andersen, 1991, Stamps and Krishnan, 2001,

Nathan et al., 2008, Avgar et al., 2013). The collection of time-series data relating to an animal's underlying physiology (e.g. stress, hunger, reproductive status, etc.) would be a valuable next step to allow construction of a more complete picture of animals in the wild, as it would allow for a better understanding of the condition of animals and how their condition is affected by changes in their environment.

1.1 Measuring Physiology

Physiology and behaviour are regulated by signalling molecules known as hormones, which are transported around the body via the circulatory system (Bentley, 1998). Therefore, one of the best means of determining an individual's physiological state is through monitoring the levels of specific hormones of interest within the body (Hill et al., 2004). For instance, the concentration of endogenous cortisol (the primary steroid hormone involved in the regulation of the mammalian stress response) has been shown to correlate with exposure to known stressors in a variety of species (Janssens et al., 1995, Adadevoh and Kwaku Adadevoh, 1971, Vélez-Marín and Velásquez, 2012). Although the primary means of obtaining hormone concentrations is from blood or saliva, these matrices have some drawbacks. Blood and saliva are not only difficult to collect in wild animals due to the need for invasive sampling and immobilisation procedures, but also these metrics only give point data, meaning they only show a snapshot of the animal's state at that moment (Sheriff et al., 2011). This is due to the concentration of hormones in these matrices constantly fluctuating over time in a pulsatile manner, being used by the animal to maintain homeostasis and exhibiting a circadian rhythm of secretion (e.g. cortisol levels have been shown to be highest shortly after awakening and gradually decrease throughout the day, in a variety of species (Pruessner et al., 1997, Heintz et al., 2011, Bohák et al., 2013)). It is therefore necessary to collect multiple saliva and blood samples, over an extended period of time, to gain a more accurate representation of underlying stress levels (Al-Gahtani and Rodway, 1991). The need to resample an animal multiple times makes this technique impractical when studying animals in the wild. However, a number of alternative sample

media exist which have been shown to contain hormone concentrations corresponding to longer time periods, minimising any effect caused to short-term measurements as a result of the collection method (i.e. handling an individual in order to take a sample) (D'Anna-Hernandez et al., 2011).

Urinary and faecal samples can be used as alternative sources from which to obtain a measure of hormone concentrations as their steroid content has been shown to correlate with the animal's mean endogenous hormone concentrations since its previous urination/defecation (Ruder et al., 1972, Möstl et al., 2002). These sample types have the advantage of being non-invasive, as well as giving a more long-term measure of hormone concentrations, without the need for repeated sampling. However, there are still some drawbacks to these sample media. It can be difficult to identify the individual who produced the sample without close monitoring, and urine samples are difficult to collect in the field (Möstl and Palme, 2002). Additionally, resampling is still necessary for the collection of truly long-term (i.e. weeks to months) physiological data. Due to all these reasons, an easier to collect, more reliable, and non-invasive means to obtain long-term data on hormone levels in the body is preferred. A relatively novel method which potentially meets all of these criteria exists; it has been discovered that several steroid hormones are present in hair (Gaudl et al., 2016, Bévalot et al., 2000, Cirimele et al., 2000) and that hair cortisol concentrations correlate with long-term blood plasma and serum (Thomson et al., 2009), urine (Sauvé et al., 2007), faecal (Accorsi et al., 2008) and salivary (Davenport et al., 2006) cortisol concentrations obtained through repeated resampling. Additionally, storage requirements such as refrigeration, are not necessary for hair samples (Accorsi et al., 2008), making their application in field studies relatively straightforward.

Hair has been used for some time in toxicology and forensic science (Kintz et al., 2015), as well as in the competitive sports industry, for the detection of banned substances (i.e. performance enhancing drugs such as corticosteroids and anabolic steroids (Thieme et al., 2000)) because of its ability to provide a retrospective, long-term measure of analyte exposure. Recent studies have suggested that hair hormone concentrations give a more precise measure of chronic physiological condition (e.g. high hair cortisol signifies chronic stress) than those obtained from blood, even when multiple blood samples are taken

(Ghassemi Nejad et al., 2014). A growing number of studies have investigated the validity of this technique in non-human species, with a few ecological studies using hair cortisol concentrations to measure stress in free-living animals (Macbeth et al., 2010, Carlitz et al., 2016, Caslini et al., 2016). These studies indicate that this form of hormone analysis shows promise as a valuable source of reliable long-term endocrine data from wild animal populations.

Of particular interest, with regards to the use of hair samples as an alternative media for the collection of physiological data, is that some hair tends to grow continuously throughout an animal's life. Blood-borne factors such as steroid hormones are incorporated into the hair through diffusion from capillaries into the cells of the hair follicle as it grows (Cone, 1996). Therefore, hair not only has the potential to give a long-term measure of several steroid hormones, but also can be used to produce multiple samples, relating to different time points, from one hair sample. This may be possible by sectioning the hair longitudinally in order to obtain multiple distinct hair segments, whose sequence reflects the time at which that specific section of hair grew. A retroactive physiological profile, covering the period the entire sample was grown, may then be obtained by plotting the levels of each hormone obtained from these sections against time; effectively converting point data into time series. However, in order to calculate the corresponding date of growth for each section of hair, hair growth rate must be measured. A few recent studies have measured stable isotope ratios in sequential sections of hair, using known life history events to validate measures of hair growth rate (Rysava et al., 2016, Kaczensky et al., 2017, Burnik Šturm et al., 2017). However, unlike stable isotopes, it is possible that hormones may become unstable in hair, over time. Furthermore, animals with little or no variation in the physiological condition of interest over time could not be used to validate the temporal element of the technique, as hormone concentrations would be the same across all sections of hair within a sample. For instance, an animal occupying a consistently sodium-abundant ecosystem would show little or no temporal variation in the relative concentration of aldosterone (the hormone responsible for the regulation of endogenous sodium). Therefore, predicted and measured aldosterone concentrations would always align, regardless of the calculated dates of growth for each section of hair, as all values would effectively be the same. On the other hand, hormone profiles from animals

experiencing temporal variation in physiological condition would only align with predicted values if the dates of growth for hair sections (calculated from the growth rate) were correct.

In order to validate this approach, the study species must therefore go through significant and predictable physiological changes (e.g. changes in reproductive state) within the timeframe which a hair sample relates. Additionally, the environment which the species of interest inhabits should change in a similarly predictable manner, so that environmentally driven changes in physiology can also be predicted (e.g. when animals occupy areas where forage is relatively low in sodium, aldosterone (the hormone which regulates sodium) should increase, to conserve endogenous sodium). Therefore, two main factors are required of the study species:

i) the seasonal movement and life cycle of the species of interest must be well defined and highly synchronous
ii) The ecosystem that they inhabit should be heterogeneous and non-fragmented (i.e. gradual changes over relatively large distances).

If the environment and underlying physiology of a population changes over time, and in a predictable manner, predicted changes in physiology can be compared to patterns measured in hair hormone profiles. It can then be determined whether predicted and measured changes in physiology are aligned, or if it is possible to correct hair hormone profiles to more accurately reflect the underlying physiology of the animal.

1.2 Study Area and Species

1.2.1 The Serengeti-Mara Ecosystem

Every year, the largest movement of terrestrial mammals on Earth, known as The Great Migration, takes place across the plains and open woodland of the Serengeti-Mara ecosystem in East Africa (Fig. 1.1) (Hopcraft et al., 2015b). The Serengeti stretches from Northern Tanzania into Southwestern Kenya, covering an area of approximately 25,000km^2 (Thirgood et al., 2004). It lies to the east of

Lake Victoria and contains two nature reserves: the almost 15,000km^2 Serengeti National Park in Tanzania, and the 1,500km^2 Masai Mara National Reserve in Kenya, to the North (Walpole, 2003). Despite its size, the Serengeti is one of the few remaining intact, protected ecosystems that retains an almost complete biota that has existed for millennia (Peters et al., 2008). Comprised of a wide variety of ecological niches, such as grassland, woodland, and swamps, the Serengeti is understandably home to a diverse range of species, including 28 species of ungulates and 10 species of large carnivores (Sinclair and Norton-Griffiths, 1979). Due to seasonal changes in the rainfall, some regions of the Serengeti are not habitable all year round. This has led some species to develop a life-history strategy of migration to compensate, leaving areas as they become unsustainable, to travel to more hospitable areas (Fryxell et al., 1988, Berger, 2004). Each year, approximately 1.3 million wildebeest (*Connochaetes taurinus*), 400,000 Thomson's gazelle (*Eudorcas thomsonii*), and 250,000 zebra (*Equus quagga*) employ this life history strategy, migrating in a clockwise direction across the Serengeti-Mara ecosystem (Fig. 1.1) (Holdo et al., 2009).

The Serengeti is therefore an ideal location for this study as it has well documented, highly predictable seasonal changes, an intact, diverse, ecosystem, and is home to species whose seasonal movement and lifecycle are well defined.

1.2.2 **Wildebeest**

Wildebeest are the cornerstone species of the Serengeti ecosystem, outnumbering all other large mammal populations in the Serengeti combined. This is believed to be a consequence of their unique biology, adaptive migratory behaviour, and the unique geomorphology of the landscape, which results in a tight feedback between the highly adapted wildebeest and an environment which closely matches their requirements (Hopcraft et al., 2015b). One of the most astonishing features of the wildebeest migration is the synchronicity between the life cycle of the wildebeest, their environment, and their spatiotemporal position along the migratory route. For instance, approximately 80% of calves are born over the course of a three-week window beginning in mid-February, as the rains and the wildebeest return to the shortgrass plains in the

most southernly region of the migratory route (Estes, 1976). This relatively short, highly productive calving period (during which, approximately 12,000 calves are born a day (Estes and Estes, 1979)), preceded by the highly synchronous rut at the onset of the dry season, allow for the approximate dates of an individual's pregnancy to be determined. Since changes in reproductive state (fertilisation/conception) incur major changes in female sex hormone concentrations, the timing of these reproductive events can be compared to physiological profiles created from hair hormone concentrations, to validate this technique. This highly synchronous breeding season, combined with the relatively long tail hair (approximately 40cm; 18 months of growth), make wildebeest an ideal study species to test a method for recreating physiological profiles from longitudinally sectioned hair samples.

1.2.3 **Resident and Migrant Wildebeest Populations**

While there can be significant interannual variation in movement patterns, two life history strategies can be identified: wildebeest who remain in one area of the ecosystem, throughout their life (i.e. sympatric residents), and wildebeest who migrate across the entire Serengeti-Mara ecosystem.

There are four subpopulations within the ecosystem that tend to remain in the same areas year-round, or perform a much smaller, local migration each year: Western Corridor, Maasai Mara, Loliondo, and Ngorongoro crater residents. These populations are only found in areas with annual rainfall that exceeds 900mm; permanent access to water; and fine-scale local heterogeneity of forage quality and quantity (Hopcraft, 2010). Genetic evidence suggests that, although the resident subpopulations were once part of a larger interbreeding pool, which included the migrant population (Georgiadis, 1995), current spatiotemporal distributions and the synchronicity of breeding in the migratory herd means that this is no longer possible, despite seasonal sympatry occurring between migrant wildebeest and all four resident subpopulations. Resident and migrant sub-populations therefore provide a good comparison to explore the links between environment, physiology, and behaviour.

Two of the key differences between migrant and resident wildebeest are their relative stature and breeding synchrony. Migrants are significantly smaller than all resident subpopulations, the largest of which is the Western Corridor residents, whose bulls are approximately 20% larger than migrant bulls (Talbot and Talbot, 1963). This size adaptation allows migrants to cover larger distances while residents are better suited to defend against predation, which is a greater threat to resident populations, since they do not leave the home ranges of resident predators and lack the security associated with larger herds (Hopcraft et al., 2015b). Additionally, residents are far less synchronous breeders; the rut generally begins much earlier, and lasts longer, relative to the migratory subpopulations (Ndibalema, 2009). These factors, as well as the ability to free themselves from the restrictions imposed by local forage abundance (Fryxell et al., 1988, Hopcraft et al., 2014), allow migratory wildebeest to vastly outnumber resident wildebeest populations in the Serengeti-Mara ecosystem.

1.2.4 **The Great Migration**

The Serengeti-Mara ecosystem has a strongly seasonal north-south rainfall gradient, with the grasslands in the south drying up as the wet season ends. This leads to a green-wave of forage quality (Boone et al., 2006, Holdo et al., 2009), which in turn causes migrant species to travel to the wetter regions in the north and west, even though the grasses in these areas are of lower quality (McNaughton, 1979). As a result, migratory wildebeest tend to follow a relatively predictable migratory route, with regards to the general area of the ecosystem they will occupy, at different times of the year.

The vast majority of migratory wildebeest are born in the nutrient rich short-grass plains of the southernmost region of the Serengeti, during a calving period lasting from February to early March. The herd remains in this area of the ecosystem (Fig. 1.1 (dark green arrows)) until the beginning of the dry season, when this area can no longer support the herd. At this point, the migration moves north-west into the Moru Kopjes and Seronera regions of the Serengeti,

where the rut takes place. Migrants arrive in the sodium-rich Western Corridor of the ecosystem around the end of June and remain here until the end of July, as the ecosystem continues to dry (Fig. 1.1 (orange arrows)). After this, the migration generally heads in a north-eastern direction, before travelling further north in the driest months, because the Mara River is the only place in the ecosystem where there is permanent water (Fig. 1.1 (brown arrows)). The herd generally arrives in the nutrient poor grasslands in the north of the Serengeti National Park and Maasai Mara, by September, when they tend to concentrate around key resources such as the Mara river, or in locations with recent rainfall, where grazing is good. They remain in this nutrient-poor area through October, before eventually returning to the south, through Loliondo on the east side of the Serengeti National Park, with the return of the rains (Fig. 1.1 (light green arrows)) (McNaughton, 1985).

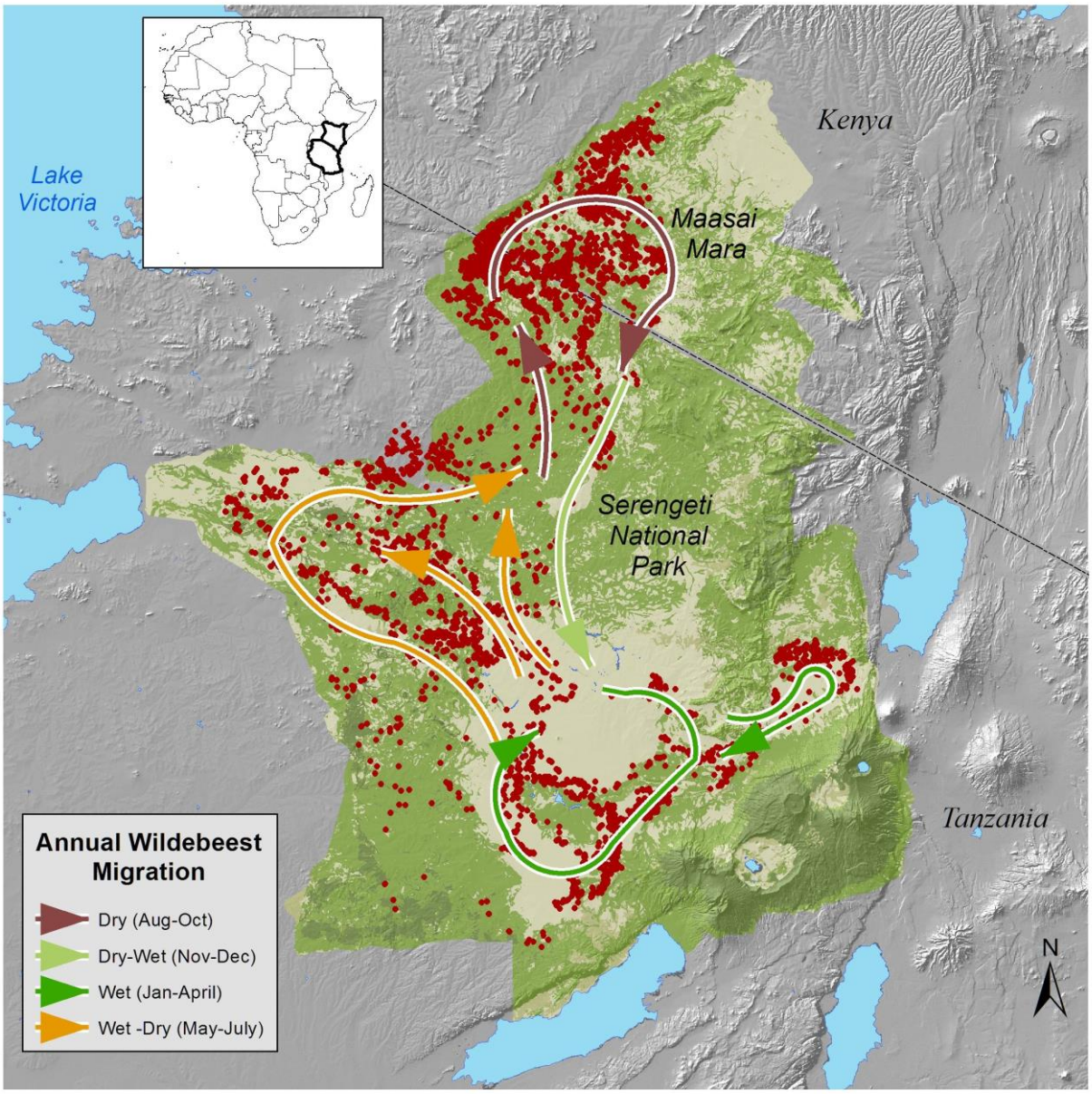


Figure 1.1 The wildebeest migration of the Serengeti-Mara ecosystem. Brown dots indicate GPS locations of 8 collared wildebeest within the Serengeti-Mara ecosystem, from 1999 to 2001 (adapted from (Torney et al., 2018))

1.2.5 **Potential Driving Factors of Wildebeest Migration**

The seasonal rainfall gradient is the primary driver of the wildebeest migration; however, it is not the only factor. If wildebeest followed only the rainfall pattern, we would expect to see direct south-east to north-west movements, however this type of linear movement is rarely observed. The more triangular movement suggests there are other factors that drive the annual movement.

Although the migration initially follows the rainfall north as the short-grass plains in the south dry up, by about May each year the migration begins travelling westward, into the Western Corridor. This area is the parallel outflow of the Grumeti and Mbalageti rivers and is fed by water running from the salt-rich areas further upriver. This detour to the west does not seem to align with the hypothesis that the migration follows rainfall gradients or food abundance directly, which travel in a strictly linear fashion, implying more complex relationships and multiple drivers of the migration (Holdo et al., 2011).

The highly complex nature of the migration, which occurs over a variety of scales, in terms of space, time, and biological complexity, makes it difficult to understand the precise, underlying mechanism behind this phenomenon (Holdo et al., 2009, Torney et al., 2018). A recent study monitoring the herd through GPS collaring and tracking of individuals within the herd suggests that the avoidance of risks and the availability of protein-rich grasses also play a role (Hopcraft et al., 2014). Although the behavioural response of these animals can be linked to broad scale environmental patterns in the ecosystem, the biggest missing link in understanding the drivers of migration is the animals' physiological response, as measured through the relative concentration of specific hormones of interest.

1.3 **Steroid Hormones**

Hormones can be divided by their general chemical structure into three main classes: steroids, eicosanoids, and amino acid/protein derivatives (Bentley, 1998). Of these classes, steroid hormones have been the focus of most hair analysis studies, with a number of steroid hormones having been measured in hair samples obtained from a variety of species (Gao et al., 2013, Koren et al., 2002, Cook, 2012). Steroids are synthesised from, and share the same basic structure as, cholesterol, a relatively small and stable biological molecule composed of four carbon rings (Bentley, 1998). This small molecular size and chemical stability allows unbound steroid hormones to cross the cell membrane of growing hair, becoming incorporated into the developing hair structure in concentrations that reflect levels in blood during the period of growth (Cone,

1996, Raul et al., 2004, Davenport et al., 2006). Additionally, steroid hormones are responsible for the regulation of a number of physiological processes, such as the stress response, reproduction, and the conservation of essential minerals (Bentley, 1998). Therefore, the focus of this study will be on the detection and quantification of steroid hormones in hair.

1.3.1 Glucocorticoids

Steroid hormones can be divided into three functional groups: glucocorticoids, sex steroids, and mineralocorticoids. Cortisol is the primary glucocorticoid hormone involved in the regulation of an individual's response to a stressor in most mammal species. It is responsible for the regulation of a number of factors including: blood sugar levels and metabolism, blood pressure and inflammation (Vining et al., 1983, Griffin and Ojeda, 1992). Cortisol is easily measured in blood and/or saliva samples (Dedovic et al., 2009, Habib et al., 2001, Möstl and Palme, 2002, Teruhisa et al., 1981). A vast number of studies have shown strong correlation between circulating glucocorticoid concentrations and stress, in a variety of species (Christison and Johnson, 1972, Barton et al., 1985, Burke et al., 2005). Cortisol has also been measured in hair obtained from a number of species, including: humans, macaques, bears, and deer (Stalder et al., 2012, Davenport et al., 2006, Macbeth et al., 2010, Caslini et al., 2016); however, no studies have investigated cortisol concentrations in wildebeest hair. Wildebeest movement may in part be determined such as to reduce exposure to potential stressors; cortisol levels would be expected to be greatest when animals are in high risk habitats that can easily conceal predators (such as woodlands (Hopcraft et al., 2005)), outside the National parks which act as protected areas and where human exposure may be greater, or in response to low nutrient availability and/or as a result of poor health.

1.3.2 **Sex Steroids**

Reproduction is one of the most important aspect of an animal's life cycle; therefore, the role of sex steroids must be considered when studying physiology. This thesis focuses only on female wildebeest because they have the largest energy demands and therefore their movement decisions are most likely to be linked to the environmental conditions that determine the distribution and availability of food and water (Hopcraft et al., 2015b).

Several steroid hormones are involved in the regulation of the female reproductive cycle. Due to differences in steroid dominance across the reproductive cycles, the reproductive state of an animal (e.g. whether they are pregnant) can be inferred from the relative concentration of these hormones (Norris and Carr, 2013). For example, a sustained elevation in progesterone concentrations can be used to indicate that an individual is pregnant (e.g. elevated progesterone levels indicates a pregnancy) (Henricks et al., 1970, Pepe and Rothchild, 1974, Smith et al., 1974, Radwanska et al., 1978). Progesterone has been measured in human and macaque hair, and hair progesterone concentrations have been shown to correlate with serum progesterone concentrations during the period of hair growth (Dettmer et al., 2015, Yang et al., 1998). Additionally, oestrogen concentrations peak around parturition in many mammal species (Romero et al., 1988, Chamley et al., 1973, Henricks et al., 1972). Both oestrone and oestradiol have also been measured in human hair (Choi et al., 2000), however, no studies have investigated changes in hair oestrogen concentration associated with different reproductive states. If these hormones are stable in hair over time, and correlate with levels in the blood, it should be possible to use the relative concentrations of these hormones to determine whether/when a female has calved.

1.3.3 **Mineralocorticoids**

Mineralocorticoids are a class of steroid hormones responsible for regulating the salt and water balance in the body. Aldosterone is the primary mineralocorticoid hormone produced in the adrenal gland and is the main regulator of essential

exogenous minerals (Na^+ (re)absorption and K+ secretion), as well as arterial blood pressure (Booth et al., 2002). A study by Gaudl et al (2016) successfully measured aldosterone in human hair using mass spectrometry, however aldosterone has not been measured in hair samples taken from any other species, nor has aldosterone been measured in any sample media taken from wild animals. Aldosterone is of particular interest when studying Serengeti wildebeest, as sodium is a critical component of herbivore diets, but is unevenly distributed across the ecosystem. This is primarily driven by variation in grass species, which is influenced by rainfall, topography, and the properties of the soil (Banyikwa, 1976, McNaughton, 1985, Hamilton III et al., 2001). The highest sodium concentrations available to the wildebeest occur in the southern plains and Western Corridor, while the grasses in the northern woodlands are sodium-poor and do not meet the minimum requirements to sustain the wildebeest for extended periods of time (Hopcraft, 2010). This sodium gradient is driven by an inverse relationship between rainfall and grass quality, with the wetter regions in the north containing lots of taller, lower quality grass species, while drier regions tend to have lower overall grass biomass, but of higher quality for grazing (Olff et al., 2002, Fritz and Duncan, 1994, Hopcraft, 2010). As salt levels within the body are depleted, aldosterone levels increase in order to trigger the retention of any remaining sodium within the body (Blazer-Yost et al., 1999). Aldosterone concentrations can therefore be used as an indicator of sodium availability within the animal's diet, and the environment. This is of value in determining potential drivers of the migration as limited salt availability is one hypothesis to explain the movement of wildebeest into the western corridor: a salt-rich region of the ecosystem (Hopcraft et al., 2015b). For example, if sodium availability were indeed a driver in the migration, then aldosterone would vary as they migrate across sodium gradients, with high aldosterone levels when the migration is in sodium-poor areas, and vice versa.

1.3.4 **Extraction and Quantification**

No single universally-accepted protocol currently exists for the extraction of steroid hormones from hair samples, however most methods contain the following general steps (e.g. (Macbeth et al., 2010, Davenport et al., 2006).

First, hair samples are washed in solvent to remove any external contaminants. Next, dried hair is ground to a fine powder, to homogenise the sample and increase the surface area for extraction. A known mass of hair is then covered with solvent (e.g. methanol) and placed into a heated shaker, to allow the extraction of hormones from the hair into the solvent (Pötsch and Moeller, 1996). The extraction solvent is then aliquoted into a glass tube and dried.

Several methods exist for the quantification of steroid hormones, the most common of which is the Enzyme Linked Immuno-Sorbent Assay (ELISA). These assays are typically carried out in a 96 well plate, wherein each well is coated with an antibody that passively binds proteins. An appropriate antibody, specific to the target molecule, is conjugated to an enzyme and added to each well, along with the sample (which are generally run in duplicate). Any target molecules present in the sample competes with the conjugate for the limited number of antibody binding sites. After incubation, any unbound reagents are washed from the plate. Substrate is then added, to allow an enzymatic reaction to occur between the substrate and the conjugated enzyme. This produces a change in either colour, luminescence, or fluorescence, which can be measured with a spectrophotometer. Optical densities in sample wells are then compared to optical densities obtained from a standard curve, to determine the concentration of the target analyte present in each sample (Engvall et al., 1971).

Commercially available ELISA kits are generally validated for the measurement of the specific molecule of interest in a variety of sample media, including blood plasma, serum, urine and saliva. Although they are not specifically designed for this purpose, it is also possible to extract and quantify biological factors from alternative sample media such as hair, if the ELISA can be validated for this purpose. Validation can be performed by extracting the factor of interest from a pooled sample, reconstituting the extract in assay buffer (included with the ELISA kit), and assaying a serial dilution of this reconstituted sample. A comparison between the optical density and relative concentrations of the serial dilution of the pooled sample extract and the standard curve of the ELISA can therefore indicate whether samples can be measured accurately in the sample media of interest. In other words, if the relative concentrations measured in the serially diluted sample extract match the expected relative concentrations from

the dilution factor, the method is validated. This process also allows for the calculation of the optimal mass of sample to be extracted so that the extract falls within the limits of the ELISA.

Hair hormone concentrations are generally expressed in *pg/mg hair* (Macbeth et al., 2010). Hormone concentrations obtained from ELISA (e.g. *pg/ml buffer*) are therefore standardized using the dilution factor of the assay buffer and extraction solvent, as well as the initial mass of hair extracted.

1.4 **Thesis Methodology and Overview**

This thesis focuses on the validation and implementation of a method for the generation of time-series physiological data using hormone concentrations measured in longitudinal sections of tail hair obtained from Serengeti wildebeest.

All wildebeest hair samples were collected from the same area of the tail, as close to the root as possible, measuring the length of any remaining stubble (to aid in the calculation of the corresponding dates of growth for each section of hair). Samples were washed and sectioned longitudinally into 8mm segments. This allowed all sections of hair from a full-length tail hair sample to fit on a single ELISA. Since inter-assay variation is higher than intra-assay variation, and differences between individuals 'baseline' values were accounted for in our statistical modelling (through the inclusion of animal ID as a random effect), by running each animal on their own ELISA we were able to effectively remove any error from our data that would be introduced through inter-assay variation. Additionally, 8mm hair sections (representing 14 days of growth) optimised the resolution of our data for this study, in which we are interested in changes in chronic hormone concentrations over ~18 months of an animal's life. Sections of hair were ground into a powder, before steroid hormones were extracted by covering them in solvent. Steroid hormones are highly soluble in lower alcohols such as methanol (Pötsch and Moeller, 1996). After overnight incubation, the steroid-rich methanol was aliquoted so that multiple assays from the same sample could be run. The methanol was removed from each aliquot via

evaporation. The dried steroid extract was reconstituted in the assay buffer (included with the ELISA kit) before the specific hormone of interest was quantified by ELISA.

Serengeti wildebeest tail hair samples were collected both from carcasses and from GPS-collared individuals who were immobilized, between 2012 and 2019. Samples taken opportunistically from carcasses were used to validate techniques (e.g. reproducibility of measures for obtaining hormone concentrations). Samples from collared individuals were used to build physiological profiles. These samples were more valuable because telemetry data obtained from GPS-collared animals was used to extract additional data on environmental variables that may account for the temporal variation of the hormone. For example, forage abundance and quality was paired with the corresponding physiological data obtained from the hair samples, allowing greater insights about the variation in hormones as a function of the environment that animal occupied at the time. Data from multiple individuals was aggregated to determine the underlying drivers behind changes in physiological state (e.g. stress, sodium-appetite), as well as whether hair hormone concentrations can be used to determine reproductive history.

This approach provides a large source of untapped information to be obtained from hair samples, enabling the creation of a physiological diary of individual animals over an 18-month period (tails hairs up to 40cm long). This procedure is akin to using tree rings or ice cores (Rysava et al., 2016) to create time-series data, and provides a novel insight into understanding the links between environment, physiology and migratory behaviours in animals.

1.5 **Thesis Organisation**

This thesis is composed of four data chapters in research article format (Chapters 2, 3, 4, and 5), as well as a general introduction (Chapter 1) and discussion (Chapter 6).

Chapter 2 focuses on the validation of the longitudinal sectioning of wildebeest tail hair samples into 8mm segments for the collection of time series

physiological data. Wildebeest tail hair growth rate is measured using the length of hair regrowth and number of days between sampling events, which is compared to previously published measures of wildebeest hair growth rate (Rysava et al., 2016). The corresponding dates of growth for each longitudinal 8mm section of hair is then calculated using the hair growth rate and each section's position along the hair sample. These dates are then used to determine the stability of cortisol in wildebeest hair.

Chapter 3 applies the method validated in Chapter 2 to produce spatio-temporal cortisol profiles for all samples collected from wildebeest previously fitted with GPS collars (n=29). Several variables are then extracted from GPS locations (e.g. forage abundance, proximity to villages, etc.) and paired with cortisol concentrations for the corresponding 8mm section of hair (n = 880). This dataset is used to determine the environmental and anthropogenic drivers of stress in wildebeest, which improves our understanding of how different factors shape this ecosystem.

Chapter 4 validates a number of steroid hormones in longitudinal sections of wildebeest tail hair for estimating the reproductive history of animals (i.e. if and when an individual has been pregnant). The data are aggregated to investigate overall seasonal trends in reproductive status in the population as a whole.

Chapter 5 validates and applies the method outlined above for the collection of time-series aldosterone data, relating to sodium appetite. Drivers of sodium appetite, as well as differences between the sodium appetite of migratory and resident wildebeest are also examined.

Chapter 6 briefly summarizes the overall results of this study and suggests improvements to the methodology. Suggestions for the implementation of this technique in other study species and ecosystems, as well as potential avenues for future research, are also outlined.

2 Inferring Individual Animal Stress over Time from Cortisol Concentration in Serially Segmented Hair

2.1 Abstract

A major issue in population ecology in animals is the lack of robust methods for the collection of physiological time-series data within individuals. Since traditional sample media (blood and saliva) only give a measure of physiological state at the time of sample collection, an individual must be repeatedly sampled in order to build a chronic profile and this is often not possible. New methods are needed which would allow longer temporal sequences of physiological data to be obtained. Establishing chronic physiological timelines would allow investigation of the underlying physiological condition of individuals that may lead to population level change.

Some substances which circulate in the blood, including drugs and endogenous steroid hormones, are stably incorporated into hair as it grows, in concentrations that reflect their circulating concentrations at that time. Since most regions of hair grow throughout the life of an individual, by sectioning hair samples along their growth axis we can create sequential, distinct samples. Measuring hormone concentrations in each of these sections therefore allows for a temporal record of hormones, covering the time the sample was grown.

To determine the validity of this technique, this chapter addresses three main questions: (1) what is the growth rate of the sampled hair, and how much does growth rate vary within and between individuals; (2) is cortisol present in the hair and does it degrade over time, and; (3) does the structure of the hair change along its growth axis (i.e. does the structure of the hair account for variation in the physiological profile).

Growth rate of wildebeest tail hair was measured using samples obtained during the deployment of GPS collars and their retrieval approximately 12 months later (n=6). This was calculated at 4.01±0.13mm/week and was relatively consistent between individuals. We were able to extract and measure cortisol in wildebeest

tail hair, as well as tail hair from a taxidermied oryx (100-year-old). Cortisol concentrations tended to increase along the growth axis of the hair when measured as *pg cortisol/mg hair*, potentially as a result of dehydration of the hair over time. However, we were able to correct for this using the change in mass/unit length to standardise the cortisol concentrations by *pg cortisol/cm hair*.

Tail hair samples are therefore a relatively straight-forward and robust media for the collection of physiological time series data and, if hair growth rate is accurately calculated, can be used to create temporal chronic stress profiles.

2.2 **Introduction**

While recent advances in geo-tagging technologies have greatly improved our ability to study the environmental context of animal movement, our understanding of how such movement may be influenced by an animals' internal physiological state is limited, particularly in wild populations (Bowlin et al., 2010, Lennox et al., 2016). The challenge of integrating physiological data into ecological studies lies in the collection of continuous metrics from wild animals with large ranges that are infrequently observed. A reliable method for the quantification of time-series physiological data over extended timeframes, such as can be obtained for population movement with GPS systems, would be hugely beneficial to build a more informed, complete picture of population movement decisions. Previous ecological studies have inferred behaviour from telemetry data such as step length (an indicator of the speed of the animal) and turn angle (change in direction from previous location) (Morales et al., 2004, Hopcraft et al., 2014), but these only give an indication of the movement, and do not provide a direct measure of the underlying physiology that may influence or drive movement decisions. More recent improvements in geotagging technologies include the addition of accelerometers to GPS collars, which can be used to infer behaviour from activity (e.g. grazing (when the head is lowered) or scanning for predators (when it is raised)) (Fehlmann et al., 2017, Giovanetti et al., 2017, Tatler et al., 2018, Wilson et al., 2018), however it is difficult to determine the

underlying physiology driving these behaviours (Leos-Barajas et al., 2017, Williams et al., 2020). Traditional methods for the direct collection of physiological data (i.e. hormone concentrations measured in blood or saliva) only give an acute 'snapshot' of an individual's condition at that moment. Therefore, multiple samples must be collected from the same individual, over extended periods of time (e.g. days-weeks apart), to obtain longitudinal physiological data. This is very time consuming and therefore costly, especially when studying wild animals that cover great distances in large herds.

Hormones regulate an animal's physiological state. Given the nature of hormones, they are most often quantified in samples of blood, its constituent parts, or immediate blood products such as saliva and urine. For example, glucocorticoids such as cortisol (the principal steroid hormone involved in the regulation of an individual's response to a stressor) are easily measured in blood and/or saliva samples (Dedovic et al., 2009, Habib et al., 2001, Möstl and Palme, 2002, Teruhisa et al., 1981). Such 'acute' hormone concentrations, however, can vary dynamically over time in response to changes in environmental variables, and as a consequence of internal processes or as the result of pulsatile release and/or circadian and circannual variations, therefore an acute measurement provides a limited view of an animal's physiology. For example, circulating concentrations of cortisol change in response to acute and chronic stressors, but also with food intake (Gibson et al., 1999), circadian rhythms (Weitzman et al., 1971), and exercise (Zelnik and Goldspink, 1981). Acute measures of stress such as those obtained from blood and saliva may also vary in response to the stress of sample collection, since this requires capture and/or immobilization of the animal (Svobodová et al., 1999, Pavlova et al., 2018). As a result, even composite values created from multiple acute measures from the same individual do not necessarily provide a reliable reflection of overall physiological state (Stalder and Kirschbaum, 2012, Sheriff et al., 2011). Hormone concentrations relating to longer periods of time can therefore give a more informative picture of the underlying physiological condition of an animal. Cortisol and/or its metabolites can also be measured in urine (Beerda et al., 1996), and faeces (Schatz and Palme, 2001). Cortisol concentrations in these samples correlate with hypothalamic–pituitary–adrenocortical (HPA) activity over the time period since the last urination or defecation, which can vary from

minutes to days, depending on the species. However, as with blood and saliva, only one value can be obtained from a single sample, and regular resampling is still required to build a temporal physiological profile for an individual over a long period of time, which can be especially difficult in wild conditions when animals move long distances or live in large groups.

Steroid hormones can also be extracted from, and therefore measured in, keratinized structures such as hair (Cirimele et al., 2000), feathers (Bortolotti et al., 2008), nails (Warnock et al., 2010), and claws (Matas et al., 2016). Although the precise method of incorporation is not fully understood, it is generally accepted that due to their small molecular size and chemical stability, unbound steroid hormones cross the cell membrane and are incorporated into the developing structure in concentrations that reflect levels in blood during the period of growth (Cone, 1996, Raul et al., 2004, Davenport et al., 2006). Since blood contains enzymes that naturally degrade hormones, most sample media must be pre-treated (e.g. blood samples spun down) and cooled/frozen, once collected, to inactivate or remove these enzymes. However, these degradation enzymes are not incorporated into keratinized structures, which also do not decompose like other body fluids and tissues, meaning no additional treatment is required to stabilise samples or the hormones present within them (Cone, 1996). Since decomposition is not an issue, these samples can be collected from an individual even if they have died, and since keratinized structures have no real nutritional value, these samples are often remaining even on carcasses who have been otherwise completely devoured. Of these keratinized tissues, hair is the most studied, with hair cortisol concentrations shown to correlate with unbound cortisol concentrations in blood, saliva, urine, and faeces, across a range of species, including: domesticated dogs and cats (Accorsi et al., 2008); sheep (Stubsjøen et al., 2015); reindeer (Carlsson et al., 2016); humans (Short et al., 2016, Papafotiou et al., 2017, Wosu et al., 2013); and cattle (Tallo-Parra et al., 2015). Compared to other sample media, hair has a number of advantages: it retains physiological information longer (months or years), can be sampled from live or dead animals and does not require frozen storage (Table 2.1).

Table 2.1 Different sample media for obtaining physiological data, the relative physiological timeframe which they relate to, and their limitations.

Sample media	Relative Time Frame	Invasive Sample Collection?	Storage Requirements
Serum/plasma/ whole blood	realtime	Yes	chilled/frozen
Saliva	realtime	requires entering oral cavity	chilled/frozen
Urine	1-2 hours	No	chilled/frozen
Faeces	10-24 hours	No	chilled/frozen
Nails/claws	weeks-years	No	none
Feathers	Duration of feather growth (previous moult)	No	none
Hair	weeks-years	No	none

Since hair grows unidirectionally, it can be sectioned along its growth axis to create distinct section of hair, each grown consecutively. If hair growth rate can be measured, the corresponding range of time it was grown over may be calculated, using the distance of the ends of each section from the root. Subsequent analysis of metabolites and hormones present within each section allows for the creation of a time series profile for an individual, from a single sample, which dates from the time when the tip of the hair sample was grown (Davenport et al., 2006). This approach has been used in studies measuring stable isotopes in hair, and relating them to seasonal variation in precipitation, and starvation patterns (Burnik Šturm et al., 2015, Rysava et al., 2016). In these studies, the measures are standardised by the ratio between two stable isotopes present in the hair. However, since it is possible that steroid hormones degrade over time, further model validation is necessary. In order to determine the reliability of this technique as a means to generate a steroidal time series, the following questions must be answered: what is the hair growth rate of the species of interest, and does the rate of growth vary significantly over time or between individuals; is cortisol present in measurable quantities that can be reliably estimated and is it stable over time (ie has a slow molecular decay

rate), and; are hormone concentrations affected by degradation of the keratin structure of the hair over time.

2.2.1 **Hair growth rate**

In order to reliably date all sections produced from each sample hair growth rate for the species of interest must be calculated, as well as both intra- and inter-individual variation in hair growth rate. Growth rate can be estimated by cutting the hair of an individual, and resampling the same area at a later date, or by measuring the length of hair in juveniles whose hair has not yet reached full length and comparing to their age. Almost all information on hair growth comes from studies on humans, which have shown that hair cycles in three distinct phases: anagen, catagen and telogen. Hair spends the vast majority of time in the growth (anagen) phase, followed by the transitional (catagen) phase when the hair follicle rests, cutting the hair off from the blood supply; and finally, the shedding (telogen) phase. Follicles in different areas of the body produce hairs of different lengths as a result of differences in relative anagen cycle length (Paus and Cotsarelis, 1999). For instance, long hairs like human head hair, spend around 2-6 years in the anagen phase and only 2-3 weeks in the catagen phase (Kligman, 1959). Coarser hairs also spend a higher percentage of the growth cycle in the anagen phase (Saitoh et al., 1970). Since longer and coarser hairs spend a higher proportion of time in the growth phase, sampling areas with longer, thicker hair maximises the proportion of hairs in a sample which are in the growth phase (and are therefore maximally aligned and easier to date). The region of the body growing the longest, thickest hairs varies from species to species; however, many animals with tails have longer, thicker hair on their tail than elsewhere. Tail hair growth rate has been measured in a variety of species, including: beef cattle (Fisher et al., 1985); African elephants (Cerling et al., 2006, Cerling et al., 2009); Przewalski's horses and domestic donkeys (Burnik Šturm et al., 2015); and even our focal species, the blue wildebeest (Rysava et al., 2016). Although tail hair growth rate varies among species, this variation decreases when comparing between individuals of the same species, especially

when the precise area of the body from which the hair is taken is identical between animals. For example, horse tail hair grows at approximately 5.8mm/week (Dunnett, 2005) while wildebeest tail hair grows at 4mm/week (Rysava et al., 2016).Intraspecific variation in hair growth rate was high in elephants, Przewalski's horses, and donkeys and therefore required overlapping isotopic profiles in subsequent hair samples to date hair sections with accuracy. Alternatively, variation in wildebeest tail hair growth rate was low, meaning growth rate alone was sufficient to calculate the date of growth of each hair section. By repeatedly resampling the same individuals from the same, precise area of the tail, we were also able to further validate previous measures of wildebeest tail hair growth rate.

2.2.2 **Presence and Stability of Cortisol in Hair**

Although a few steroid hormones have been measured in several different species, no published studies have measured any hormones in the hair of our focal species, the blue wildebeest (*Connochaetes taurinus)*. Furthermore since each section of hair will have been exposed to the environment for a different length of time (i.e. the closer to the root the less time the hair will have been exposed to the environment), it must also be determined if environmental exposure affects the relative concentration of any hormone of interest. A study on hair cortisol in rhesus macaques (Davenport et al., 2006) found that hair cut into root and tip sections had no trend along the hair when macaques were kept in a stable environment. A similar study on grizzly bears also found hair cortisol concentrations were not affected by environmental exposure (no trend between proximal, middle, and distal sections of hair) or prolonged laboratory storage (over a year) (Macbeth et al., 2010). However, Kirschbaum et al (2009) found a strong monotonic decline in cortisol concentration along the growth axis of human hair, with a 30-40% decrease from one 3cm section to the next. This trend was suggested to be a "washout effect", caused by regular washing and treating of human hair which may gradually degrade the hormone or facilitate hormone loss from the hair over time. Although such a "washout effect" would be expected to be minimal in wild animals, the possibility that species-specific corrections may still be necessary has to be considered, especially in animals

which live in environments which are more extreme e.g. reaching high temperatures and could be more likely to cause structural damage to the hair and any hormones contained within it.

2.2.3 **Structural Stability of Hair**

Hair has three constituent parts: the cuticle, cortex, and medulla. The cuticle is the outermost layer of the hair shaft, which protects the hair and anchors it to the follicle. Underneath the cuticle is the cortex, a structure of keratin fibres which make up the bulk of the hair. The medulla is the centremost region of the hair and is made up of loosely packed cells that dehydrate over time, leaving empty spaces along the central axis of the hair shaft (Talukdar et al., 1972; Harkey, 1993; Chatt and Katz, 1989; Dunnett, 2005). Figure 2.1 shows a transverse cross-section of a wildebeest tail hair, taken from near the root end of the hair, containing all three regions.

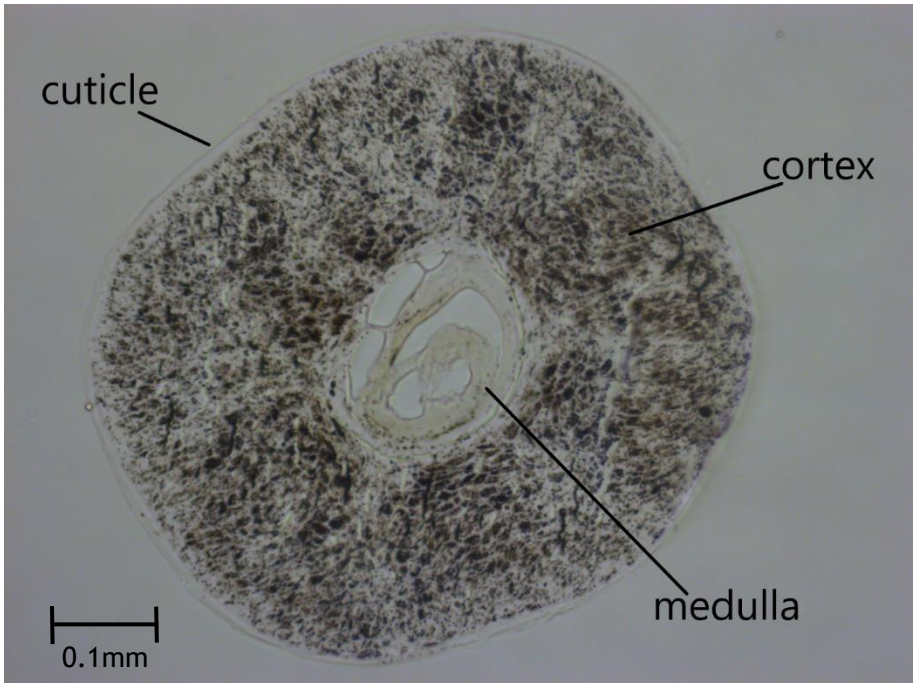


Figure 2.1 Transverse cross-section of wildebeest tail hair taken from near the root end of the hair.

The proportion of the hair relating to the medulla increases as the diameter of the hair increases, so while thinner hairs may not even contain a medulla, thicker hairs may have a noticeable decrease in mass along its length as a result

of dehydration. Therefore, while hair cortisol concentrations have previously been standardised by expression as *pg cortisol/mg hair* (e.g. Davenport et al., 2006; Sauvé et al., 2007) a further correction to account for this dehydration may be required, in thicker hairs, when comparing between sections of hair cut from different positions along the growth axis of the sample. Measuring the mass of sections of hair at different positions along a sample may allow for a method of correcting hair cortisol concentrations by unit length of hair, to account for this dehydration.

2.2.4 **Objectives**

The objectives of this research are to determine:

- the growth rate of wildebeest tail hair;
- if cortisol is present and stable in wildebeest tail hair
- if degradation of the hair along its growth axis affects cortisol concentrations, and whether it is possible to correct for this.

2.3 **Materials & Methods**

2.3.1 **Study Area and Animals**

Hair samples were collected from 12 free-ranging migratory wildebeest occupying the Serengeti Mara ecosystem, East Africa (1°15’ to 3°30’S, 34° to 36°E), during the period June 2013 - December 2018. Samples were collected evenly throughout the year, in order to avoid correlation between season and position along the hair’s growth axis. Eight of the samples were collected from GPS-collared wildebeest; 2 during deployment of a collar and 6 from animals that were recaptured approximately 12 months later. Animals were immobilized by veterinarians from the Tanzania Wildlife Research Institute (TAWIRI) using an injectable dart containing 4-6 mg of etorphine hydrochloride and 80–100 mg of azaperone tartarate, delivered remotely by a rifle from a stationary vehicle near the animal. Veterinarians followed the handling and care protocols established

by TAWIRI. All animals were in a healthy condition at the time of initial collar deployment. All hair samples were collected from the same region of the tail and tied into bundles in order to keep the hair aligned at the root. Superficial contaminants (e.g. mud, faeces, grass) were removed by washing samples with water in the field. Samples were then dried and stored at room temperature. Additional tail hair samples (n=4) were collected from wildebeest carcasses showing no signs of chronic stress, determined through body score and bone marrow density and colour. All study animals were reproductively active females between 3-10 years old.

2.3.2 **Hair Growth Rate**

During routine immobilization of wildebeest (n=6) for deployment of a GPS collar, all hairs from an approx. 3cm x 3cm region of the tail were cut, as close to the root as possible, with hairdressing scissors. Six hair roots were plucked from this region and their mean length recorded, to determine the length of hair still attached to the animal after clipping (i.e. the remaining stubble). Approximately 12 months later, wildebeest were re-immobilized and a hair sample taken from the same area of the tail, using the method outlined above. Mean length of the regrown hair was determined for every individual by measuring the length of the twenty longest hairs. Hair growth rate was estimated by dividing the mean length of regrown hair by the total number of days between collar deployment and recapture for that individual. Mean length of regrown hair was corrected slightly after accounting for the stubble remaining after sample collection during both the initial and secondary hair clippings. Intra-individual variation in growth rate was assessed by comparison of the lengths of individual hairs within a sample; inter-individual variation in growth rate was assessed by comparison of growth rates between six individuals.

2.3.3 **Sample Preparation for laboratory analysis**

Hair samples were washed twice with a 2:1 chloroform:methanol solution in a heated water bath for 10 minutes and then rinsed with MilliQ UltraPur water, in order to allow for future isotopic analysis (Burnik Šturm et al., 2015). Samples were dried overnight in a fume hood. Bundles of hair were aligned precisely at the root-end and held in place using thread and haemostats. Hair samples were carefully inspected along their length, and any damaged hair removed or trimmed to a length where no visible damage could be identified (i.e. cutting off the most distal ends of all hairs, where it changes from thick black hair to thin light-brown hair). Full length samples taken from carcasses were cut to a maximum length of 32 cm, which represents approximately 18 months of growth (Rysava et al., 2016). Sections of hair were cut to 8mm, so that all sections of hair from a full-length tail hair sample could fit on a single ELISA plate. Since inter-assay variation is higher than intra-assay variation, and differences between individuals 'baseline' values were accounted for in our statistical modelling (through the inclusion of animal ID as a random effect), by running each animal on their own ELISA we were able to effectively remove any error from our data that would be introduced through inter-assay variation. Additionally, 8mm hair sections (representing 14 days of growth) was an optimal resolution for this study, in which we are interested in changes in chronic hormone concentrations over ~18 months of an animal's life. Aligned hair was cut into 8mm sections using hairdressing scissors, recording each section's position along the hair. Sections were placed in 2ml metal lysing tubes, containing ¼ inch x ½ inch grinding cylinders (MP Biomedicals, Cambridge, UK), frozen in liquid nitrogen and powdered using a Retsch MM400 mixer mill (Retsch, Hope Valley, UK) set to 30Hz for 3 minutes to optimise extraction efficiency by increasing the relative surface area of the sample and break up the cuticle to expose the potentially hormone-rich medulla and cortex of the hair.

2.3.4 **Validation of Cortisol ELISA Kit**

A test for parallelism was carried out to validate a commercially available cortisol Enzyme-Linked ImmunoSorbent Assay (ELISA) for measuring cortisol in

wildebeest hair sample extract. This involved assaying a serial dilution produced from 100mg of a pooled sample of wildebeest tail hair (extracted using the method outlined below). The percentage bound for all dilutions which fell between the Ed_{20} and ED_{80} values for the assay were then plotted against the log of their relative concentration, along with the standards for the assay. Parallelism between the line of best fit for the standard curve and samples indicates that the relative concentrations of the serial dilution are being measured accurately using the assay. This analysis also allowed us to determine the optimal mass of hair and dilution of the extract to accurately estimate cortisol using the kit.

2.3.5 **Extraction and Quantification of Cortisol**

For each sample, 35-40mg of powdered hair was weighed in a borosilicate glass vial and 2ml of 100% HPLC Grade Methanol added. Vials were capped and placed into a heated orbital shaker (Stuart orbital incubator S150 (Cole-Parmer, Staffordshire, UK)) at 52°C, 200rpm, for 16 hours. Vials were weighed before and after the extraction process, to allow subsequent correction for any evaporation that may occur during the extraction process. In order to allow for multiple assays three 550ul aliquots of solvent were dried down (Savant SC210A speedvac concentrator, Thermo Fisher Scientific, Renfrew, UK, medium heat for three hours) in separate borosilicate glass tubes. When required for assay, dried samples were resuspended in 150ul of assay buffer, and cortisol concentrations were determined using the Cortisol Express ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. The manufacturer reports that the cross-reactivity of the antibody is: cortisol (100%), dexamethasone (15%), prednisolone (4.01%), cortexolone (1.58%), 11-deoxycorticosterone (0.23%), 17-hydroxyprogesterone (0.23%) cortisol glucuronide (0.15%), corticosterone (0.14%), cortisone (0.13%), androstenedione (<0.01%), enterolactone (<0.01%), estrone (<0.01%), pregnenolone (<0.01%), 17-hydroxypregnenolone (<0.01%), testosterone (<0.01%). All samples from the same individual were measured in duplicate in the same assay. Assay Zap

software (Biosoft, Cambridge, UK) was used to interpolate cortisol concentration of unknowns from the standard curve. Across the 50 cortisol assays, intra- and inter-assay coefficients of variation averaged 2.95% and 5.15%, respectively. Cortisol concentrations were initially standardized using the dilution factor and mass of hair used in the extraction, and initially expressed in ng/gram of hair (Macbeth, Cattet, Stenhouse, Gibeau, & Janz, 2010).

2.3.6 **Hair Degradation**

To account for any degradation of the hair over time, the change in mass along the hair was measured. Individual hairs were taken from a sample and sectioned with a scalpel (to increase precision) into 8mm segments. The tips of the hair sample where hair began to become visibly damaged (e.g. split ends) were discarded. Longer hair samples were sectioned to a maximum length of 32cm (40 sections). This was repeated for a total of 20 individual hairs per sample and a mass/unit length was calculated for each position along the growth axis by dividing the mass of the 20 sections by their length (20*8mm sections = 16cm). This was repeated with tail hair samples taken from 5 different wildebeest (n=180), in order to calculate variation between samples. This allowed us to calculate hair degradation over time, as well as intra- and inter-individual variation in hair degradation. If this change in mass was as a result of dehydration of the medulla, then the hormones obtained within the hair should remain intact, suggesting that the concentration of *cortisol / mg* would increase over time simply due to the loss of water. Therefore, measuring cortisol by unit length (rather than by unit mass) should account for this bias and give a more accurate representation of the underlying physiology of the individual. Based on this measure of hair degradation cortisol measurements were corrected from *pg cortisol/mg hair* (the standard units used in hair hormone analysis) into *pg cortisol/cm hair*.

2.3.7 **Cortisol Stability**

To establish the stability of cortisol in hair over time we performed two tests. First, hair cortisol concentrations were compared (paired sample t-test) between two tail hair samples collected from the same individual two months apart, such that the second sample contained hair equivalent to the root of the first sample, but which had been exposed to the environment for an additional two months. Both tail hair samples were sectioned so that the date of growth was aligned between samples. If hair cortisol is unstable over time because of heat, UV radiation or any other environmental exposure then we would expect cortisol concentrations in the sample exposed to the environment for a longer period to differ across the length of the hair. If there is no difference between the corresponding date-aligned sections of the two samples, this verifies the hair growth rate (used to align sections between samples) as well as cortisol stability.

The second test of cortisol stability examined if cortisol concentration was related to section number along the length of hair samples. This test utilised samples that were collected at times spread evenly throughout the year, so that there was no overall correlation between the position of a section along its sample and any variation caused by seasonal-environmental factors. For example, the first section of hair from two different animals sampled 6 months apart would be from opposite seasons. If cortisol is unstable in the hair (i.e. it degrades over time) then the position of the section within the sample should be a significant explanatory variable when predicting cortisol. However, if cortisol in the hair is stable, then position along the sample should not account for any variation between subsequent sections.

Finally, to determine if cortisol remained in hair for very long periods of time, we were able to acquire an oryx tail hair sample from the Hunterian Zoology Museum at The University of Glasgow, which was taxidermied approximately 100 years ago. Initially, two full length tail hairs were used to produce a pooled sample to test for parallelism between sample dilutions and the standard curve, to assess if cortisol could still be detected and accurately measured. A further 10 full-length hairs were sectioned into 3cm segments to create a cortisol profile and to calculate a mean cortisol concentration for the sample over time.

2.3.8 Statistical Analysis

All statistical analyses were performed using R version 3.5.1 (R Core Team, 2018).

2.3.8.1 Hair growth rate

Levene's test of equality of variances was performed using package “car” (Fox and Weisberg, 2019), to determine if variance in growth rate across hairs differed significantly between samples from different individuals. Since no significant difference in variance between samples was found, a one-way ANOVA was performed on hair growth rates to determine if there was significant intra- and/or inter-individual variation in growth rate, or if a single mean hair growth rate could be applied to all samples.

2.3.8.2 Hair degradation

Mass/unit length was log transformed, to more closely fit a normal distribution and a general linear model fitted to determine the effects of position along the growth axis of the hair (and therefore time exposed to the environment) on relative hair mass (mg/cm). Animal ID was also included in the model as a fixed effect and as an interaction with position along the hair, to determine if there were significant differences between individuals, and whether individual-specific corrections would be necessary.

2.3.8.3 Cortisol Stability

The effect of environmental exposure on cortisol (standardised by both *pg cortisol/g hair* and *pg cortisol/cm hair*) was determined by fitting a linear mixed effects model using package lme4 (Bates et al., 2014), with animal ID included

as a random intercept. A linear mixed effects model was also fitted to determine if age of the sample had any effect on cortisol, with animal ID included as a random intercept.

2.3.8.4 Reproducibility of technique

Through this study we were able to collect two samples from the same individual two months apart, as it died two months after initial sampling and being fitted with a collar. This allowed us to perform a paired sample t-test to determine if cortisol concentrations between hair samples collected from the same individual two months apart differed significantly.

2.4 Results

2.4.1 Hair Growth Rate

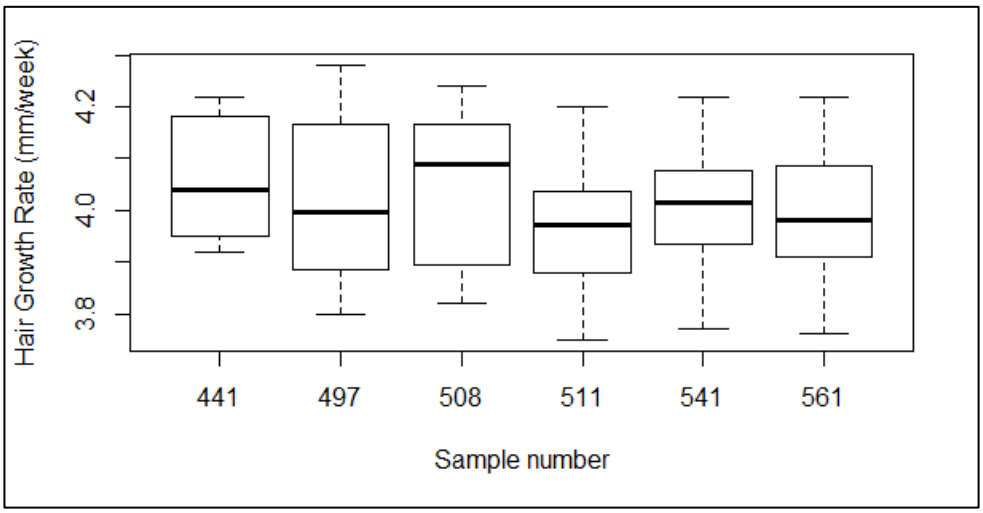


Figure 2.2 Boxplot of calculated growth rates for 6 different wildebeest tail hair samples suggest there is no significant difference in the growth rate of hairs between individuals. Data points are growth rate measurements calculated from 20 individual strands of regrown hair per animal. Boxes and whiskers represent quartiles while bold line represents median.

The results from Levene's test of equality of variances showed no significant difference in variance between hair growth rates across individual wildebeest (p

= 0.344) (Fig. 2.2). This allowed us to perform a one way ANOVA of hair growth rates, which showed no significant difference between samples ($F_{(5, 114)} = 1.243$, $p = 0.294$).

Mean tail hair growth rate was calculated at 4.01±0.13mm/week. This growth rate matched previous measurements of hair growth rate in Serengeti wildebeest (Rysava et al., 2016) and was therefore used to calculate dates of growth for all sections of hair.

2.4.2 Hair Degradation

Figure 2.3 shows the change in relative mass along the length of each hair sample. Position along the length of a hair sample is a significant predictor of relative mass of hair and explained most of the variation, with hair halving in mass over a change in 350mm hair length ($n=180$; $p < 2x10^{-16}$; $R^2 = 77.5\%$) (Table 2.2). Animal identification was not a significant predictor. Since most of the variation in mass along hair samples can be predicted by position, a single correction for the change in mass along the length of a sample can be applied to all samples.

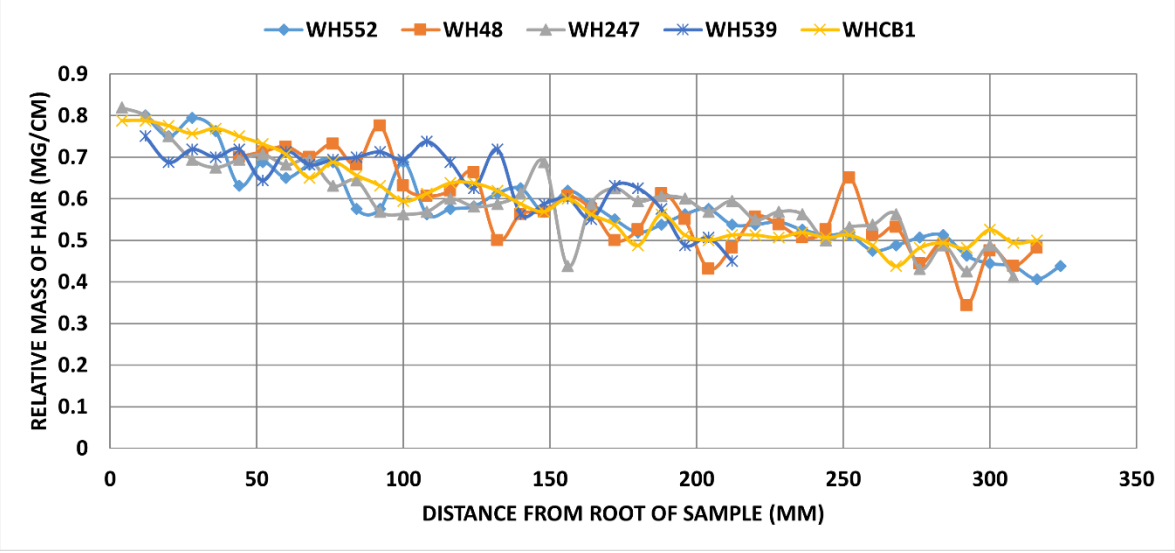


Figure 2.3 Change in relative mass of wildebeest tail hair along its growth axis suggests there is a constant rate of hair degradation over time with little variation between individuals.

Table 2.2 Output from general linear model of the effect of position along the length of the sample and animal ID on relative mass of tail hair. Results show distance from root is a significant predictor, and that there is no significant difference between individuals.

	D.F.	F value	p-value
Distance from root	1	597.754	$<2x10^{-16}$
Animal ID	4	0.321	0.864
Distance from root * Animal ID (interaction)	4	0.560	0.692

2.4.3 Validation of ELISA Kit for Wildebeest Tail Hair Samples

The serial dilution from a pooled wildebeest hair extract exhibited parallelism with the standard curve (Fig. 2.4), as confirmed by regression analysis (r^2 = 0.933, $p < 0.01$). This indicates that wildebeest hair cortisol concentrations can be measured accurately, across a range of concentrations, using this assay.

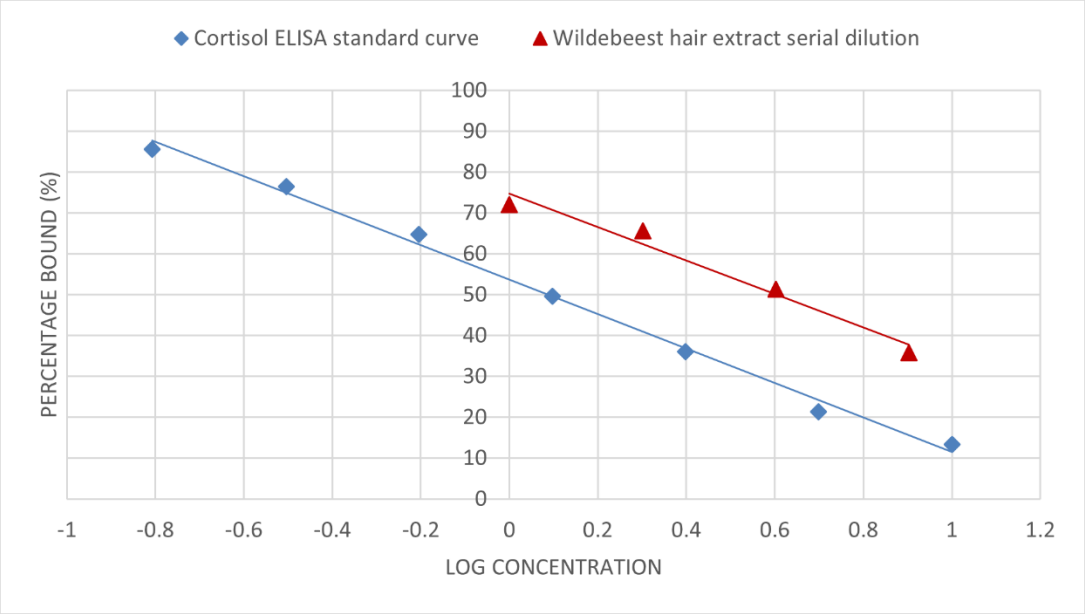


Figure 2.4 Plot of the log concentration of cortisol against percentage bound. A comparison of the serial dilution of wildebeest tail hair extract and the standard curve

confirms parallelism and suggests the cortisol concentration in the tail hair falls within the quantitative range of the assay and the concentration can be estimated with accuracy.

2.4.4 Cortisol Stability along Hair Axis

Hair cortisol concentrations were initially expressed as *pg cortisol/mg hair*, but this resulted in a small but significant increase along the growth axis of the hair (Mixed effects model results; β = 0.004 ± 6.153x10^{-4}; t = 7.201; p = 5.860x10^{-13}). This trend was expected since wildebeest occupy a subtropical, dry environment and it is known that the medulla dehydrates over time, lowering the relative mass of the hair, and therefore increasing the relative proportion of cortisol within the hair. When expressed as *cortisol/cm hair* environmental exposure time was no longer a significant predictor of cortisol (Fig. 2.5) (β = 0.0001 ± 0.0003; t = 0.317; p = 0.736). We also tested whether time between hair growth and steroid extraction had any effect on cortisol; no effect was found (β = 0.0001 ± 0.0003; t = 0.416; p = 0.664).

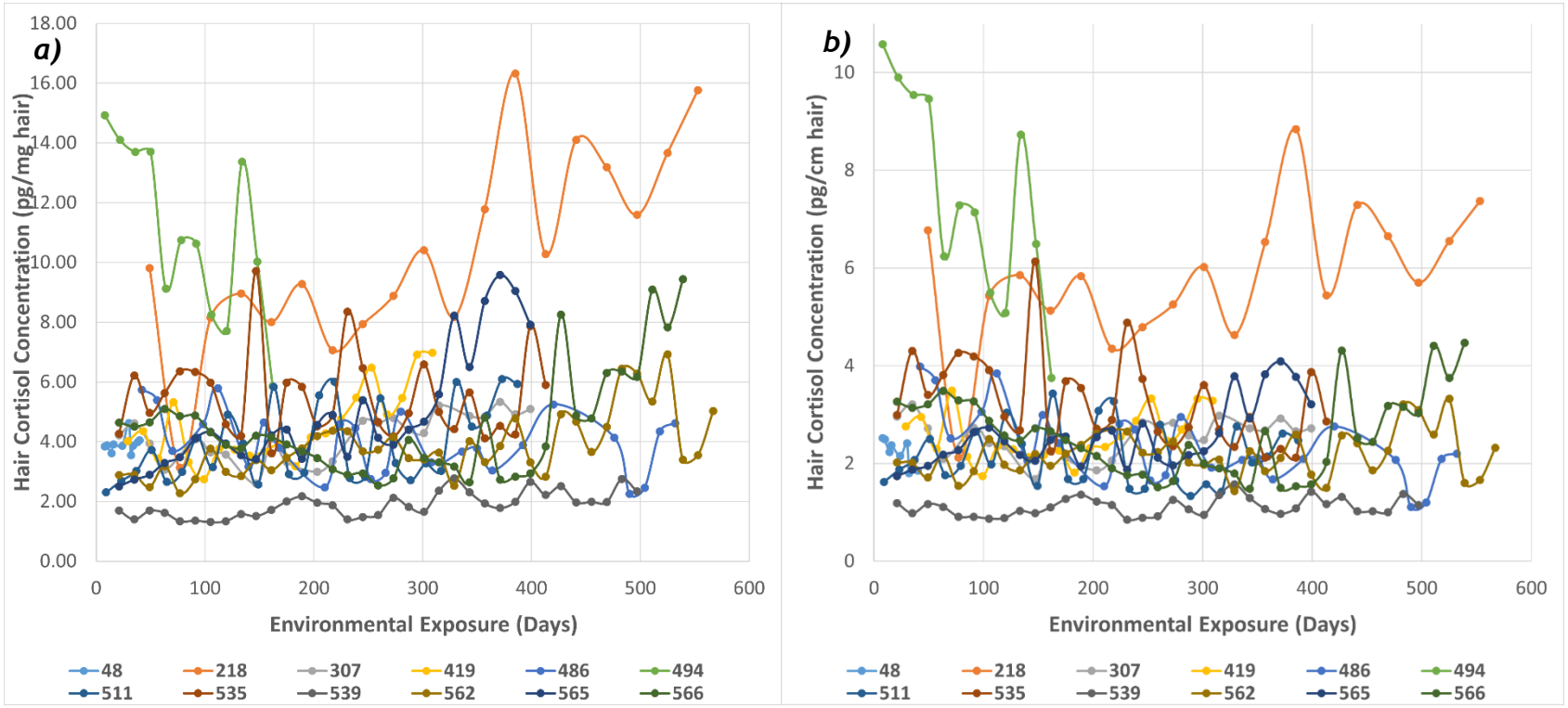


Figure 2.3 Hair cortisol concentrations measured in sequential 8mm sections of hair sampled from 12 different individuals, measured in: a) *pg cortisol/mg hair (standard units) and b) pg cortisol/cm hair*, illustrating that hair cortisol concentration is unrelated to time spent in the environment, when standardised by unit length.

When hair cortisol concentrations, measured in *pg cortisol/cm hair*, were compared between hair samples collected two months apart (Fig. 2.6) we found no significant difference between pairs of date-aligned sections (t = 1.62, df = 11, P = 0.13).

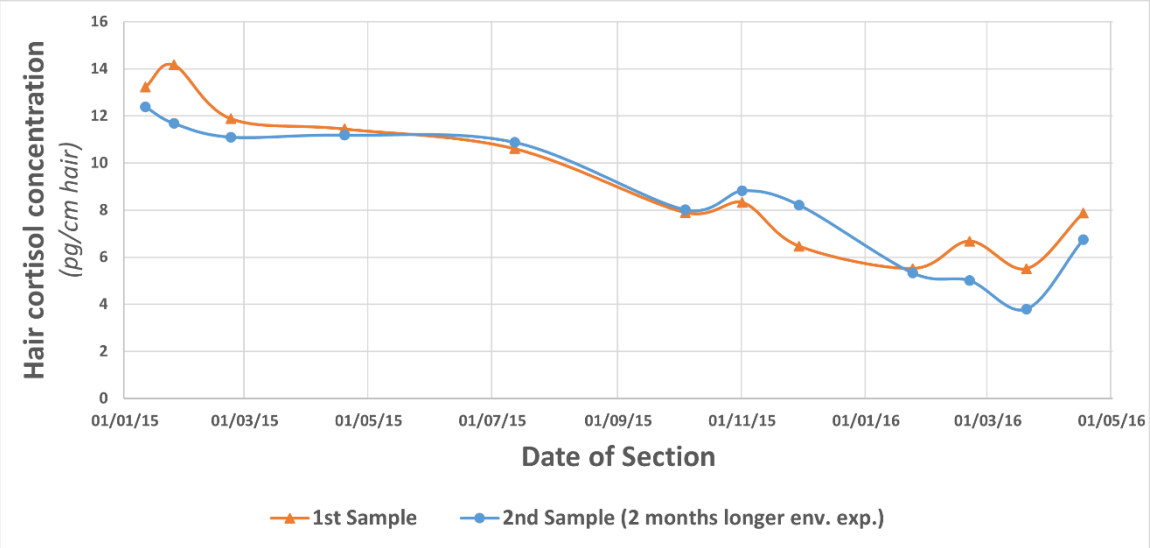


Figure 2.4 Hair cortisol concentrations of 8mm sections from two wildebeest tail hair samples collected from the same individual two months apart, aligned by date of growth suggests there is no significant effect of environmental exposure on cortisol stability in the hair.

2.4.5 Measuring Cortisol in Museum Hair Samples

The extract of the ~100-year-old oryx tail hair exhibited parallelism in the ELISA. It was possible to extract and measure cortisol from the tail hair of an oryx and generate a cortisol profile (Fig. 2.7). Cortisol varied along the length of the sample, suggesting a temporal signal. Mean hair cortisol concentration for the oryx were similar to those found in our wildebeest samples (*Oryx* = 4.63 ± 1.16 pg/mg; *Wildebeest* = 4.73 ± 2.66).

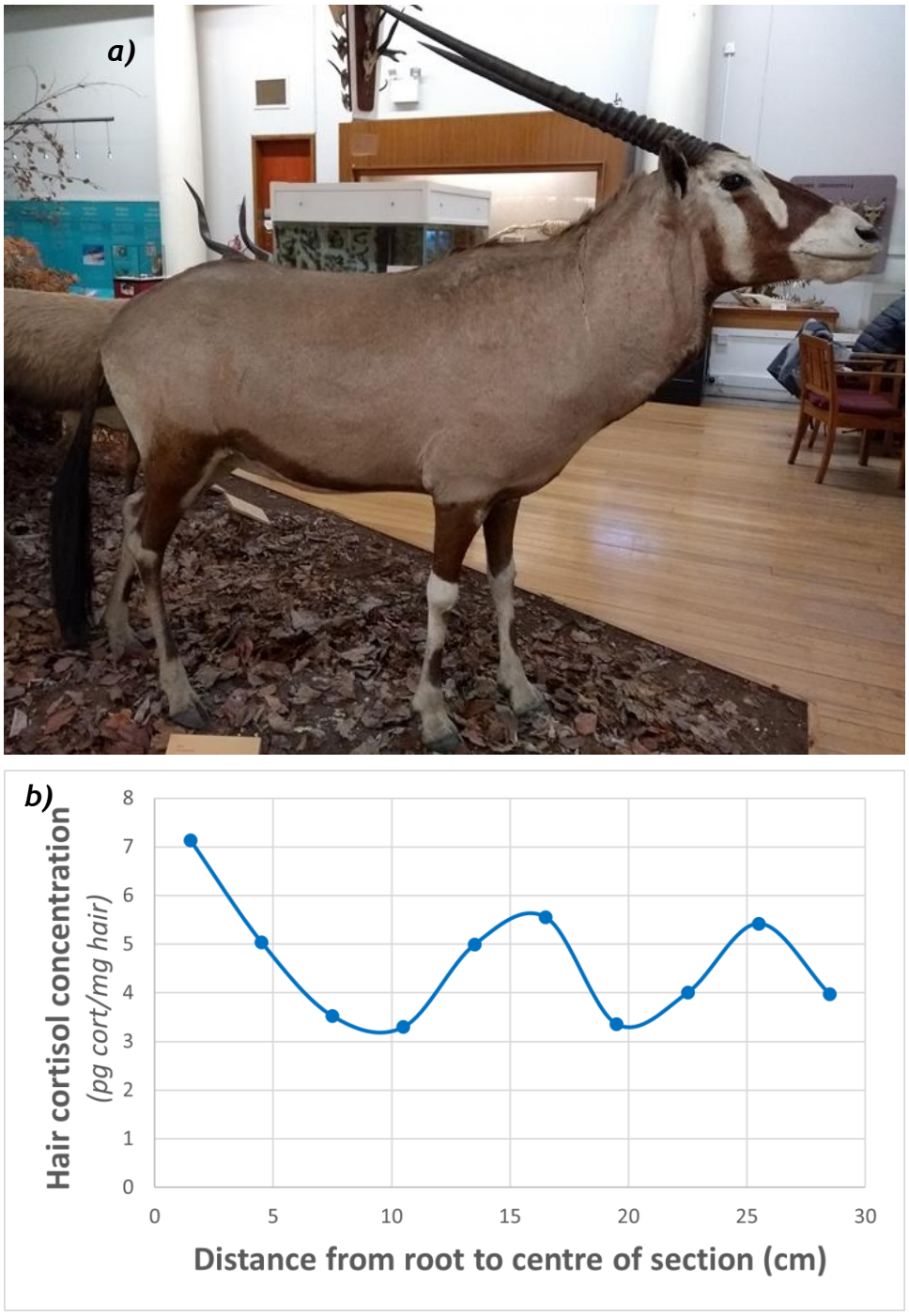


Figure 2.5 **a) Image of the ~100yr old taxidermied oryx that was sampled and b) cortisol concentrations for sequentially sectioned 3cm segments of tail hair, produced from this sample. The data illustrate that cortisol concentrations are similar to those measured in extant wildebeest and there is variation over time, suggesting cortisol in hair is both stable and can provide reliable profiles from historic samples.**

2.4.6 **Minimum Mass of Sample Required**

This study was able to detect and measure cortisol in hair samples taken from a variety of species. Through optimisation of the cortisol ELISA kit for each species, we were able to calculate the minimum mass of sample required, using our method (outlined in section 2.3.3). Minimum mass of milled hair required for each species are: 2-3mg for dogs, 8-10mg for sheep and humans; 10-12mg for wildebeest and oryx; 12-14mg for zebra; and 20-25mg for rabbit.

2.5 **Discussion**

The two most important findings from this work are that (i) wildebeest tail hairs grow at a constant and consistent rate over time, and (ii) cortisol is present and stable over the length of the hair. This enables us to create a data time-series for an animal and effectively recreate its physiological profile over an 18-month period. Although other studies have produced hormone profiles over time from point data, by consecutively resampling from individuals, our sampling technique can be used to obtain temporal physiological data in wild animals, where regular resampling is not possible. The ability to measure changes in cortisol concentrations that occur in wild animals as they move through changing environments is an entirely novel contribution to ecological research, providing information on biotic and abiotic drivers of stress, as well as bottlenecks of stress in animal movement studies.

2.5.1 **Growth and the estimation of time**

Hair growth rate was found to be consistent between individuals and was calculated in this study at 4mm/week. While our sample size was relatively small, this growth rate matched previous measurements of hair growth rate in Serengeti wildebeest (Rysava et al., 2016), and was similar to tail hair growth

rate measured in cattle (4.06+-0.07mm/week) (Fisher et al., 1985) and thus can be used to date each section of hair. This gives us a methodology which we can implement to calculate the corresponding date of growth for each section of wildebeest tail hair, without the need to resample individuals. This shows promise for the application of this technique in other studies, since, if growth rate is constant for the study species (like in the case of the wildebeest), hair growth rate only needs to be calculated once, not for every individual, greatly reducing the amount of work required to obtain this physiological data.

2.5.2 **Stability of cortisol over time**

Our hair samples showed a significant decrease in mass along their length, which appears to be a result of progressive dehydration of the hair over time spent in the environment. However, our results indicate that hair cortisol concentrations, when measured in *pg cortisol/cm hair* are not affected by environmental exposure. Since position along the growth axis of the hair has no effect on cortisol concentration when standardised by *cortisol/cm hair*, cortisol must be stable in the hair. The increase in concentration seen when standardising hair cortisol by unit mass of hair is therefore due to dehydration of the hair and can be corrected using the change in mass of the hair as it spends longer in the environment. As we have demonstrated that cortisol itself in hair is stable these results suggest that accuracy of physiological characterisation may be increased when cortisol measures are expressed as *pg cortisol/cm hair*. This correction appears to be animal or ecosystem specific as macaque and grizzly bear studies show no trend in cortisol along the length of hair when expressed as *pg cortisol/mg hair* (Davenport et al., 2006; MacBeth et al., 2010). However, this may be due to the shorter length of hair in these species relating to a shorter period of time spent in their respective environments, as well as these animals inhabiting an environment with a relatively cool climate. The Serengeti-Mara ecosystem which the wildebeest inhabit can regularly exceed temperatures of 40ºC, therefore cortisol should be stable in hair exposed to temperatures at least as high as this. The samples included in this study were collected over

multiple years, and as such we were also able to model the effects of the age of the sample on cortisol. The results indicated that age of sample (measured as time between when the hair was grown and cortisol extracted and measured) had no effect on cortisol, suggesting that cortisol remains stable in wildebeest hair samples for at least several years after collection.

To further test cortisol stability in hair, we also obtained a tail hair sample from an approximately 100-year-old museum specimen of an oryx (*Oryx beisa*). We were able to quantify cortisol, which varied along the length of the tail hair, and was in similar concentrations to those found in wildebeest hair (oryx: 3.3 - 7.1ng/g hair; wildebeest: 1.5 - 31.7ng/g hair) (Buchanan, unpublished data). Although taxidermy, at the time our sample was produced, involved the use of harsh chemicals such as arsenic, this does not appear to have stripped cortisol from the hair. Previous studies have successfully measured corticosteroid concentrations in: feathers obtained from a variety of bird species, ranging from 35-70 years old (Bortolotti et al., 2009); 100 year old polar bear fur (Bechshøft et al., 2012); and even in human hair obtained from archaeological sites, dating from AD550–1000 (Webb et al., 2010).

2.5.3 **Method transferability**

The results of our test for parallelism show that cortisol is present, and can be accurately measured, in the extracts from wildebeest tail hair samples using the *Cayman Cortisol Express ELISA kit*. Since steroid hormones are not species-specific on a molecular level (e.g. the molecular structure of cortisol is identical, regardless of the species), if hair growth and degradation rates can be measured, this technique should be directly transferable to other species.

Since hair samples can be collected from immobilized animals with relative ease and with no detrimental effect to the animal, any study which involves handling an animal (e.g. fitting a GPS collar) could benefit from collecting hair samples using the method outlined above. Even if the project does not have time/resources to prepare and extract hormones from these samples in the

immediate future, our results indicate that the stability and lack of any refrigerated storage requirement for these samples allows physiological data to be obtained years later. This is also true for any hair samples that can be taken from carcasses, opportunistically.

Hair growth rates vary across different regions of the body, therefore we recommend always sampling from the same region, to ensure consistency. We also recommend that hair is taken from the area of the body growing the longest hair on the animal, since longer hair spends a longer proportion of its time in the growth phase, maximising alignment of hairs within a sample.

Future studies could be improved by resampling the study animals before the region of hair originally sampled has grown back entirely, allowing samples to be used to determine hair growth rate (and therefore calculate the corresponding dates for each section of hair). This can be done by collecting two hair samples at the second sample collection; a regrowth sample, taken from the same region as the original sample (to be used to calculate hair growth rate); and a full-length hair sample, taken from immediately adjacent to the initial sample collection region. The regrowth sample can be used to calculate hair growth rate by dividing the length of the sample (in mm) by the number of days between sampling events, to get a mean hair growth rate, in *mm/day*. This mean hair growth rate can then be validated by performing sequential analysis of the initial sample and the second full-length sample collected from the same individual, to produce a hormone profile for each sample, using the date of sample collection to temporally 'anchor' each sample at the root. Since the growth of these samples partially overlaps, their profiles should also overlap. Therefore, a comparison can be made between the overlapping regions of the hormone profiles, to determine how well the profiles align, and how reliable our method is for calculating the date of growth for each section of hair. Additionally these samples can also be used to determine the stability of the hormone of interest over time (since sections from the second full-length sample will have spent more time in the environment than their date-aligned sections from the original sample), as well as reproducibility of the technique. If the hormone of interest does not appear to be stable over time (i.e. the hormone concentration of date-aligned sections of hair differ) then it may be possible to correct for this by

including time spent in the environment as an additional explanatory variable in statistical models.

2.5.4 **Future work**

Wildebeest live in a relatively extreme environment, in terms of high temperatures and frequent periods of starvation (Mduma et al., 1999), which could affect hormone stability and hair growth, respectively. However, our results illustrate this is not the case, suggesting this method could be applicable to other species, environments and ecosystems. Additionally, as more studies implement this technique, more information will be available on hair growth rates for different species, making implementation of the method easier.

These approaches are not only useful for collecting vital physiological information from free-living wild animals but can also be used to retroactively inform physiology from historic specimens.

We validated the method outlined in this study by resampling individuals within the time it takes for the hair to grow to its full length, collecting hair from a new area immediately adjacent to the area that the initial hair sample was cut from. The root sections of the initial sample and tip sections of the second samples would therefore overlap in terms of date of growth. By sectioning these samples and comparing between hormone concentrations in each of these overlapping regions, growth rate and stability of the molecules of interest can be measured more directly.

The use of this technique in collecting physiological data from museum hair samples (as demonstrated with the cortisol profile created from our 100 year old oryx hair sample) shows a lot of promise for unearthing historic physiological information retroactively, making natural history museums a vast, untapped source of physiological data previously believed to be impossible to obtain. Additionally, the comparison of contemporary hair samples to hair obtained from historic samples of the same species would also allow for the study of populations over much larger time-frames (e.g. hundreds of years), to determine

the physiological response to events occurring on a greater temporal scale, such as long-term anthropogenic disturbances and climate change.

2.6 Conclusions

We have shown hair growth rate to be constant over time and between individuals, in our study species, the blue wildebeest (*Connochaetes taurinus*). We have also developed a method for obtaining chronic cortisol profiles from tail hair samples. Although dehydration of the medulla of the hair over time caused an increase in hair cortisol concentration relative to mass of hair, we were able to correct for this by measuring the change in mass along the hair growth axis. Since this method was successful with a species which inhabit an environment as arid as the Serengeti, this method shows promise in other species; especially species living in less arid ecosystems, which may not require a correction for dehydration of the hair. Since the molecular structure of steroid hormones are all very similar, this technique may be directly transferable to measure other steroid hormones. This technique has the potential to drastically reduce the ease with which long term physiological data can be obtained, similar to the effect that recent advances in GPS technology have had on the collection of location data. By assaying hair from animals previously fitted with GPS-collars and pairing cortisol data with environmental data extracted from locations (e.g. food availability, quality, proximity to humans), the roles of each of these factors on chronic stress can also be determined; a valuable avenue of study for eco-physiology research.

3 Determinants of Stress in GPS-collared Wildebeest

3.1 Abstract

The environmental context in which animals live, such as their exposure to predation or availability of food, can strongly shape their physiological state, and their subsequent behaviour. However, longitudinal measures of physiological state within individual animals are rarely studied, particularly in wild populations, because they are difficult to collect.

Hair has been shown to contain the stress hormone cortisol in concentrations that reflect the levels of stress experienced by the animal over time. When collected from geo-tagged animals, these cortisol signatures from hair can be linked spatially to the set of environmental and anthropogenic conditions experienced by an animal over time.

This chapter implements the method described and validated in Chapter 2 for the longitudinal characterisation of cortisol in tail hair over its length, using 29 hair samples collected from GPS-collared wildebeest (*Connochaetes taurinus*) in the Serengeti-Mara ecosystem, sectioned longitudinally into 8mm segments (n = 880). Cortisol data is then paired with a variety of environmental and anthropogenic factors extracted from telemetry data to determine drivers of stress. Differences in the stress response between resident and migrant wildebeest, as well as samples taken from living animals and carcasses, were also examined.

Hair cortisol concentrations were found to be negatively related with *NDVI* (a remotely sensed metric of vegetative productivity) and *proximity to villages*. Cortisol was generally higher in samples taken from animals found dead than those that were live captured, suggesting chronic stress impacts negatively on survival. Interestingly, the effect of NDVI on cortisol differed between animals found dead and those captured. These results suggest that chronic stress increases when wildebeest are in locations where food availability is low and where villages are nearby.

The ability to link spatial information with an individual animal's physiology over long time periods opens exciting new avenues for research relevant to movement ecology, conservation management and animal welfare across a wide range of taxa and settings.

3.2 Introduction

The movement decisions of animals in response to resources and risks such as food, shelter, predation and competition are the foundation of studies that address animal distribution and abundance (Hopcraft et al., 2005, Holdo et al., 2009, Middleton et al., 2013). However, an overlooked mechanistic link between the environmental context and the behavioural response of the animal is how an animal's behaviour may be modified by its internal state, such as the degree of hunger or stress. For example, animals may forego access to high quality resource patches either because of a perceived threat or because they are satiated (Johnson et al., 2013). While recent advances in geo-tagging technologies have greatly improved our ability to study the environmental context of animal movement, our understanding of animals' internal physiological state is less well known (Hooten et al., 2017). The challenge in integrating physiological data into ecological studies lies in the collection of continuous physiological metrics, especially for wild animals with large ranges that are infrequently observed and where repeated sample collection is not possible.

The underlying physiological status of an animal can be determined by measuring the relative concentrations of specific hormones within the body, which can in turn be indicative of behaviour (Nelson, 2005). For instance, glucocorticoid hormones such as cortisol, the hormone responsible for regulating an individual's stress response, can routinely be measured in blood, saliva, urine and faeces (Habib et al., 2001, Möstl and Palme, 2002, Dedovic et al., 2009). However, these samples only give a snapshot of the animals' physiological stress levels in the preceding minutes to hours (dependent on sample media) (Sheriff et al.,

2011). In the case of blood and saliva, the cortisol concentrations can vary due to the pulsatile nature of its release and can also be affected by the sample collection procedure itself which often requires capture and immobilization of the animal (Davenport et al., 2006). Furthermore, multiple samples must be collected from the same individual in order to generate a temporal physiological profile, which is very expensive, for many wild populations of animals.

Due to the small molecular size and stability of steroid hormones, these molecules are integrated directly into hair as it grows (Cone, 1996). Therefore, an alternative approach for characterizing the chronic physiological state of an animal is to measure cortisol in hair. Hair cortisol concentrations have been shown to correlate with cortisol concentrations in blood, saliva, urine, and faeces across a range of species, including primates (Davenport et al., 2006); domesticated dogs and cats (Accorsi et al., 2008); sheep (Stubsjøen et al., 2015); reindeer (Carlsson et al., 2016); humans (Papafotiou et al., 2017, Wosu et al., 2013); and cattle (Tallo-Parra et al., 2015). Therefore, by uniformly sectioning hair samples along the length of the growth axis, it should be possible to generate a sequential record of an individual animal's physiological state from a single sample. This principle has been used to obtain sequential stable isotope data (Burnik Šturm et al., 2015, Kaczensky et al., 2017, Rysava et al., 2016), as well as in a limited number of studies measuring cortisol in hair (Dettenborn et al., 2012, Davenport et al., 2006, Macbeth et al., 2010). Producing a time-series of physiological information requires stability of the relevant molecules in the hair over time, as well as an accurate measure of hair growth rate.

An animal's physiological response to stress is mediated by the hypothalamic-pituitary-adrenal (HPA) axis. Detection of a potential threat to homeostasis (a stressor) triggers increased activity in the HPA axis, causing an increase in circulating glucocorticoid concentrations (Habib et al., 2001, Tsigos and Chrousos, 2002). Known stressors include life history events, natural environmental factors, and anthropogenic factors. For example, a peak in maternal and foetal cortisol has been shown to precede and initiate parturition in a variety of mammals, including humans (Knapstein et al., 1975), dogs (Concannon et al., 1978), and cows (Hudson et al., 1976). Environmental factors such as variation in the food supply (Pride and Ethan Pride, 2005, Behie et al., 2010), exposure to predation (Barcellos et al., 2007), or deviations from

expected seasonal cycles (Cavigelli, 1999, Alila-Johansson et al., 2003) also act as stressors in animals. In addition, human land-use change (Creel et al., 2013, Fokidis et al., 2009), proximity to and interaction with humans (Tingvold et al., 2013, Rangel-Negrín et al., 2009, Behie et al., 2010, Dehnhard et al., 2001, Mason, 2010), pollution (Skomal and Mandelman, 2012), and anthropogenic noise (Sierra-Flores et al., 2015) have all been shown to act as stressors in a number of species. In addition, prolonged activation of the HPA axis (i.e. chronic stress) can have a detrimental effect on immune processes, reproductive output and survivorship in wild vertebrates (Wingfield et al., 1998), and also inhibits cortisol's ability to mediate the stress response (Barton et al., 1986).

Migration enables animals to increase their access to variable food supplies, and can result in superabundant populations such as are seen with regard to the blue wildebeest in the Serengeti (Hopcraft et al., 2014, Fryxell and Sinclair, 1988). Migratory species, however, are vulnerable to human impact as their movement strategy relies on large, unfragmented landscapes with clear environmental gradients. This has led to a widespread decline in ungulate migrations over the previous two centuries as a result of habitat loss, habitat degradation and overexploitation (Bolger et al., 2008, Harris et al., 2009). Given that migratory animals move across large environmental gradients and any given individual will be exposed to a diverse array of anthropogenic and environmental stressors throughout its annual movement cycle there is a need to identify how anthropogenic factors may exacerbate stress and ultimately affect individual survival. Hair cortisol could be a practical, non-invasive biomarker for assessing how the movement patterns of migratory animals shape their physiological state.

The annual movement of wildebeest in the Serengeti affects virtually every aspect of the ecosystem, from biodiversity of flora and fauna (Anderson et al., 2007) to socioeconomic status of local people (Sinclair et al., 2009). Although the migration is contained within the boundaries of the Serengeti National Park and Massai-Mara National Reserve, it remains vulnerable to human disturbance (Rentsch and Damon, 2013, Hopcraft et al., 2015a). For example, changing land use in pastoral ranches that border the Massai-Mara National Reserve, as a result of exponential human population growth and increasing livestock numbers, has caused a marked decline in six ungulate species throughout the Maasai-Mara region (Ogutu et al., 2009). More recently, this region has also come under

increasing pressure from agricultural expansion and the fencing in of communal grazing land, which hinders access to vital resources by wildlife and could lead to further declines in their population (Serneels and Lambin, 2002, Løvschal et al., 2017). Indeed, fencing and infrastructure development have been shown to cause substantial declines and even extinctions in other migratory populations globally (Bolger et al., 2008, Harris et al., 2009). Effects of other forms of human infrastructure such as the effect of tourism have been less well studied. Although tourism constitutes a large source of revenue for many conservation areas, including the Serengeti-Mara ecosystem, the impact of large-scale mass tourism (i.e. hundreds of thousands of visitors) on wildlife is not well-studied. .

The objective of this research is to understand how environmental and anthropogenic factors in the landscape affect variation in cortisol in Serengeti wildebeest. The aim is to generate spatiotemporal profiles of the activity in the hypothalamo-pituitary-adrenal stress axis by assaying cortisol in tail hair from animals whose movements are known (GPS-collared). We predict that hair cortisol concentrations will be higher in samples obtained from carcasses, compared to samples taken from living animals. We also predict the most stressful time in the annual cycle is at the height of the dry season, when food availability and water are most scarce and potential anthropogenic stressors (e.g. agricultural fences, tourist infrastructure) are most abundant. Finally, we expect higher variation in cortisol concentrations over time in migrating wildebeest, relative to western corridor residents.

This thesis focused on the collection of data pertaining to three distinct physiological metrics: stress (cortisol); reproduction (progestogens and oestrogens), and electrolyte and fluid homeostasis (aldosterone). Studies in animal physiology tend to focus on the measurement and analysis of a single hormone, as a physiological metric (e.g. stress studies generally only measure either cortisol or corticosterone concentrations). However, it is often the case that multiple hormones play a role in the regulation of certain physiological events, requiring the measurement of multiple hormones. For instance, studies on dominance and risk-taking behaviours in humans found that both cortisol and testosterone jointly modulate these behaviours (Mehta and Josephs, 2010, Mehta et al., 2015).

All cortisol and aldosterone data obtained in Chapters 3 and 5 of this thesis were collected from the same samples (while different samples were used for Chapters 2 and 4). Therefore, it was possible to perform statistical analysis to determine the relationship between cortisol and aldosterone, and whether this differed, between resident and migrant wildebeest. Independently, each of these metrics provides valuable physiological insight, however the relationship between these hormones may shed further light on wildebeest physiology.

3.3 Materials and methods

3.3.1 Study area

The study was conducted in the Serengeti-Mara ecosystem, East Africa (1°15' to 3°30'S, 34° to 36°E) (Fig. 3.1(a))(Sinclair et al., 2015). A strong soil fertility and rainfall gradient creates a natural pattern of high-quality grazing that is seasonally available, and which is the underlying driver of the migration of Serengeti herbivores (Holdo et al., 2009). The nutrient-rich high-altitude volcanic soils in the south coincide with a rainfall shadow created by the Ngorongoro highlands (<400mm rain / year). These areas support nutrient rich grasses that are only available to migratory herbivores for a short period of time during the wet season. These are the calving grounds for the wildebeest. The Western Corridor is defined by two rivers (Mbalageti and Grumeti) that drain westerly towards Lake Victoria, creating heterogeneous alluvial floodplains between the neighbouring valleys composed of large grass meadows mixed with open woodland and separated by closed canopy riverine forests. The leached and eroded granite soils in the north are dominated by mixed *Acacia* and *Commiphora* woodlands with nutrient poor grasses below the canopy, however, the north has the highest annual rainfall (>1200mm rain/year) and the only permanently flowing river. The northern extent of the ecosystem is the dry season refuge for the migration. In general, the migration of wildebeest moves annually between the south, west, and north in a clockwise fashion (Hopcraft et al., 2014).

3.3.2 GPS collars

Wildebeest tail hair was collected from 26 free-ranging wildebeest equipped with GPS-collars, between 2012 and 2018. Three migratory wildebeest were also resampled approximately 18 months after they were initially sampled, giving a total of 29 collection events (i.e. n=29 hair samples from n=26 individuals). All study animals were reproductively active females between 3-10 years old and in outwardly healthy condition at the time of collaring. GPS collars (Tellus collars from Followit, Sweden) were deployed as part of a larger effort to understand the movements of migratory wildebeest in the Serengeti, as described by Hopcraft et al (2014). Collars were programmed to collect GPS fixes every 4-6 hours. Collared wildebeest were immobilized by veterinarians from the Tanzania Wildlife Research Institute (TAWIRI) using an injectable dart containing 4-6 mg of etorphine hydrochloride and 80–100 mg of azaperone tartrate, delivered remotely by Dan-Inject rifle from a stationary vehicle near the animal. Veterinarians followed the handling and care protocols established by TAWIRI.

3.3.3 Life History

3.3.3.1 Age

The age of individual wildebeest was determined using width and height of mandibular incisors (Attwell, 1982).

3.3.3.2 Carcass Samples

Several starvation/predation related mortalities occurred to collared animals during the study period (n= 6). These samples were grouped together as ‘carcass’ samples, which may show greater chronic stress than samples collected from animals that had not starved or been predated.

3.3.3.3 Reproductive Status

Wildebeest are a precocious species, with strong filial imprinting and follower instincts, staying with their mother for the first nine months of their life. In addition, migratory wildebeest are also highly synchronous breeders, with over 80% of all calves born in a three-week window beginning in late February (Estes, 1976). Presence and size of a calf at sample collection was therefore used as confirmation that an individual had calved during the previous calving season. These hair samples containing a known parturition event were used to determine whether hair cortisol profiles could be used to infer reproductive state (i.e. could a significant peak in cortisol be observed in hair sections relating to the calving season, in comparison to sections of hair relating to periods of time immediately preceding/proceeding calving).

3.3.3.4 Movement Strategy

Visual inspection of location data showed four of the wildebeest in this study were not migratory, belonging to a subpopulation resident to the western corridor region of the Serengeti.

3.3.4 Collection of Tail Hair Samples

Tail hair was collected from carcasses 0-3 days after being notified by an inbuilt mortality sensor on the collars of the mortality event. In order to collect wildebeest tail hair, study animals were recaptured 18 months (n=26) or 3 years (n=3 resampled individuals) after initial collar deployment. Hair was pulled from carcasses and clipped as close to the root of the tail hair as possible in living animals, recording the length of the stubble remaining on the animal. Samples were then tied into bundles, keeping the hair aligned at the root end. Superficial contaminants (e.g. mud, faeces, grass) were removed by washing samples with filtered rainwater in the field. Samples were then dried and stored at room temperature.

3.3.5 **Laboratory protocol**

Hair bundles were washed twice with a 2:1 chloroform:methanol solution in a heated water bath for 10 minutes and rinsed with MilliQ UltraPur water(Logan and Lutcavage, 2008). Samples were then dried overnight in a fume hood before being aligned precisely at the root end. Bundles of hair were cut to a maximum length of 32 cm, representing approximately 18 months of growth (Rysava et al., 2016). Using hairdressing scissors, hair samples were cut into 8mm sections along the length of the sample, and a note taken of each section's position along the hair (i.e. 1st, 2nd, 3rd … 40th). Sections were placed in 2ml metal lysing tubes, containing ¼ inch x ½ inch grinding cylinders (MP Biomedicals, Cambridge, UK), frozen in liquid nitrogen and powdered using a Retsch MM400 mixer mill (Retsch, Hope Valley, UK) set to 30Hz for 3 minutes. This procedure homogenises each section and optimises extraction efficiency by increasing the relative surface area of the sample and destruction of the cuticle to expose the potentially hormone-rich medulla and cortex of the hair. Dates that corresponded to the period of time over which each section of hair was grown were calculated based on the measured wildebeest tail hair growth rate of 4mm/week, the date of sample collection, and the distance from the root (as outlined in Chapter 2).

For each sample, 35-40mg of powdered hair was weighed in a borosilicate glass vial and 2ml of 100% HPLC Grade Methanol added. Vials were capped and placed into a heated orbital shaker (Stuart orbital incubator S150 (Cole-Parmer, Staffordshire, UK)) at 52°C, 200rpm, for 16 hours. To allow for any potential assay repeats, three separate 550ul aliquots of solvent were placed into borosilicate glass tubes and dried down in a Savant SC210A speedvac concentrator (Thermo Fisher Scientific, Renfrew, UK) set to medium heat for three hours. Dried samples were resuspended in 150ul of assay buffer, before carrying out an Enzyme-Linked ImmunoSorbent Assay (ELISA), as specified in the manufacturer's instructions (Cortisol Express ELISA kit, Cayman Chemical, Ann Arbor, MI). The reported cross-reactivity of the antibody is as follows: cortisol (100%), dexamethasone (15%), prednisolone (4.01%), cortexolone (1.58%), 11-

deoxycorticosterone (0.23%), 17-hydroxyprogesterone (0.23%) cortisol glucuronide (0.15%), corticosterone (0.14%), cortisone (0.13%), androstenedione (<0.01%), enterolactone (<0.01%), oestrone (<0.01%), pregnenolone (<0.01%), 17-hydroxypregnenolone (<0.01%), testosterone (<0.01%). All samples from the same individual were measured in duplicate in the same assay. Assay Zap software (Biosoft, Cambridge, UK) was used to interpolate cortisol concentration of unknowns from the standard curve. Across the ten cortisol assays, intra- and inter-assay coefficients of variation averaged 2.95% and 5.15%, respectively. Cortisol concentrations were standardized using the dilution factor, and initial mass of hair used. The number of days each section of hair had spent in the environment was also included in all statistical modelling, to account for dehydration/degradation of the hair over time (as highlighted in Chapter 2).

3.3.6 **Environmental layers**

GIS layers were created by estimation of the quality and quantity of forage available to migrating wildebeest, as well as factors associated with predation risk and anthropogenic infrastructure (Fig. 3.1). All layers were created at a spatial resolution of 1 km (Hopcraft et al., 2014). NDVI and ΔNDVI data were extracted from a chronological series of 16-day NDVI composites, using both the location and the date of growth for each section of hair to extract dynamic NDVI values, while all other GIS variables were extracted from static layers.

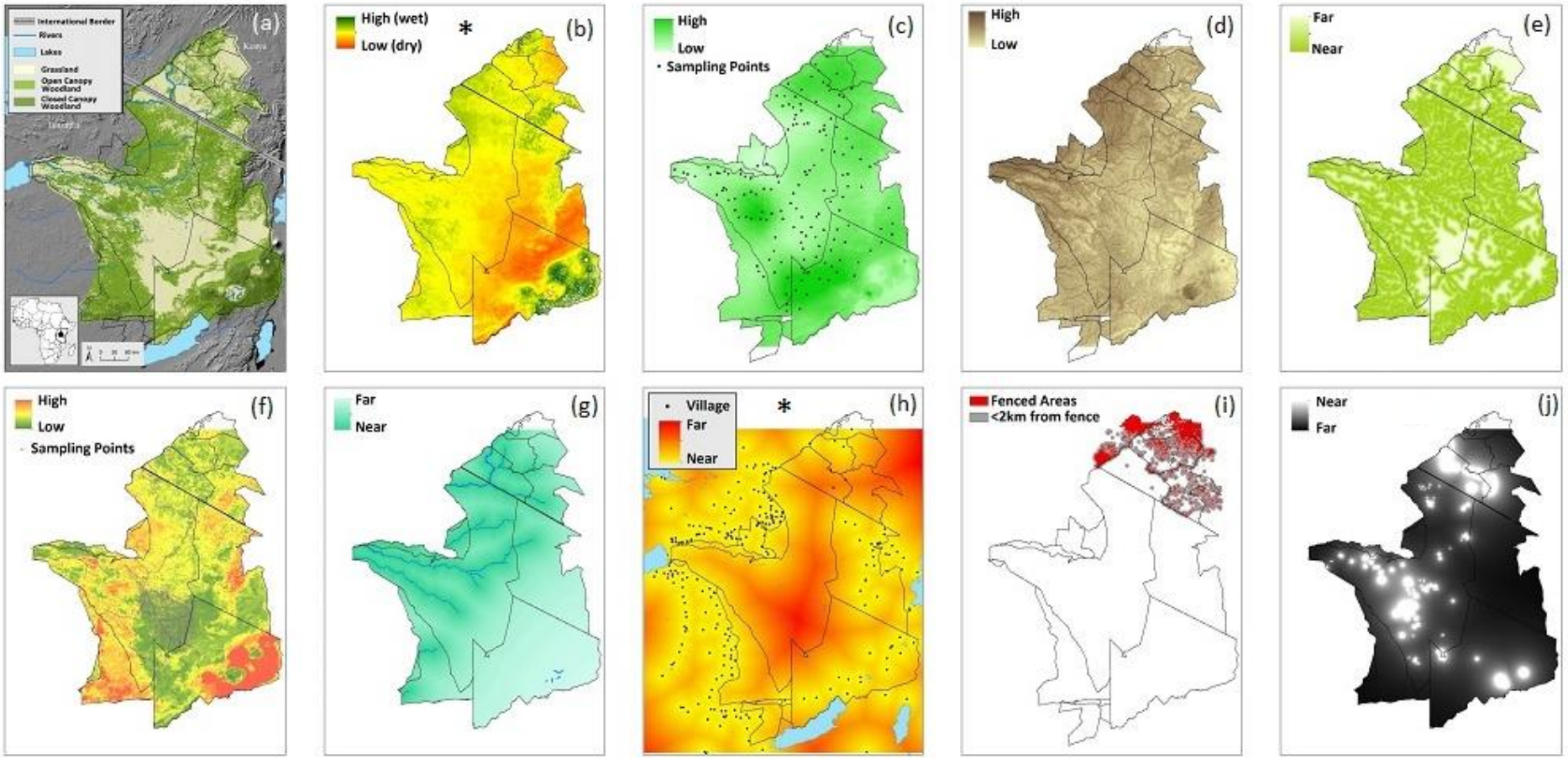


Figure 3.1 (a) Map of the Serengeti ecosystem with the covariates used to explore the spatial and temporal patterns in wildebeest cortisol. Covariates related to food quality and abundance are (b) NDVI*; (c) grass nitrogen; and (d) potential grass biomass. Covariates that either conceal predators or provide predictable locations where prey are found are (e) distance to drainages (f) proximity to thick vegetation; and (g) distance to water. Covariates associated with anthropogenic factors are proximity to (h) proximity to villages*; (i) agricultural fences, and (j) tourism intensity. Asterisk (*) denotes significant covariates in optimal model.

3.3.6.1 Forage quality and abundance

NDVI: The Normalized Difference Vegetation Index (NDVI) is a commonly used metric of vegetation greenness. High NDVI values indicate live green vegetation whereas low NDVI values indicate dry or senescent vegetation. 16-day NDVI composites from the MODIS Terra satellite were used to estimate the condition of the vegetation (Fig. 3.1(b)). NDVI correlates both with vegetation biomass and with quality (Pettorelli et al., 2005, Hopcraft et al., 2011), therefore in addition to green versus dry we also estimated grass quality and quantity independently as grass nitrogen and potential grass biomass, respectively.

ΔNDVI: To estimate the rate of change of vegetation condition (i.e. to capture greening or drying processes), we also measured change in NDVI, by subtracting the previous 16-day's NDVI from the current NDVI at that location.

Grass Nitrogen: The spatial distribution of the grass quality was estimated by regression kriging the grass nitrogen values measured at 148 vegetation plots distributed across the Serengeti-Mara ecosystem with a long-term NDVI layer. The vegetation plots were stratified to capture all major habitat types across the soil and rainfall gradient (Fig. 3.1(c)). In each vegetation plot grass was clipped from five 25cm^2 areas, dried and ground to 2mm particle size using a Cyclotec 1093 cyclonic grinder (Foss Analytics, Warrington, UK). Grass nitrogen in each sample was measured using near infrared spectrophotometry (Bruker Optik GmbH, Ettlingen, Germany) and the average grass nitrogen per plot calculated (as per (Hopcraft et al., 2011)). Previous studies show that grass nitrogen is inversely correlated with long-term NDVI; therefore, a 9-year composite NDVI layer was used to regression krige (Hengl et al., 2007, Bivand et al., 2013) the spatial distribution of grass nitrogen over the entire ecosystem. Cross validation suggests this method has an acceptable level of accuracy (Hopcraft et al., 2011).

Potential Grass Biomass: Grass biomass in semi-arid savannah systems is highly dynamic and depends on soil moisture, cumulative rainfall, fires (over 30% of the ecosystem routinely burns every year) and herbivory (the wildebeest migration alone consumes about 4,500 tonnes / day), amongst other factors. Therefore, instead of measuring grass biomass directly, the landscapes' capacity to grow

grass was estimated as the potential grass biomass: a metric that combines the topographic wetting index and mean annual rainfall (Fig. 3.1(d)). The topographic wetting index is a metric of the landscapes' capacity to retain water and is determined by flow direction, catchment area, and local topography such as concave versus convex. Cells with the largest potential grass biomass have a high topographic wetting index, are concave, and are located in the low reaches of large catchments with high rainfall. Cells with lowest potential grass biomass have a convex shape and are located near the headwaters in low rainfall areas.

3.3.6.2 Risk of Predation

A herbivore's exposure to predators is associated with landscape features that either increase their rate of detection or improve a predator's chance of capturing its prey(Lima and Zollner, 1996, Valeix et al., 2009). Previous research has shown that landscape features such as erosion terraces associated with drainages or thick vegetation conceals ambush predators and compromises a herbivore's ability to detect a predator (Hopcraft et al., 2005). In addition, features such as rivers provide predictable locations where predators are most likely to encounter prey searching for water or crossing points during the migration. Most rivers in the Serengeti are ephemeral and flow only for short periods during the wet season but may contain pools for several months in the dry season. These drainages improve the success rate of ambush predators because: they are associated with erosion terraces that conceal predators from vigilant prey; confluences and meanders form natural traps in the landscape; and pools may attract prey, especially in the dry season. Studies on the effects of the lunar cycle on predator behaviour have found an increase in the hunting activity of lions in the weeks following a full moon, when moonlight is most faint (Packer et al., 2011). Therefore, predation risk was measured indirectly using three proxy metrics: mean horizontal cover, distance to rivers, and lunar phase.

Rivers: For each animal GPS location we estimated the distance of the animal to rivers classified as having incised banks and frequent pools for at least 2 months into the dry season (Fig. 3.1(e)) (i.e. class 3 and greater of the Rivers shape layer from the Serengeti GIS Database (version 6)). Drainages that did not

meet the criteria were ignored. Proximity to permanent water sources (Fig. 3.1(g)), was also estimated as a proxy for river crossings, and the associated increase in predation pressure.

Vegetation Cover: The vegetative cover available to ambush predators was estimated from 1,882 ground-truthing points that estimated the horizontal cover for each structural vegetation type identified by(Reed et al., 2009). At each point the percentage of vegetative cover greater than 40cm high at 15m from the observer was recorded in the four cardinal directions. The mean percent cover for each structural vegetation type was calculated and used to classify Reed *et al*'s vegetation map to estimate the horizontal cover across the entire landscape (Fig. 3.1(f)). As wildebeest may move in a predator sensitive manner(Sinclair and Arcese, 1995), the distance for each recorded animal GPS location to the nearest high density cover was calculated (i.e. areas where vegetation cover is above 85th percentile), rather than the average vegetative cover at each location.

Lunar Phase: Proximity of a full moon to the midpoint of the date range for each section of hair was calculated. Since nights are brightest when the moon is full, the closer the midpoint date is to a full moon, the brighter the nights will have been during that section of hair's growth. Since increased brightness means increased visibility at night, this may increase predator detection, minimising predation risk.

3.3.6.3 Anthropogenic layers

Potential anthropogenic effects on cortisol were estimated in three ways: proximity to villages, weighted by the human population (Fig. 3.1(h)); proximity to agricultural fences (Fig. 3.1(i)), and tourism intensity, weighted by bed-nights (Fig. 3.1(j)).

Villages: Human-induced risks, such as poaching, were estimated using a point file of village locations from which a raster of distance to the nearest village was created. Low values represent areas that are close to villages, whereas cells with high values are areas far from villages.

Fences: Agricultural fencing occurs in the northern extent of the wildebeest range in Kenya and migratory animals may only be near fences for short periods of the annual cycle. For the majority of the year most wildebeest are in Tanzania, far from fences. For these periods, fences are unlikely to be within the sensory range of wildebeest. Therefore, rather than a continuous distance measurement, a fence encounter was defined when an animal was within 2km of a fence (shapefiles of fences as per (Løvschal et al., 2017) which corresponds roughly to <1 day mean displacement distance of wildebeest during the dry season. Total number of fence encounters were then calculated for each hair section (two-week period).

Tourism Intensity: Proximity to tourist infrastructure such as lodges and semi-permanent tented camps was weighted by the bed-night capacity of the facility. Tourist facilities were classified in five size categories: <15, 15-50, 51-100, 101-150, and 151-200 beds / night. The inverse Euclidean distance to each class of tourist facility was multiplied by the maximum bed-nights for the class and summed across all classes. High values in the resulting layer represented areas that are close to large facilities whereas small values are areas that are far from small facilities.

3.3.7 **Statistical Analysis**

Telemetry data was paired with cortisol profiles, to determine the effects of environmental and anthropogenic factors in the landscape on cortisol levels in wildebeest. Data relating to potential stressors was obtained for every GPS location the animal occupied (recorded every 6-12 hours), by extracting values from spatiotemporal layers relating to each factor. Average values were calculated to correspond with each section of hair, from all GPS locations visited throughout the growth of the corresponding 8mm section of hair (i.e. 14 days growth). This alignment of temporal resolutions of cortisol data and environmental and anthropogenic variables allowed for the relationship between these variables to be analysed. Cortisol concentrations were measured in 880 sections of hair, generating profiles for 29 hair samples, taken from 26 individual wildebeest.

For each GPS location we extracted metrics of forage availability (grass nitrogen content, potential grass biomass, NDVI and the rate of change of NDVI), predation risk (vegetative cover, proximity to rivers and water, and lunar phase) and anthropogenic factors (proximity to villages, tourism intensity and fence encounters). Mean values for each of these factors were then calculated for the period of time corresponding to every section of hair and paired with matching cortisol concentrations. Season (measured as the number of days to the end of the wet season) was included as an explanatory variable in the preliminary analysis, to determine whether wildebeest exhibit annual variation in baseline cortisol. Age and movement strategy (e.g. resident or migrant) were also included as explanatory variables.

Cortisol concentrations were log transformed prior to analysis to conform to assumptions of normality. All variables were scaled (by subtracting the mean and dividing by their standard deviation) to facilitate model convergence and to allow for a direct comparison between the effect of covariates.

All statistical analyses were performed using R version 3.5.1 (R Core Team, 2018).

A linear mixed effects model was fitted to the data using package lme4 (Bates et al., 2014) in R (R Core Team, 2018) to test the effects of the explanatory variables indicated above. All explanatory variables were assessed for collinearity and were not significantly correlated (i.e. correlation coefficient $r < 0.6$ for all covariates). AIC was used to select the top model amongst a suite of candidate models (Table 3.2 (Supplementary)). First, the general model containing all potential explanatory variables and their interactions was fitted to the data. The weakest predictor of cortisol was then removed from this model; If this caused a decrease in AIC of 3 or more, the new model was better and would replace the original model. The next weakest predictor was then removed and a comparison between the AIC of these models was used to determine which was better. This process was continued until removing covariates no longer improved the model, indicating the most parsimonious model (Table 3.1).

A second mixed effects model was fitted to a subset of the data, composed of animals with a calf at sample collection. Since it was known these individuals gave birth in the previous calving period, we compared between sections of hair

grown before, during, and after parturition, to determine whether the peak in cortisol that occurs at parturition could be detected in the hair samples.

Animal ID was included as a random effect in all models, to control for pseudo-replication.

Figures were produced using the R packages ggplot2 (Wickham, 2016) and effects (Fox and Weisberg, 2019).

Finally, to determine the relationship between aldosterone and cortisol, a linear mixed effects model (Bates et al., 2014, R Core Team, 2018) was fitted to the subset of our data with both cortisol and aldosterone data (n = 813) and position along the hair growth axis was included as a fixed effect, to account for environmental exposure. Sample ID was also included as a random effect.

3.4 **Results**

3.4.1 **Cortisol Profiles**

Cortisol concentrations were measured in 880 sections of hair, generating profiles for 29 hair samples, taken from 26 individual wildebeest. Cortisol profiles varied both temporally and spatially within individuals, as shown for an example individual in Figure 3.2, as well as between individuals (Fig. 3.6 (Supplementary)).

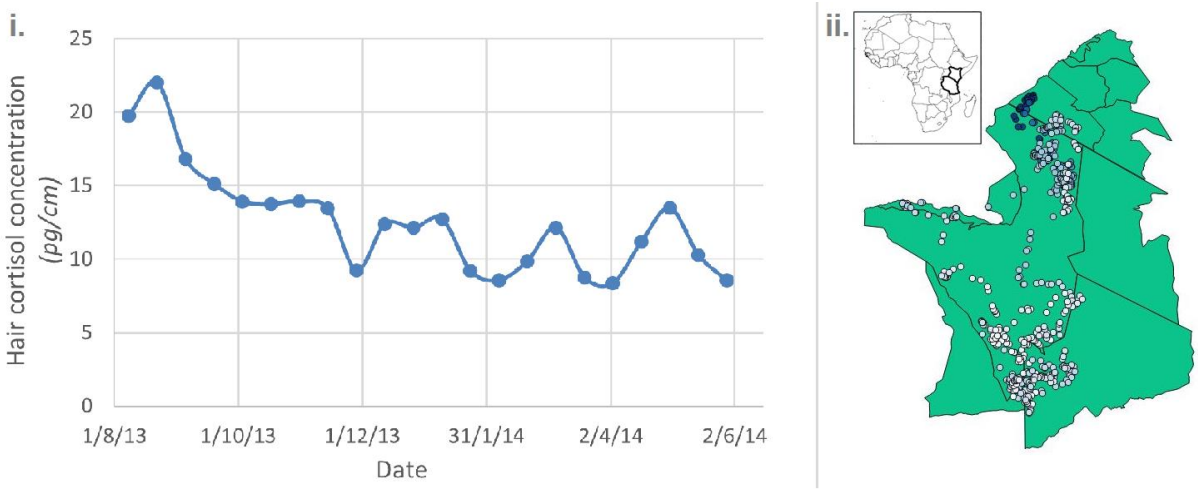


Figure 3.2 Hair cortisol concentrations from a single hair sample (WH418) plotted temporally (i.) and spatially (ii.), using the corresponding locations the animal visited during

that time, superimposed onto a map of the Serengeti-Mara Ecosystem. Darker points on map represent higher cortisol concentrations.

The results of our AIC-optimised model indicate that a number of factors correlate with cortisol in our wildebeest tail hair samples. Overall, our AIC-optimal model has an R^2 coefficient of 0.777, with an R^2 coefficient of 0.390 when only accounting for fixed effects. Since all variables have been standardised, continuous variables' covariates can be directly compared for a comparison of effect size.

3.4.2 Life History Covariates

3.4.2.1 Cortisol Peak at Parturition

A mixed effects model, fitted to a subset of our data composed of all samples taken from individuals who were known to have given birth in the previous calving period (n=11), allowed us to determine if the peak in cortisol which occurs at parturition could be detected in our hair samples. Although a peak in cortisol is known to occur around parturition in a variety of species, we were unable to detect a difference between sections of hair grown when the individual calved and sections of hair immediately preceding/proceeding parturition (n = 74; β = 0.07 ± 0.06; t = 1.25). This may be explained by the disparity in temporal resolution between the cortisol peak that occurs for a few hours to days around parturition and our 8mm hair sections, which relate to mean cortisol concentrations over a two-week period.

3.4.2.2 Additional Life History Covariates

Movement strategy (migratory or western corridor resident) and the age of wildebeest at the time of sample collection were included in the preliminary analysis but had no significant effect on cortisol, nor did they have any significant interactions with any other covariates, so were dropped from the most parsimonious model (Table 3.1). Season was also included in preliminary

analysis, to determine whether there is annual variation in baseline cortisol, however this was also not a significant predictor of cortisol and was dropped from the most parsimonious model.

Table 3.1 Output from the AIC-optimised linear mixed-effects model, suggesting there are important determinants of cortisol concentration in wildebeest tail hair in the Serengeti-Mara Ecosystem. Environmental exposure (i.e. length of time each section of hair spent in the Serengeti) was included, to account for dehydration/degradation of the hair over time. Sample ID was included as a random intercept and all covariates were normalized to allow direct comparison of effect size. Covariates retained in the top candidate model were: *Village Distance; NDVI* (i.e. relative greenness of vegetation); *Dead/Alive* (i.e. whether the sample came from a living animal or carcass); as well as an interaction between *NDVI * Dead/Alive*

Covariate	Coefficient Estimate	Standard Error	T-statistic
(Intercept)	-0.177	0.143	-1.233
Village Distance	-0.045	0.019	-2.340
NDVI	-0.058	0.021	-2.774
Dead/Alive: Dead	1.528	0.316	4.829
NDVI * Dead/Alive	0.148	0.057	2.596
Environmental Exposure	0.482	0.019	25.173

3.4.3 **Forage Quality and Abundance**

NDVI (an index of live, green vegetation) was negatively related to hair cortisol concentration (β = -0.058 ± 0.021; t = -2.774)) (Figure 3.5). However, Δ*NDVI, Grass nitrogen*, and *potential grass biomass* were not significantly correlated with changes in the cortisol profiles of the animals. This relationship also

differed between samples taken from living animals and carcasses (section 3.4.6).

3.4.4 **Risk of Predation**

None of the predation covariates (*distance to drainages*, *proximity to thick vegetation*, *distance to large rivers with permanent water*, and *lunar phase*) were significant predictors of changes in hair cortisol concentrations and were dropped from the final model.

3.4.5 **Anthropogenic Stressors**

Village Distance remained in the AIC-optimised model as a significant predictor of hair cortisol concentration (Table 3.1). Cortisol concentrations were negatively related to *Village Distance* (i.e. cortisol was highest when animals were near villages) (β = -0.045 ± 0.019; t = -2.340) (Fig. 3.3). Although this effect size was small, removal of *Village Distance* did not improve the model, indicating significance. Fences and tourism did not have a significant effect on the changes in cortisol seen in the sampled animals.

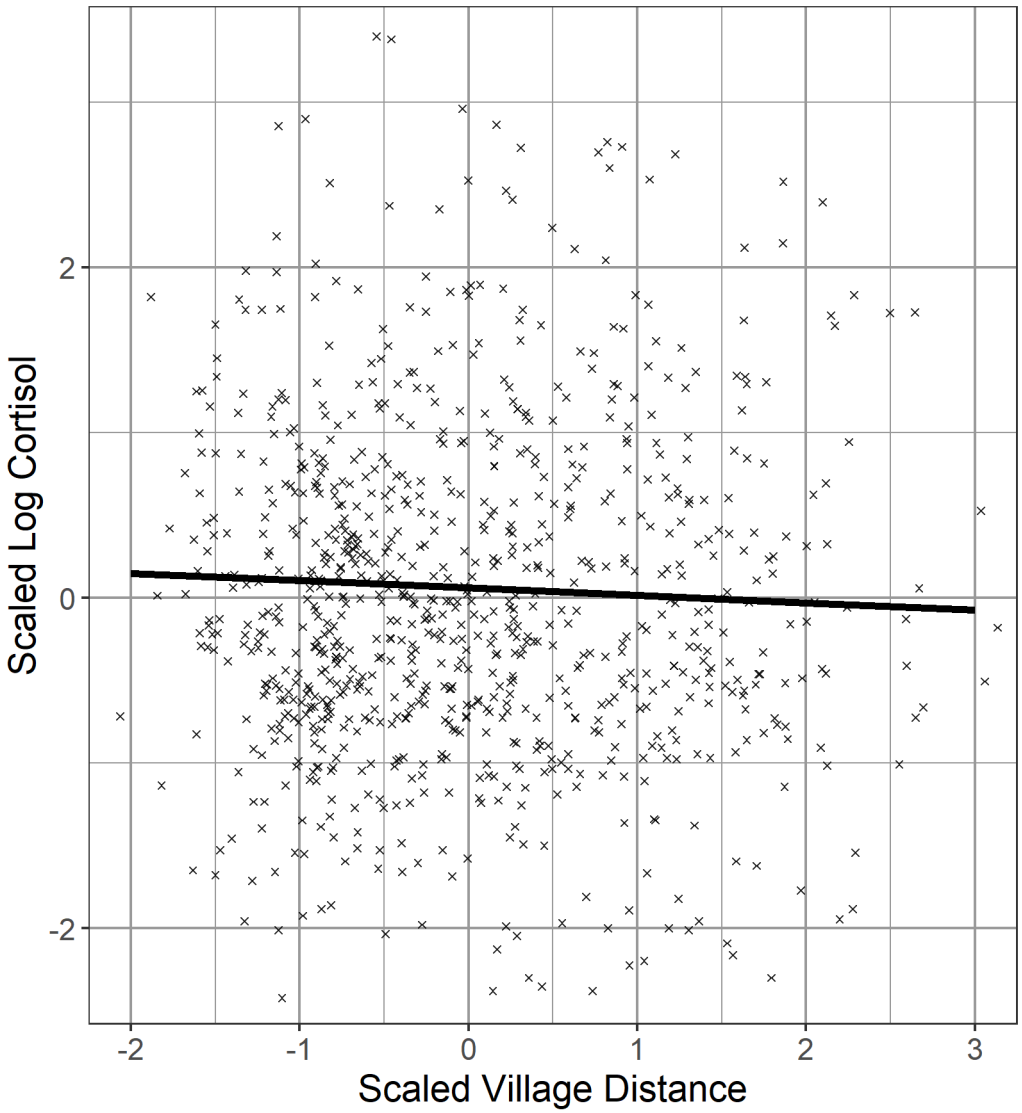


Figure 3.3 Scatterplot of cortisol against village distance with line of best fit showing small but significant negative correlation between proximity to villages and cortisol.

3.4.6 **Carcasses and Living Animals**

The results from the optimal linear mixed effects model reveal that cortisol concentrations were significantly higher in samples obtained from carcasses (n=6) than from living animals (n=23) (β = 1.528 ± 0.316; t = 4.829) (Fig. 3.4).

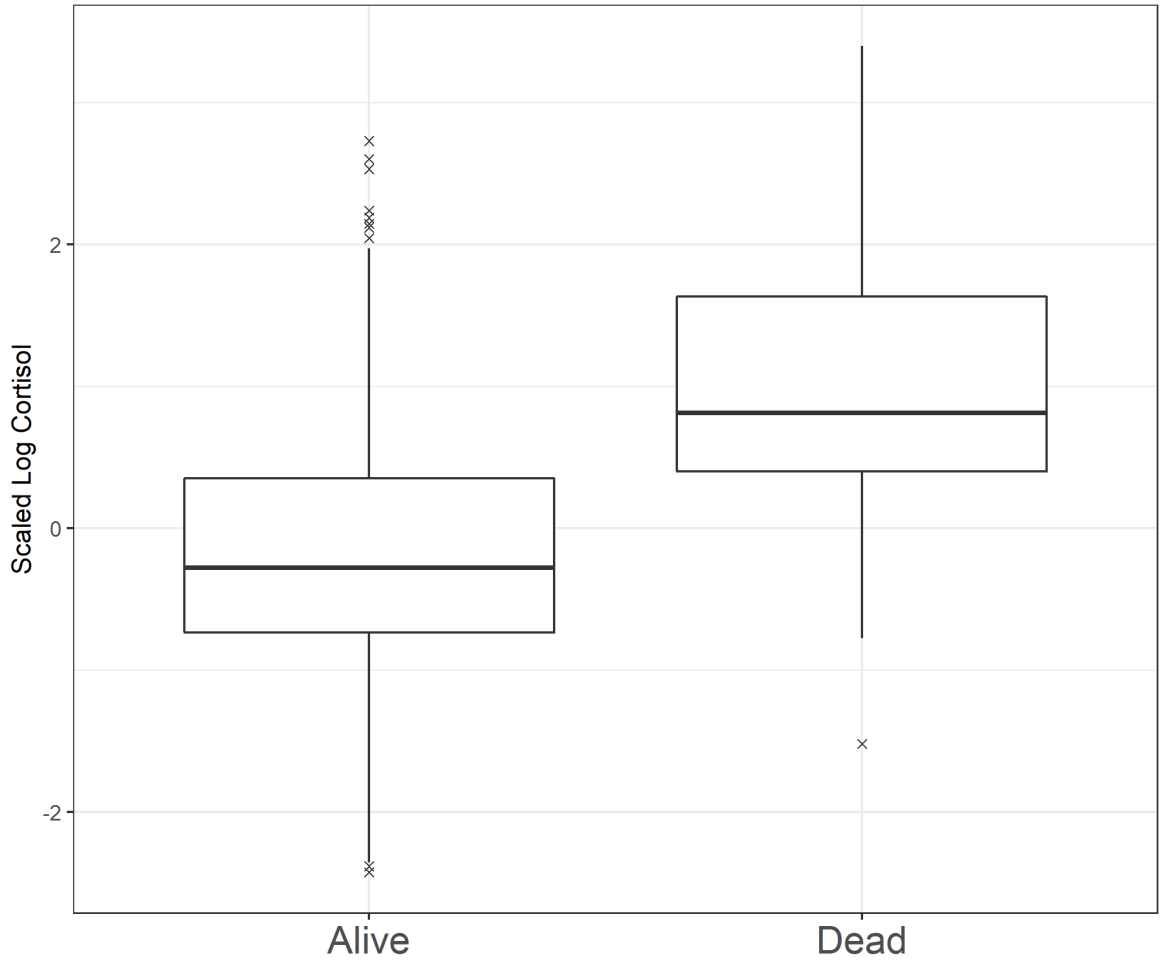


Figure 3.4 Boxplot showing differences in scaled cortisol between samples taken from living animals and carcasses. Samples from carcasses have significantly higher overall cortisol, a sign of chronic stress, which likely contributed to the death of the animal.

An interaction between *NDVI * Dead/Alive* was also present in the most parsimonious model (β = 0.148 ± 0.057; t = 2.596) (Table 3.1). *NDVI* is negatively correlated with cortisol in living animals and positively correlated with hair cortisol concentration in samples obtained from carcasses (Fig. 3.5). Although the effect size is smaller in samples taken from living animals, NDVI remained in the AIC-optimal model fitted to a subset of our data containing only living animal samples (β = -0.052 ± 0.021; t = -2.465), indicating that NDVI is a significant predictor of cortisol in both groups, independently.

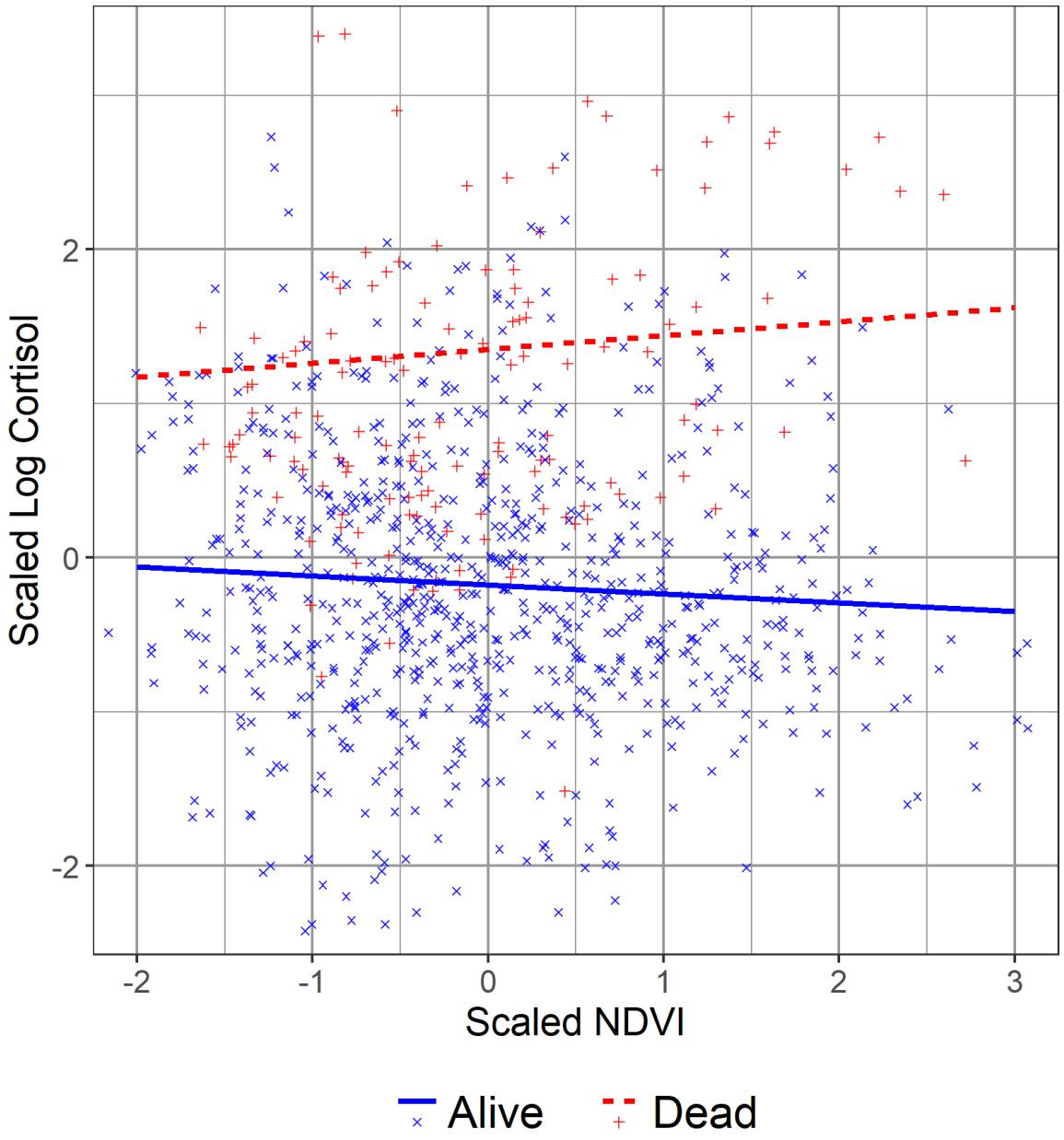


Figure 3.5 The interaction of NDVI and mortality status on cortisol concentrations. Living individuals showed a negative relationship between NDVI and cortisol, while samples from carcasses showed a positive relationship.

3.4.7 **Examining Interactions Between Aldosterone and Cortisol**

The results of a linear mixed effects model fitted to a subset of our data containing both cortisol and aldosterone values indicated that hair cortisol concentrations were positively correlated with aldosterone concentrations (β = 0.05 ± 0.02; t = 2.80). This relationship was approximately 5 times greater in

wildebeest resident to the sodium-rich Western corridor region of the ecosystem (β = 0.27 ± 0.05; t = 5.15), compared to migrants (Figure 3.6).

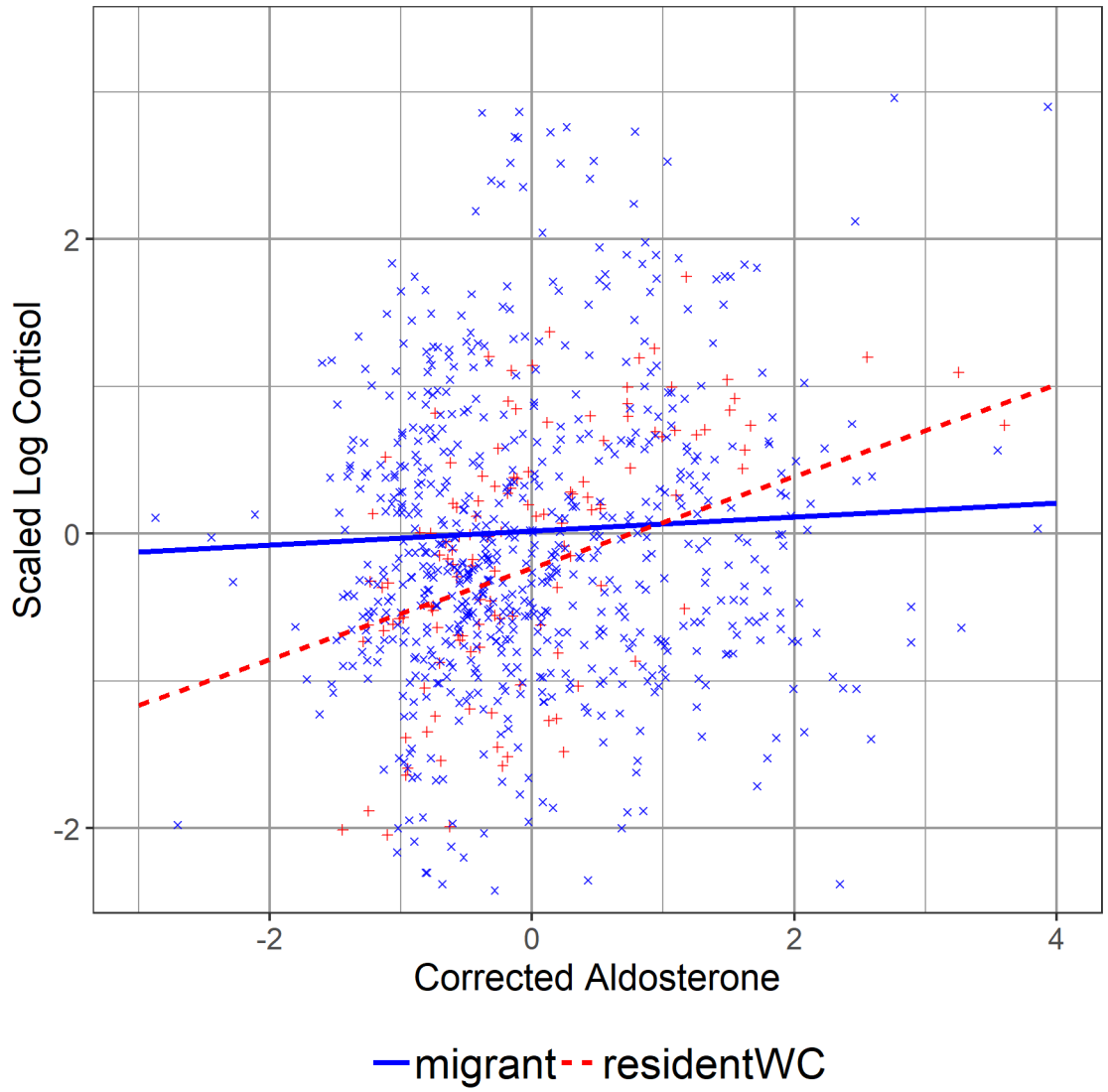


Figure 3.6 **The relationship between aldosterone and cortisol in resident and migrant wildebeest. Overall, aldosterone and cortisol were positively correlated; however, the effect size was five times higher in residents, compared to migrants.**

3.5 **Discussion**

Differentiating between anthropogenic versus natural causes of stress in wild free-living animals is essential when trying to minimize or mitigate sources of

anthropogenic stress. By retrospectively matching the time series of hair cortisol concentrations with the locations of each animal we were able to investigate the spatial and temporal patterns of chronic stress in animals as they moved through different landscapes. The ability to link physiological variables with location data provides the ability to gain unique insights about the internal motivations behind animal movement, such as the migration of wildebeest, as well as the impact that human activities may have on population health and viability.

Creating chronological cortisol profiles from hair samples shows promise as an alternative means for monitoring physiological changes in wild animals for several reasons. For example, it reduces the need for locating and sampling individuals multiple times; it is relatively non-invasive, having no detrimental effects on the animal after the sample is taken; samples can be stored at room temperature, with no complex storage method necessary; samples can be collected from carcasses and analysed retrospectively, as hair is often left even after a carcass has been scavenged, and; a single sample can be used to obtain time-series data. Therefore, exploring the application of these techniques in a model system such as the Serengeti wildebeest migration has considerable value, especially if the approach could be expanded to other species in other ecosystems.

3.5.1 Causes of chronic stress in Serengeti wildebeest

The results of this work illustrate that variations in cortisol concentrations are correlated with both anthropogenic (Proximity to villages) and habitat (NDVI) factors that the animal experiences as it moves through a landscape. Overall, our AIC-optimal model explained 77.7% of the variation in cortisol concentrations observed in our samples, with 39.0% of the variation explained by fixed effects.

3.5.1.1 Forage Quality and Abundance

Overall, cortisol was highest when food availability (as measured by NDVI) was low. This result suggests that low food availability incurs a physiological stress response and corroborates previous findings from other species, such as pigs (*Sus*

domesticus), chimpanzees (*Pan troglodytes*), and kittiwakes (*Rissa tridactyla*), in which cortisol peaks during periods of nutritional deficit (Parrott and Misson, 1989, Muller and Wrangham, 2004, Kitaysky et al., 1999). Although other covariates that provided a measure of forage quality (grass nitrogen) and abundance (potential grass biomass) were assessed, NDVI was the only significant predictor of hair cortisol concentration. This is likely due to NDVI being the only covariate that contains a temporal element (i.e. NDVI values were obtained by reduction of a series of NDVI layers to the specific 16-day raster layer that corresponded with the dates over which the hair section was grown), while estimates of grass nitrogen and grass biomass were extracted from a single static layer. NDVI, therefore, accounts for seasonal differences in forage and may incorporate aspects of grass quality and quantity to give a more precise measurement of food availability in the highly dynamic Serengeti grasslands.

3.5.1.2 Anthropogenic Disturbance

The results demonstrated that hair cortisol concentrations were higher when animals were closer to villages. Although villages had the smallest effect size in the AIC-optimal model, removing villages from the model did not improve the model, therefore it remained a significant predictor of cortisol. This supports the findings from other studies that have investigated the relationship between proximity to humans and stress. For instance, a study on African elephants (*Loxodonta africana*) reported that faecal glucocorticoid metabolite levels were higher when animals ventured outside the boundary of the Serengeti National Park (Tingvold et al., 2013). It is theorised that long-ongoing hunting activity has led the elephants to associate humans with detrimental effects. Additionally, a study on African lions (*Panthera leo*) by Creel, Christianson and Schuette (Creel et al., 2013) found faecal cortisol concentrations were negatively correlated with proximity to humans, and that lions respond to seasonal movements of people, by moving away from human settlements as they become occupied.

We did not detect an effect of fences on stress within a 2km buffer of the fence. This implies that either interactions with fences do not increase chronic stress, or that they did increase stress but only for a short period of time that was insufficient to be detected. Furthermore, the relatively small number of animals

that currently run into and become ensnared in fences are likely to die as a result. Therefore, cortisol concentrations may not be the most effective method for determining the effect of fences on animals. As the number of fences erected in the Maasai Mara increases (Løvschal et al., 2017), the number of animals becoming entangled in fences is also expected to increase, making this an issue of growing importance.

3.5.1.3 Predation Risk

It was not possible to collect any data directly on predator density within the Serengeti and Maasai Mara ecosystem, therefore predation risk was inferred through indirect measures of environmental variables known to correlate with predator density (*distance to drainages*, *proximity to thick vegetation*, *distance to large rivers with permanent water*, and *lunar phase*). Although a number of predation risk covariates were included in the analysis, none were significant predictors of hair cortisol concentrations. However, this may be a result of differences in the resolution between the stressor and our measure of stress. Acute changes in stress associated with predation may be too brief and infrequent to show a difference in such chronic measures as those obtained from hair. For instance, a study on the effects of the reintroduction of wolves (*Canis lupus)* on elk (*Cervus elaphus*) populations in the Greater Yellowstone Ecosystem found changes in predator avoidance behaviour (e.g. increased time spent vigilant, decreased time spent grazing), but no change in faecal glucocorticoid metabolite (FGM) concentrations (Creel et al., 2009). The authors suggest that, since elk typically detect wolves and take action to avoid them, perhaps the hypothalamic-pituitary-adrenal response associated with predation is too small to be detected in more chronic measures of stress, such as FGM concentrations. Of additional interest is Creel's control of risk (COR) hypothesis: that proactive responses to predictable aspects of risk generally have food-mediated costs, while reactive responses to unpredictable aspects of predation risk generally have stress-mediated costs (Creel, 2018). Finally, our variables are only proxies of risk, developed for lions (*Panthera leo*) (Hopcraft et al., 2005). It may be possible that our proxies do not sufficiently capture the threat of predation by lions, or that only considering lions is insufficient when measuring predation

threat. Spotted hyenas (*Crocuta crocuta*) outnumber lions in the Serengeti and are also successful predators of wildebeest, with around a third of all hunting attempts resulting in prey capture (Holekamp et al., 1997). However, since hyenas are cursorial predators rather than ambush predators and hunt by chasing prey, we were unable to infer hyena predation based on landscape predictors, so we may be missing additional factors that contribute to a wildebeest's perception of risk.

Lunar phase (a proxy of night-time brightness and predation risk) was also investigated as a potential source of stress, but this did not influence hair cortisol concentrations in wildebeest. Lunar phase is known to cause changes in the nocturnal anti-predator behaviours of wildebeest (Palmer et al., 2017). However, our results indicate that either anti-predator behaviours that are associated with the lunar phase are not driven by activation of the HPA axis, or that the size of the effect is too small to be detected in chronic measures of stress.

3.5.1.4 Life History Stressors

Neither age, reproductive status, nor parturition had detectable effects on hair cortisol concentrations. However, our data on reproductive status were incomplete. Due to the abundance of carnivorous animals in the Serengeti, most carcasses are reduced to only hair, skin and bone within hours of death. Therefore, it was not possible to obtain data on reproduction for our samples taken from carcasses (such as pregnancy, or luteal structures in the ovaries).

3.5.1.5 Carcass Samples

Hair cortisol concentrations were found to be higher in samples obtained from carcasses, compared to those collected from living animals. This result could indicate that there is an increased risk of mortality in chronically stressed animals. Such a relationship between stress and risk of mortality is supported by studies in humans (Ohlin et al., 2004). This may be further amplified in juveniles, with research on zebra finches showing exposure to elevated stress

hormones in early life causes a reduction in adult lifespan and increases stress reactivity (i.e. an exaggerated and prolonged response to acute stress) (Monaghan et al., 2012). In this regard it is interesting to note that in addition to the hair collected from carcasses having higher cortisol concentrations, these animals also differed in how they responded to environmental variables associated with nutrition and human disturbance. Specifically, in samples collected from live animals, cortisol concentrations decreased with increasing vegetative greenness (NDVI), as might be expected if abundant food reduced stress. However, in samples taken from carcasses the opposite trend was observed (Fig. 3.5). While this may seem counterintuitive, as carcass samples had higher HPA activity when there was more vegetation, it has been reported that wildebeest move larger distances when grazing during the wet season (when the quality of grazing is at its peak) (Hopcraft et al., 2014). This increased movement is likely driven by large, dense groups of competing individuals (up to 1.65 million grazers), which rapidly deplete localized grazing opportunities. The positive correlation between NDVI and hair cortisol in samples taken from carcasses may therefore be explained by the need to move constantly between patches of high quality, low abundance food in the wet season (when wildebeest occupy high-NDVI areas), which may have induced additional stress in chronically stressed or unhealthy individuals (carcass samples), while these mobility costs may be negligible in individuals that were alive and in an outwardly healthy condition at the time of sampling.

Although living and carcass samples and their interaction with NDVI remained in our AIC-optimal model, these results should be interpreted with caution, due to our limited number of samples when comparing between living animals (n=23) and carcasses (n=6). Additional measurements of animal health (body fat condition, disease status, etc.) could provide additional information about an animals' physiological status and their reaction to different situations, in future studies.

3.5.2 **Ecological knock-on effects of elevated cortisol**

The Serengeti is under increasing pressure particularly due to unprecedented human modification of the landscape (Veldhuis et al., 2019). The results of this study indicated that proximity to villages was significantly correlated with hair cortisol concentration in wildebeest. While this is an important finding, as it indicates a quantitative effect of human presence on wildebeest physiology, it does not provide a complete picture as there will be other factors that we did not measure that will also contribute to the observed variation in hair cortisol concentrations. For example, the detrimental effects of roads on large mammals (especially animals with large movement ranges), specifically as a result of landscape fragmentation, is well documented (Fahrig and Rytwinski, 2009). Resident wildebeest populations in ecosystems neighbouring the Serengeti avoid roads, which amplifies habitat loss and is a likely mechanism contributing to widespread population declines across much of their range over the past few decades (Stabach et al., 2016). Despite this, plans for the construction of a road across the northernmost region of Serengeti National Park are underway (Hopcraft et al., 2015a). This would not only disrupt access to the essential dry season refuge in the northernmost region of the ecosystem but would also likely lead to an increase in road traffic deaths and poaching. Additionally, since this restriction of movement would occur at times when chronic stress in wildebeest is inherently high, any detrimental effects caused by the road would be inflicted at a time when animals are most vulnerable, potentially with catastrophic population consequences.

Our data suggests the northern Serengeti and Masai Mara is the location of greatest concern for conservation and the location where conservation efforts should be concentrated in order to protect the migration. This is due to these areas being close to villages, and also the area where wildebeest experience lowest NDVI (since migrating wildebeest only occupy this region in the dry season); all of which cause a significant increase in chronic stress in samples taken from living individuals. It is possible that this large source of stress could alter the behavioural decisions of the animals, further modifying their migratory path, especially as fencing in the Masai Mara continues to reduce the dry season

range for both the Kenyan and Serengeti wildebeest populations (Serneels and Lambin, 2002, Løvschal et al., 2017).

3.5.3 **Future Work**

The results of our analysis could be improved by analysing metabolites at finer temporal scale. Each 8mm section of hair provides a single measure of cortisol integrated over a 14-day period and as a result we may not be able to detect fine scale effects. For example, since the effect of some stressors tends to be binary (e.g. presence or absence of fences, predators, etc.), the disparity in the detection of acute versus chronic stressors could be mitigated by reducing the size of each hair section, to get finer temporal resolution. However, this could increase the error involved with sectioning the hair (for example, 0.5mm error in cutting a section of hair equals 6.25% error in an 8mm section, but 12.5% error in a 4mm section). In addition, less biomass per sample may result in issues related to assay sensitivity, as cortisol is deposited in relatively low concentrations in hair.

The accuracy of explanatory variables such as fences could be improved by including a temporally variable element (as with NDVI, which is measured every 16 days). Future studies could benefit by measuring these variables at different timepoints to create dynamic, rather than static, layers. Additionally, by comparing the effects of some variables (e.g. fences) over shorter timeframes (e.g. effect of proximity to fences when in the Mara), or by incorporating movement decisions into models, may give a better understanding of how the wildebeest are affected by these variables.

Other factors which could be significant explanatory variables but were not measured in this study include metrics of fitness, such as disease status; as well as alternative predation metrics, such as proximity to roads or kopjes (rocky outcrops selected for by lions). Including these covariates may further improve the power of future models of stress. Additionally, stable isotope data from the same hair samples (sample preparation is the same) would provide further metrics of physiological condition. For instance, nitrogen stable isotope can be

used to inform about starvation patterns (Rysava et al., 2016). This would enable us to disentangle the effect of hunger from other environmental or anthropogenic stressors such as proximity to villages.

Additionally, the results of our statistical model determining the link between cortisol and aldosterone shows value in studying multiple hormones in one study. We found aldosterone and cortisol to be positively correlated, with resident wildebeest showing an effect size five times greater than migrants. This indicated that prolonged periods of sodium-stress (i.e. high aldosterone concentrations) are a source of chronic physiological stress in wildebeest (i.e. high cortisol concentrations), and that sodium-stress is a far greater source of physiological stress in resident (compared to migrant) wildebeest. Since resident wildebeest are always in areas containing sodium-rich grasses, sodium-stress may be indicative of a lack of forage in general (i.e. that an animal is starving). On the other hand, migratory wildebeest are more likely to experience periods of high volume, low sodium forage (i.e. depleted sodium, but not starving). This is the first study to discover a link between sodium availability and physiological stress, as measured in aldosterone and cortisol concentrations, respectively. These preliminary results highlight the value in investigating the relationship between different physiological markers that can be measured in hair.

3.6 **Conclusions**

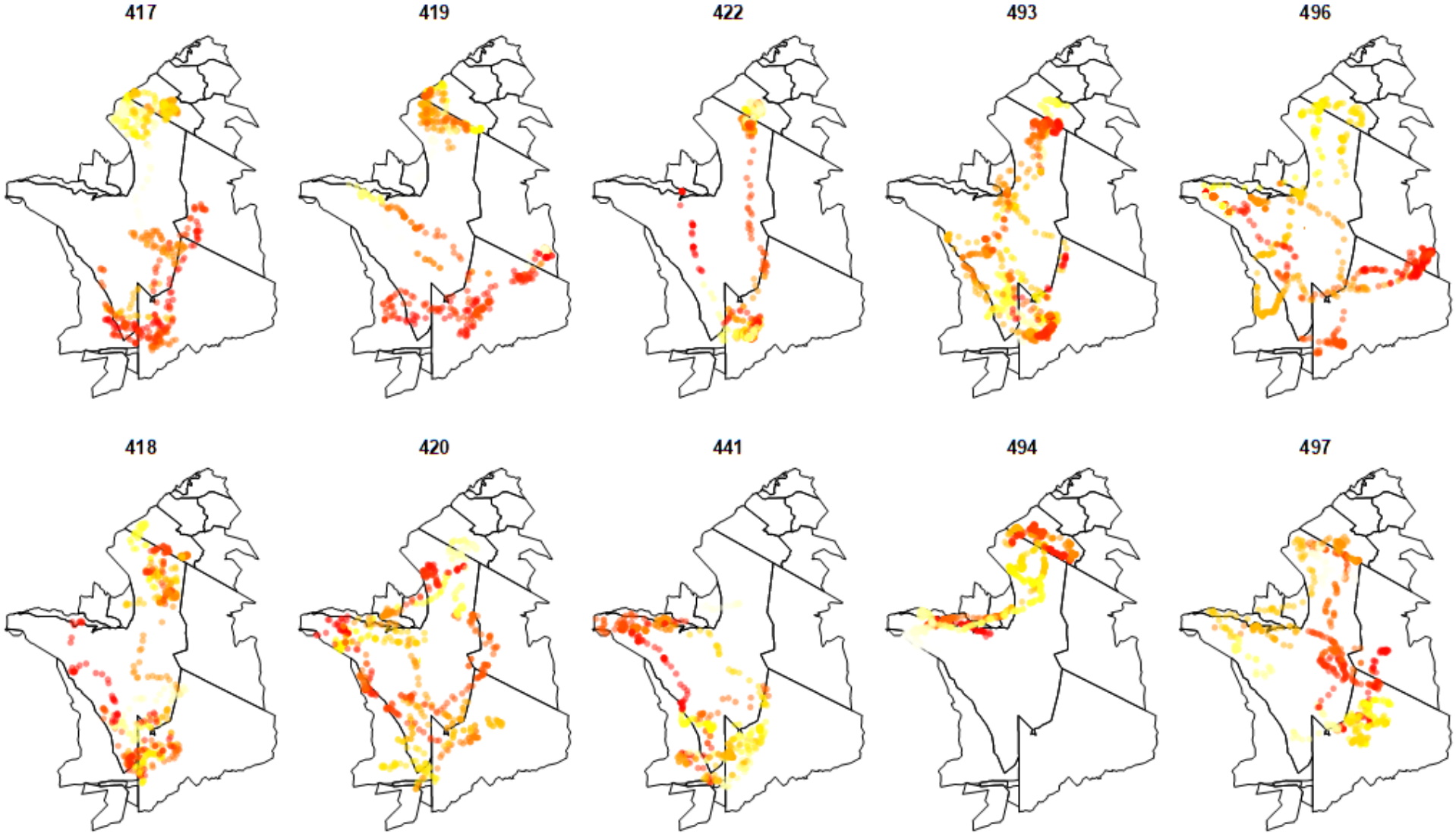
The results of this study show variations in cortisol concentrations in wildebeest tail hair over time are associated with both environmental and anthropogenic factors. Notably, we found that proximity to villages represents an important anthropogenic source of chronic stress in our wildebeest. Hair cortisol concentration was also found to be elevated in samples obtained from carcasses and these individuals responded differently to NDVI, further supporting the use of hair cortisol analysis to assess physiological wellbeing and health. Additional steroid hormones (e.g. progesterone, aldosterone) involved in the regulation of a variety of physiological processes can also be extracted from tail hair (Buchanan,

unpubl. data), providing a more detailed picture of an animal's physiological state over time and space.

3.7 **Supporting Information**

Table 3.2 (supplementary) Comparison of models explaining variation in hair cortisol levels in 880 sections of hair from 26 female wildebeest in the Serengeti Ecosystem, Tanzania. Sample ID was also included as a random effect. Models ranked by AIC and log likelihood, with the optimal model in bold.

Model	AIC	ΔAIC	Log Likelihood
Village + NDVI + Dead.Alive + NDVI*Dead.Alive + Days.Exp.Env. + (1|sample.ID)	**1483.921**	**-**	**-733.960**
Village + NDVI + Dead.Alive + Days.Exp.Env. + (1|sample.ID)	1484.741	0.820	-735.370
Days.Exp.Env. + (1|sample.ID)	1492.409	8.488	-742.205
*Village + NDVI + ΔNDVI + Dead.Alive + AvgNDVI*Dead.Alive + ΔNDVI*Dead.Alive + Days.Exp.Env. + (1|sample.ID)*	1493.537	9.616	-736.769
NDVI + Days.Exp.Env.+ (1|sample.ID)	1496.249	12.328	-743.124
*Biomass + SeasonWater + Village + NDVI + ΔNDVI + Dead.Alive + NDVI*Dead.Alive + ΔNDVI*Dead.Alive + Days.Exp.Env. + (1|sample.ID)*	1504.874	20.953	-740.437
*Biomass + River + VegCov + Water + Village + NDVI + ΔNDVI + Age + Dead.Alive + VegCov*Dead.Alive + NDVI*Dead.Alive + DeltaNDVI*Dead.Alive + Days.Exp.Env. + (1|sample.ID)*	1526.935	43.014	-747.467
*GrassN + Biomass + River + VegCov + Water + Village + Fence + Tourism + NDVI + ΔNDVI + LunarPhase + Age + Res.Mig + Dead.Alive + GrassN*Dead.Alive + Biomass*Dead.Alive + River*Dead.Alive + VegCov*Dead.Alive + Water*Dead.Alive + Village*Dead.Alive + Fence*Dead.Alive + Tourism*Dead.Alive + NDVI*Dead.Alive + ΔNDVI*Dead.Alive + LunarPhase*Dead.Alive + Days.Exp.Env + (1|sample.ID)*	1602.866	118.945	-772.433
1 + (1|Sample.ID)	1980.938	497.017	-987.469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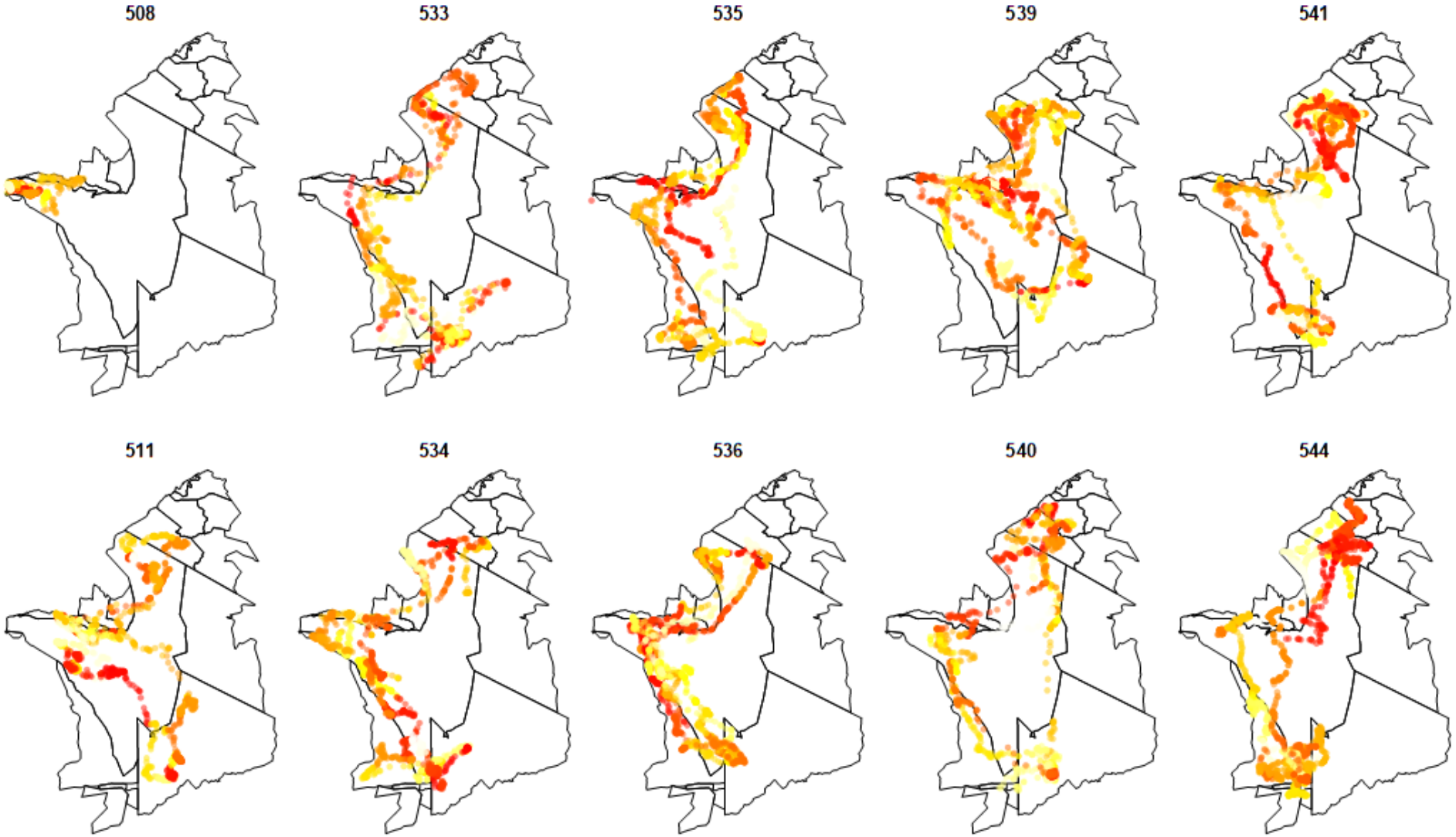
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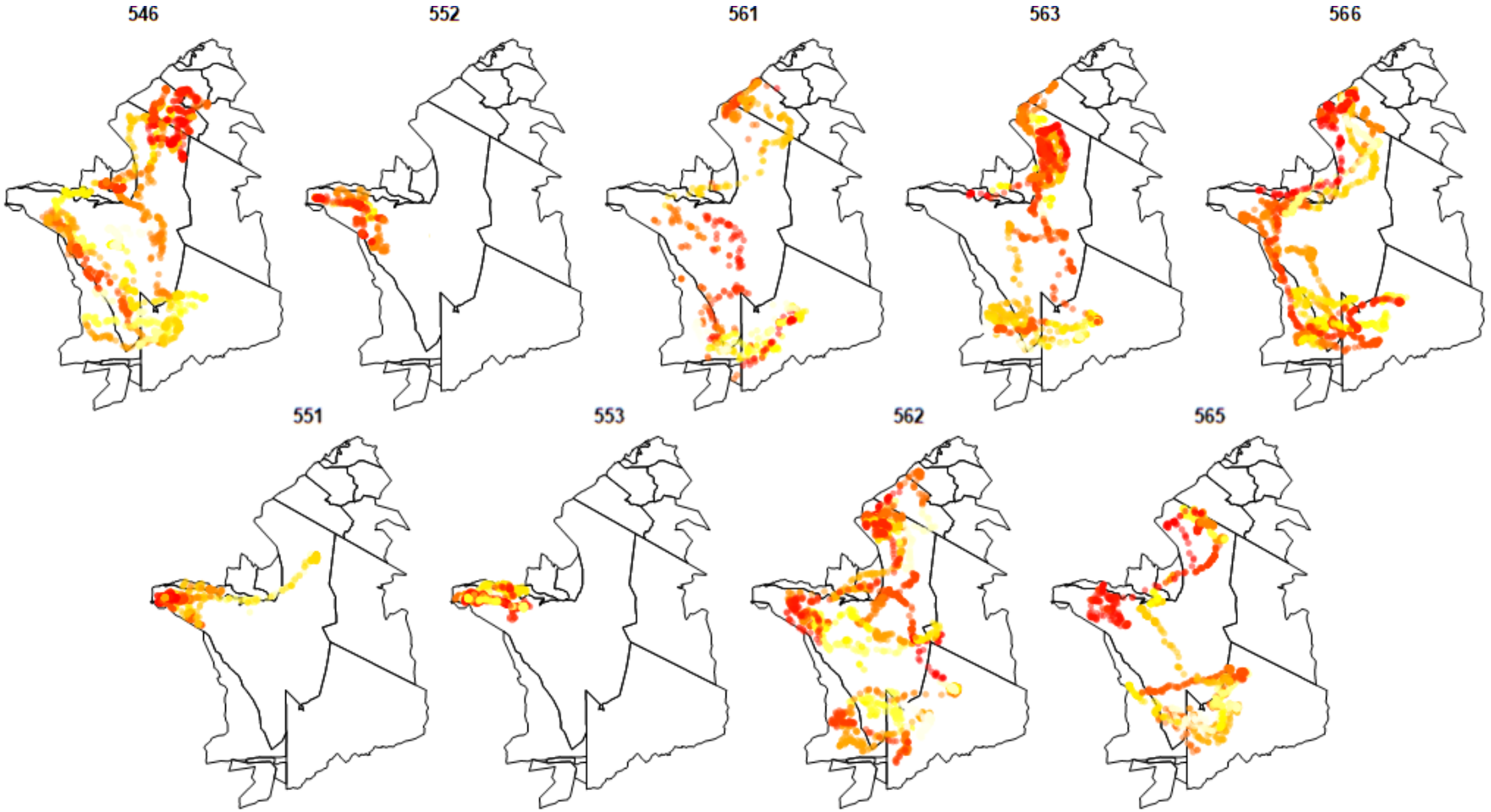


Figure 3.7 (supplementary) **Sample number and corresponding map of GPS-collared wildebeest hair cortisol concentrations plotted relative to their locations within the Serengeti-Mara ecosystem, for all samples (n=29). Dark red indicates high cortisol; light yellow indicates low cortisol.**

4 Inferring Reproductive Status from Hormone Concentrations in Segmented Wildebeest Tail Hair

4.1 Abstract

Some of the most significant changes in the endocrine environment of female mammals occur as a result of changes in reproductive state. Information on reproductive history is highly valuable when studying physiology, however it is difficult to obtain in wild animals. By measurement of the concentration of hormones which regulate reproductive function, in longitudinal sections of a hair sample, it may be possible to produce profiles of reproductive history from a single sample. This chapter tested whether progesterone, oestradiol, oestrone, and prolactin could be profiled in tail hair samples taken from free-living adult wildebeest (*Connochaetes taurinus*) in the Serengeti-Mara Ecosystem, East Africa. Progesterone and oestradiol were measurable, and varied in concentration, along the length of each hair sample. Prolactin and oestrone were not measurable in our system. Oestradiol showed a slight peak in concentration at the predicted time of parturition in only two of five animals sampled. This is likely due to disparity in the duration of the oestradiol peak that initiates parturition (hours-days) and the temporal resolution of our hair sections (two weeks). The results showed that hair progesterone concentrations tended to increase as a result of exposure to heat and light in the environment but that it is possible to correct for this post hoc, using relative metrics compared against a long-term mean. Corrected progesterone concentrations were significantly higher during the time between the rut and calving period ($n=444$; $\beta = 0.22 \pm 0.08$; $t = 2.67$), which corresponds to the time when most migratory wildebeest are pregnant. This technique shows promise for determining reproductive trends in wild animals, which may be more reliable in animals that occupy cooler environments, where reproductive steroids should be more stable.

4.2 **Introduction**

Hormones regulate a wide variety of developmental and physiological processes in the body. The relative concentrations of specific hormones can be measured in a variety of sample media (e.g. blood, tissue, hair, etc), which can give information relating to a variety of underlying physiological processes. Most hormones serve a variety of functions, which can vary with life history stage. For instance, one of the most significant factors that influence changes in the endocrine environment in a female mammal is reproductive state. Reproductive hormones are typically low during the prepubertal period and rise (and in some species, cycle) when animals become reproductively competent. However, some of the largest changes are associated with pregnancy. Relatively soon after conception, the placenta is formed. In addition to provisioning nutrients to the foetus, the placenta also secretes hormones that alter the physiology, nutrient metabolism, and anatomy of the mother. This supports foetal growth and development, maintains maternal homeostasis, and prepares for lactation (King, 2000). Placental hormones also affect the secretion of additional maternal hormones, including cortisol, which has been shown to be three times higher than baseline levels, during the third trimester in humans (Gardner and Shoback, 2011). Further, hormones produced by the fetal-placental unit also alter the function of a range of maternal endocrine glands. Pregnancy can cause changes in nutrition, both in terms of overall intake and specific micronutrient requirements (Lammi-Keefe, 2009), and can have important effects on animal behaviour. Therefore, any study which seeks to investigate nutrition and behaviour in females would benefit from measures that can indicate the animal's reproductive status. Therefore, in order to understand the underlying physiology of an individual, the first big question which should be answered is whether or not an animal is pregnant.

Although the primary literature on the reproductive physiology in domestic and captive mammals is extensive, much less is known about reproduction in wild populations; primarily as a result of difficulties in sampling. While observational data can be a valuable source of information on reproductive status in wild mammals, in many cases it is difficult to visually assess an individual's reproductive state. For instance, since wildebeest calves follow their mother

until they are 8-12 months old, observations of a female wildebeest with a calf indicates that she has given birth within the last year, and the size of the calf can be used to determine the approximate parturition date. Approximately 20% of wildebeest calves die within the first month of life (Estes, 1991); therefore, the absence of a calf does not confirm absence of a pregnancy. In previous chapters we have shown how wildebeest tail hair samples can be sectioned and cortisol can be measured to create stress profiles for the preceding 18 months. If this technique can be validated for hormones which regulate reproduction, it may also be possible to create 18-month profiles of reproductive history. This would inform if, and when, individuals became pregnant, calved, or miscarried.

A number of different hormones regulate reproductive function and may be detectable in sufficient concentrations to allow inference of reproductive state. Hormones can be split into four main classes: catecholamines, eicosanoids, steroids, and protein derivatives. Steroids are the smallest and most stable group of hormones, and the only group which has been shown to be able to pass from the circulatory system and become incorporated into hair, as it grows. Steroid hormones are synthesised from cholesterol and include: the corticosteroids and mineralocorticoids (e.g. cortisol, aldosterone), which are generally synthesised in the adrenal cortex; and the sex steroids (e.g. progesterone), which are typically produced in the gonads, placenta, and adrenal glands. Cortisol is the only corticosteroid which has been shown to correlate reliably with changes in reproductive state. A spike in cortisol has been shown to occur immediately preceding parturition, in a variety of species, including cattle (Hudson et al., 1976), sheep (Magyar et al., 1980), dogs (Concannon et al., 1978), and humans (Ohrlander et al., 1976). Unfortunately, it was not possible to detect such a brief cortisol peak in our samples (Chapter 3); likely due to the temporal resolution of our data being too low (averaged across fortnights), as well as the high variation in cortisol over time because of its primary role in the stress response. No studies have successfully demonstrated a link between mineralocorticoid concentrations and reproductive state. However, several sex steroids play a role in the regulation of the reproductive system, which allows a variety of information on reproduction to be inferred from their relative concentrations in the body. Sex steroids can be sorted into three main structural groups: androgens, oestrogens, and progestogens. The primary

function of androgens is to regulate the development and maintenance of male characteristics, so they cannot be used to inform of female reproductive state. However, both oestrogens and progestogens are involved in the regulation of female reproduction.

4.2.1 **Oestrogens**

There are four main endogenous oestrogens in mammalian physiology: oestradiol, oestrone, oestriol, and oestetrol; all of which are composed of an 18-carbon skeleton. These undertake a variety of roles in the female reproductive system, including: triggering and maintenance of oestrus (or menstrous); stimulation of follicular growth; and optimisation of uterine conditions for embryonic development (Gruber et al., 2002, Garverick and Smith, 1993). The role of oestrogens in the regulation of metabolic, behavioural and morphological changes during the different stages of reproduction makes them potential biomarkers of reproductive status. During periods of ovarian cyclicity oestrogen concentrations would be expected to fluctuate in association with the growth of ovarian follicles but it is likely that such variation may not be detectable in an integrated 2-week sample although it may be elevated relative to the anoestrous season. However, a more definitive marker may be provided by the dramatic changes in oestradiol concentrations that are required for the initiation of parturition. The spike in cortisol that occurs immediately preceding parturition induces synthesis of placental enzymes that convert progesterone into oestradiol (the primary oestrogen). This switch from progesterone dominance during pregnancy to oestradiol dominance at parturition enables muscles in the uterus to contract (which were previously inhibited by progesterone), inducing labour (Sjaastad et al., 2010). A number of studies have successfully detected changes in reproductive state by measuring oestrogen concentrations, in several sample media, including plasma (Henricks et al., 1972), urine (Mellin et al., 1966), faeces (Chapeau et al., 1993), and even milk (Heap and Hamon, 1979). Both oestrone and oestradiol have been measured in human hair (Choi et al., 2000, Yang et al., 1998), however no studies have investigated changes in hair oestrogen concentration associated with different reproductive stages/states. If oestrogens are stable in hair, and the peak that occurs at parturition is sustained

for enough time, it may be possible to detect it in sections of hair grown during parturition.

4.2.2 **Progesterone**

Progesterone is the primary mammalian progestogen (and major female sex hormone), named for its essential role in maintaining pregnancy. It is responsible for a variety of reproduction-related activities, which include promotion of development of a thick lining of blood vessels and capillaries in the uterus to sustain the growing foetus and changes in thermoregulation and carbohydrate, protein and lipid metabolism (Siiteri et al., 1977). Progesterone increases at conception and remains elevated throughout pregnancy, before finally returning to lower, baseline levels at parturition (Radwanska et al., 1978, Sjaastad et al., 2010). Therefore, if progesterone is stable in hair samples, measurement of hair progesterone concentrations should inform approximate conception and parturition dates, as well as gestation length. Additionally, since increases in progesterone occur over long periods of time (gestation), in comparison to oestrogens and cortisol (parturition), these changes should be easier to detect in sample media such as hair, which reflects chronic hormone concentrations. Since progesterone also increases during the luteal phase of the oestrous cycle (short, shallow peaks), changes in hair progesterone concentration may also be detectable in sections of hair grown when the animal is in the breeding season. A study on rhesus macaques (Dettmer et al., 2015) found higher hair progesterone concentrations in samples collected during pregnancy, in comparison to samples collected post-pregnancy. Hair progesterone has also been measured (and appears to be stable) in humans (Yang et al., 1998), where concentrations of progesterone along the hair were found to be correlated with serum progesterone concentrations during the period of hair growth. While these studies would support the proposition that hair progesterone may be a useful longitudinal measure of reproductive state, a study on dairy cattle found no significant correlations between hair progesterone concentrations and reproductive status, days of gestation, number of lactations, or days in milk (Tallo-Parra et al., 2018).

Although cortisol is known to be stable in wildebeest tail hair over time (Chapter 2), this is not necessarily the case for all steroid hormones. For example, cortisol is not a precursor to any additional steroids in the steroid metabolic pathway. However, progesterone is a direct precursor to several other progestogens, all of which are very similar to progesterone, in terms of molecular structure. Although progesterone is mainly secreted by the corpus luteum, it can also be produced by the extracellular conversion of cholesterol, cholesterol esters, pregnenolone, pregnenolone sulfate, and adrenal steroids (Ramaley, 1972). Therefore, it may be possible, given enough energy and time, that some progestogens lose chemical groups gained during steroidogenesis, and effectively revert back to their precursor molecule. If these reactions occur at a constant rate, this effect would introduce trends in our data, with regards to time spent in the environment (e.g. a decrease in progesterone if it reverts to its precursor (pregnenolone), and an increase in progesterone if its products reverse), which would have to be corrected.

A variety of protein-derived hormones are also involved in regulating reproductive function, and their quantification could also be used as indicators of reproductive status. For instance, the presence of human chorionic gonadotropin (hCG) (produced by the placenta following implantation) in urine or blood, is extensively used to detect human pregnancies. However, hCG, (a glycoprotein hormone composed of 247 amino acids) is much larger and much less chemically stable than the steroid hormones and there are no available published studies which have documented protein-derived hormones in hair.

4.2.3 **Prolactin**

In many species mortality rate is highest during the postnatal life stage. For example, wildebeest calf mortality rates within the first month after birth average 25.1% in aggregations and 58.9 % in small herds (Estes and Estes, 1979). Since mothers will cease lactation when suckling stops, the nutritional stress associated with lactation will not be present in individuals who gave birth, but whose offspring were predated. Measurement of hormones associated with

lactation, would therefore be advantageous as they could allow offspring survival, as well as maternal nutritional stress, to be inferred.

The primary hormone which regulates lactation is prolactin. In general, prolactin increases at parturition, remains elevated throughout lactation, and returns to baseline levels after weaning. It has been measured in pituitary tissue, urine, serum, and milk samples (Lewis et al., 1971, Gala et al., 1975) and exists in three structural isoforms, the smallest of which is a single polypeptide chain composed of 198 amino acids. Although the molecular mass of prolactin is approximately 70 times larger than steroid hormones, no smaller alternative potential indicator of lactation is known. Additionally, since prolactin is a protein hormone, there may be small interspecific variations in its molecular structure (i.e. human prolactin likely differs from bovid prolactin). Therefore, it may be necessary to obtain an ELISA containing a species-specific variant of the antibody, in order to quantify prolactin.

4.2.4 **Serengeti Wildebeest**

The Serengeti-Mara wildebeest migration is an ideal system to study the time-series of reproductive history using hair samples. Wildebeest are highly synchronous breeders. Mating occurs late May to early June (Sinclair, 1977), with only 5% of adult female wildebeest estimated to remain unbred each year (Estes and Estes, 1979). 250,000 calves (over 80% of all calves) are born in a three-week window beginning in late February (Estes, 1976). Faecal progestogen concentrations in semi-free-living wildebeest have been documented to vary in association with ovarian activity and pregnancy, and have been used to determine the average duration of the luteal, oestrous, gestation, and post-partum anoestrous phases (14.3 ± 0.5, 22.6 ± 1.0, 240.8 ± 11.7, and 104.1 ± 15.6 days, respectively (Clay et al., 2010). Therefore, it is likely that hair samples obtained from adult female wildebeest (which contain approximately 18 months of growth) should contain hair grown during each stage of reproduction (e.g. pregnant, not pregnant, cycling). Through analysis of sequential progesterone concentrations along a hair sample, and using the growth rate to date each section, it should be possible to obtain baseline concentrations from sections of

hair grown between the calving period and the rut (i.e. when the animal is not pregnant) and compare these values to periods when the animals are pregnant.

Calf survival is an important metric that determines the population's capacity to grow, however it is difficult to infer without direct observations of mothers and their offspring. For example, the massive, synchronous pulse of wildebeest calves born in a relatively short period of time undermines the ability of predators to regulate wildebeest recruitment (Hopcraft et al., 2015b). However, mean annual proportional calf survival rates are relatively low; 0.84 and 0.44 in migratory and resident populations, respectively (Ndibalema, 2009). Therefore, a method to infer calf survival for individual mothers, such as metrics of lactation, would be hugely valuable insight for population ecology and could be used to identify critical bottlenecks in population recruitment.

4.2.5 **Aims and Hypotheses**

This study aims to determine whether oestrogens, progesterone, and prolactin occur in measurable quantities in the tail hair of wildebeest and whether the sequential deposition of these hormones in the tail hair can be used to create the reproductive history for individual animals in time-series.

We hypothesise that oestrogens will be highest in sections of hair grown at parturition. We also hypothesise that progesterone will increase at the rut (June), as females begin cycling. Small increases in progesterone may also be detected as the wildebeest go into oestrus, before becoming pregnant. Once conception occurs, progesterone concentrations will increase further and remain elevated until the calving season (February-March), when they will return to baseline levels, in individuals who carried their calf to term. In animals who miscarry, progesterone will drop to baseline levels before expected parturition (approximately 240 days after conception). Prolactin will increase at parturition, and remain elevated until the calf is predated, or weaned (8-12 months later), when it will return to baseline levels.

4.3 Materials & Methods

4.3.1 Study Area and Animals

Tail hair samples were collected from 23 (22 female; 1 male) free-ranging blue wildebeest (*Connochaetes taurinus*) that occupied the Serengeti Mara ecosystem, East Africa (1°15' to 3°30'S, 34° to 36°E). Samples were obtained from both carcasses (n=10) and living animals (n=13), from 2012-2018. All animals sampled were adults, although we did not have data on the specific age for all animals, so age could not be included in statistical modelling. Samples were collected, washed, cut into 8mm sections, and ground, as described in Chapter 2. Dates of growth for each section of hair were also calculated using the hair growth rate and method outlined in Chapter 2.

4.3.2 Validation of ELISAs

Since no ELISA kits were specifically validated for quantification of hormones extracted from our sample media (hair) and species (wildebeest), each type of kit used was first validated. For all ELISA kits that were designed to measure steroid hormones, approximately 300mg of powdered wildebeest tail hair was extracted for 16 hours in 2ml of 100% HPLC Grade Methanol, in a heated orbital shaker (Stuart orbital incubator S150 (Cole-Parmer, Staffordshire, UK)) set to 52°C, 200rpm. Vials were weighed before and after the extraction process, to allow subsequent correction for any evaporation that occurred during the extraction process. A 1700ul aliquot of solvent was dried down (Savant SC210A speedvac concentrator (Thermo Fisher Scientific, Renfrew, UK; medium heat for six hours) in a borosilicate glass tube. Once dried, the extract was resuspended in 500ul of assay buffer, which was then used to create a serial dilution, with a total of ten dilutions. These samples were assayed in duplicate in the ELISA kit, according to the manufacturer's instructions. Dilutions which fell within the measurable range of the ELISA (between ED_{20} and ED_{80} values) were checked for parallelism to the standard curve by plotting percentage bound against log

concentration. Parallelism between the gradient of the line of best fit for the standards and the serial dilution produced from the samples indicates that the hormone of interest can be measured accurately using this method (as described in chapter 2.3.4). The results of the serial dilution of the extract also indicated the optimal mass of hair and dilution of the extract required to accurately estimate the concentration of the hormone of interest, using each ELISA kit. All hormones were expressed in pg of hormone per cm of hair.

4.3.2.1 Oestrogens

An oestrone ELISA was obtained from IBL International GMBH (Hamburg, Germany), and tested for parallelism as described above. The manufacturer reports that the cross-reactivity of the antibody is: oestrone (100%), oestrone-3-sulfate (4.9%), 17ß-oestradiol (2.2%), oestrone-3-glucoronide (1.2%), 17ß-oestradiol-3-glucoronide (0.14%).

An oestradiol ELISA kit was obtained from R&D Systems (Abingdon, UK). The manufacturer reports that the cross-reactivity of the antibody is: oestradiol (100%), 17α-ethynyloestradiol (0.07%), 17ß-oestradiol 3-benzoate (0.03%), oestriol (0.86%), oestrone (0.26%), progesterone (0.06%). The following substances were also assayed at 50ng/ml, and showed no cross-reactivity: 5α-androstan-17ß-ol-3-one, 6ß-hydroxytestosterone, 11α-hydroxytestosterone, ß-oestradiol 3-(ß-D-glucuronide), ß-oestradiol 3-sulfate, androsterone, aldosterone, cortisol, cortisone, deoxycorticosterone, epitestosterone, oestrone 3-sulfate, prednisolone, prednisone, testosterone, trans-dehydroandrosterone.

No commercially available/suitable ELISA kits were identified for oestriol or oestetrol.

4.3.2.2 Progesterone

A progesterone ELISA was purchased from Enzo Life Sciences (Lausen, Switzerland). The manufacturer reports that the cross-reactivity of the antibody is: progesterone (100%), 5α-pregnane-3,20-dione (100%), 17-OH-progesterone (3.46%), 5-pregnen-3ß-o1-20-one (1.43%), corticosterone (0.77%), 4-androstene-

3,17-dione (0.28%), deoxycorticosterone (0.056%), dehydroepiandrosterone (0.013%), 17ß-oestradiol (<0.001%), oestrone (<0.001%), oestriol (<0.001%), testosterone (<0.001%), hydrocortisone (<0.001%), 5α-pregnane-3α,20α-diol (<0.001%), danazol (<0.001%).

In comparison to other hormone ELISAs, progesterone ELISA kits tended to have higher cross-reactivity with other steroids. The antibody in the progesterone ELISA used in this study showed 100% cross-reactivity with both progesterone and 5α-pregnane-3,20-dione (an endogenous progestogen), however, these molecules have been shown to correlate strongly in humans (Milewich et al., 1977). Therefore, since we are only interested in determining information on reproductive history, it was decided that combined measures of progesterone and 5α-pregnane-3,20-dione should still be indicative of changes in reproductive state.

4.3.2.3 Prolactin

Since prolactin is a peptide hormone, it is possible that the molecular structure of prolactin is species specific. Unfortunately, no bovid prolactin ELISA kits are commercially available, therefore a human prolactin ELISA was purchased from Cayman Chemical (Ann Arbor, MI, USA) and its transferability determined. A potentially prolactin-rich pooled wildebeest tail hair sample was produced, by combination of sections of wildebeest tail hair which were grown when circulating prolactin concentrations are highest (during lactation). A similar potentially prolactin-rich human hair sample was also obtained from an individual who had been breastfeeding for 9 months preceding sample collection. The human hair sample was prepared using the same method used for the wildebeest hair.

Since protein hormones are less stable than steroids, the extraction protocol was altered, to minimise degradation of any prolactin that may be present in the hair (i.e. all samples were extracted and dried down at room temperature). Additionally, five different solvents were tested; four of which have been shown to extract prolactin from a variety of sample media, as well as the solvent used for our steroid extractions. These were: 2.5M urea (Haggi and Aoki, 1981); 60%

ethanol (pH 9.9) (Ellis, 1961); 99.8% MeOH:0.2% HCl; 95% EtOH:5% HCl (Fleischer, 1943); and 100% HPLC-grade methanol. The manufacturer reports that the cross-reactivity of the antibody is: prolactin (100%), luteinizing hormone (none detected), FSH (Follicle-Stimulating Hormone) (none detected), hCG (Human Chorionic Gonadotropin) (none detected), TSH (Thyroid-stimulating hormone) (none detected), HGH (Human Growth Hormone) (none detected).

4.3.3 Using Oestradiol Concentrations to Detect Parturition

If the increase in circulating oestradiol that occurs at parturition is large enough (i.e. a large increase in concentration, for a sustained period of time), oestradiol concentrations should be elevated in the section of hair grown at parturition. To test this, oestradiol profiles were created from hair samples grown at the time of parturition, as well as the three sections preceding and proceeding parturition (n=35) for female wildebeest (n=5) who were known to have given birth during the previous calving season (observed with calves at sample collection). Only five of our samples were taken from a wildebeest which we had a record of being with a calf at sample collection, therefore only these animals were used for preliminary oestradiol analysis (as the approximate date of parturition could be estimated).

4.3.4 Using Progesterone Concentrations to Infer Reproductive History

Progesterone profiles were produced for 19 wildebeest (18 female; 1 male), using a total of 448 8mm sections of wildebeest tail hair. Samples were collected from 18 female wildebeest (to determine pregnancy status) and one male (as an additional indicator of the effects of environmental exposure). It was predicted that hair progesterone concentrations would exhibit short, shallow peaks as

animals enter the breeding season, would rise and remain elevated during gestation, and then would fall to the lowest levels during the anoestrous season. Migratory wildebeest are highly seasonal breeders, therefore the largest increase in progesterone was expected to occur between the time of the rut (mid-June) and the calving period (mid Feb - early Mar), after which it would return to baseline levels. Reproductive season was included in our model, with sections relating to dates between the rut and calving seasons as *Possibly Pregnant* and sections grown between the calving season and the following rut as *Not Pregnant*. A hair sample taken from a male wildebeest was also assayed, in order to further test the stability of progesterone in the hair over time (as males should have less variation in progesterone concentrations).

4.3.5 **Statistical Analysis**

All statistical analyses were performed using R version 3.6.1 (R Core Team, 2018).

For each section of hair, we used the hair growth rate to calculate the corresponding dates of growth. Sections of hair were divided into *possibly pregnant* and *not pregnant* dates, relating to whether they were grown between the rut and calving period, or calving period and rut, respectively.

We calculated environmental exposure as the number of days between sample collection and the date that section of hair was grown (i.e. the end nearest the root was grown most recent and therefore was exposed to the environment for a shorter time than more distal sections).

A linear mixed effects model was fitted to the data using package lme4 (Bates et al., 2014) to test the effects of the explanatory variables indicated above. Animal ID was included as a random effect to control for interindividual differences in baseline values.

4.4 Results & Discussion

4.4.1 Validation of ELISA kits for wildebeest hair

4.4.1.1 Oestrogens

Oestradiol was detectable in wildebeest hair extract, and could be quantified accurately, using a commercially available oestradiol ELISA kit (R&D Systems, Abingdon Science Park, Abingdon, UK) (Fig. 4.1(a)) ($r^2 = 0.922$, $p < 0.01$).

Oestrone was measured in wildebeest hair extract, but a lack of parallelism between the serial dilution of samples and standards indicated that the kit could not be used to accurately quantify oestrone in wildebeest hair extracted with our methods (Fig. 4.1(b)) ($r^2 = 0.41$, $p = 0.78$). Wildebeest hair oestrone cannot, therefore, be used to inform of reproductive status.

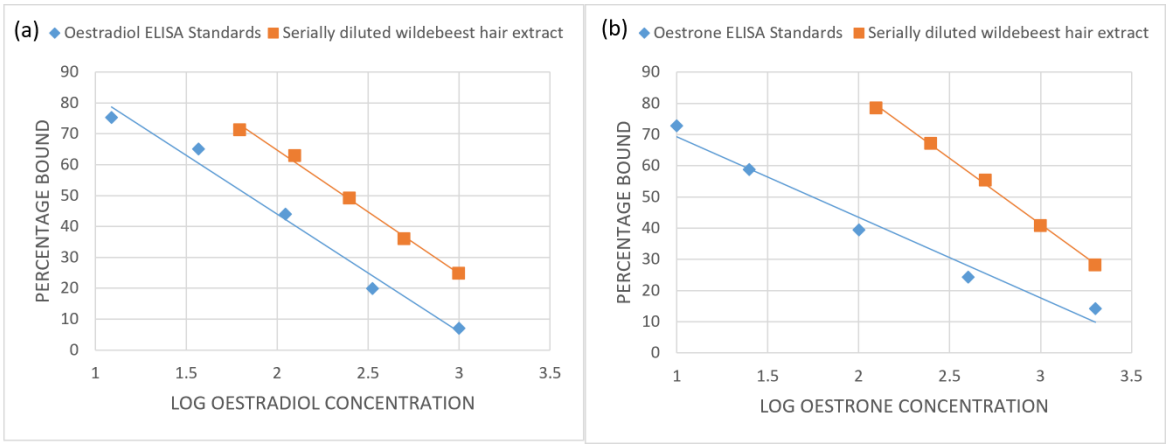


Figure 4.1 Plots of log concentration against percentage bound for the serial dilution of wildebeest tail hair extract and the standard curve for (a) oestradiol and (b) oestrone ELISA kits. Our samples show parallelism with the standards included in the oestradiol ELISA, but not the oestrone ELISA, suggesting only oestradiol concentrations can be measured with accuracy in our samples, using these ELISAs.

4.4.1.2 Progesterone

Progesterone could be measured with accuracy in our wildebeest hair sample extracts, using an Enzo Life Sciences progesterone ELISA (Lausen, Switzerland) (Fig. 4.2), as confirmed by regression analysis ($r^2 = 0.984$, $p < 0.01$).

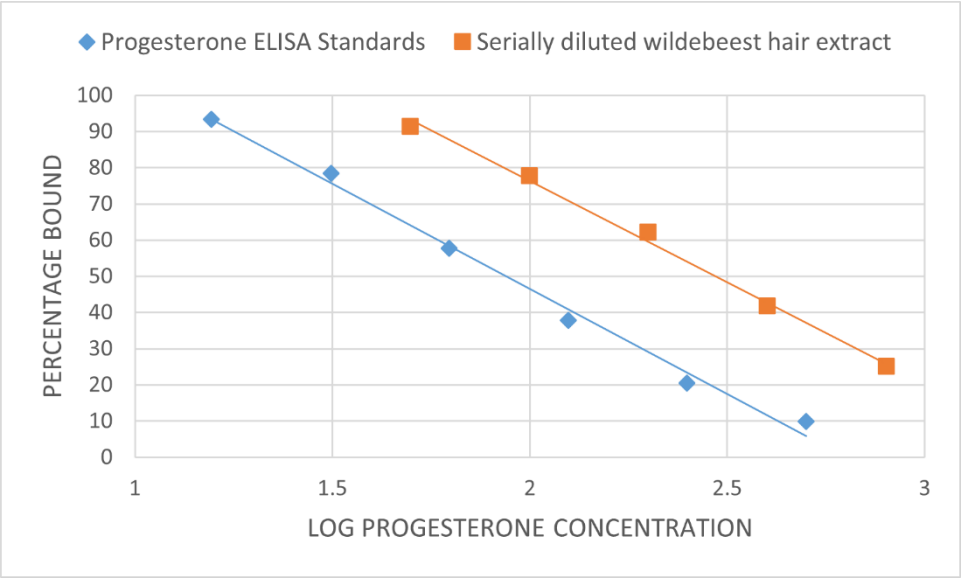


Figure 4.2 Test for parallelism between percentage bound of progesterone standards supplied with ELISA kit and a serial dilution of a pooled wildebeest tail hair extract. Parallelism between the gradient of the lines shows the kit can accurately measure progesterone in our samples, along a range of concentrations.

4.4.1.3 Prolactin

Prolactin could not be detected in either wildebeest or human hair, using any of the five extraction methods outlined above. This suggests that prolactin is not able to pass from the blood and become stably incorporated into hair. Wildebeest hair prolactin cannot therefore be used to inform of reproductive status.

4.4.2 Using Oestradiol Concentrations to Detect Parturition

Hair oestradiol concentrations varied between samples and between different sections from the same sample.

Tail hair samples were collected from five female wildebeest with a calf that was born in the previous calving period. Seven sections of hair estimated to have been grown around the time of parturition were assayed, for each of five samples taken from a female with a calf. The presence and size of a calf was recorded at sample collection, to determine whether an individual had calved

during the previous calving season (3-week window beginning in mid-late February). Samples were collected from five females who were known to have calved during the previous calving season, and hair was sectioned to produce pre-, during-, and post-calving samples. Only two of the five samples showed highest hair oestradiol concentrations in the section that was predicted to span the time of parturition (Fig. 4.3). A mixed effects model was fitted to the data to determine whether oestradiol concentrations differed significantly between sections of hair estimated to have grown before, during, or after parturition. A comparison of AIC between this model and the null model indicated that time of hair growth (i.e. before, during, or after parturition) was not a significant predictor of hair oestradiol concentrations (ΔAIC = 0.48).

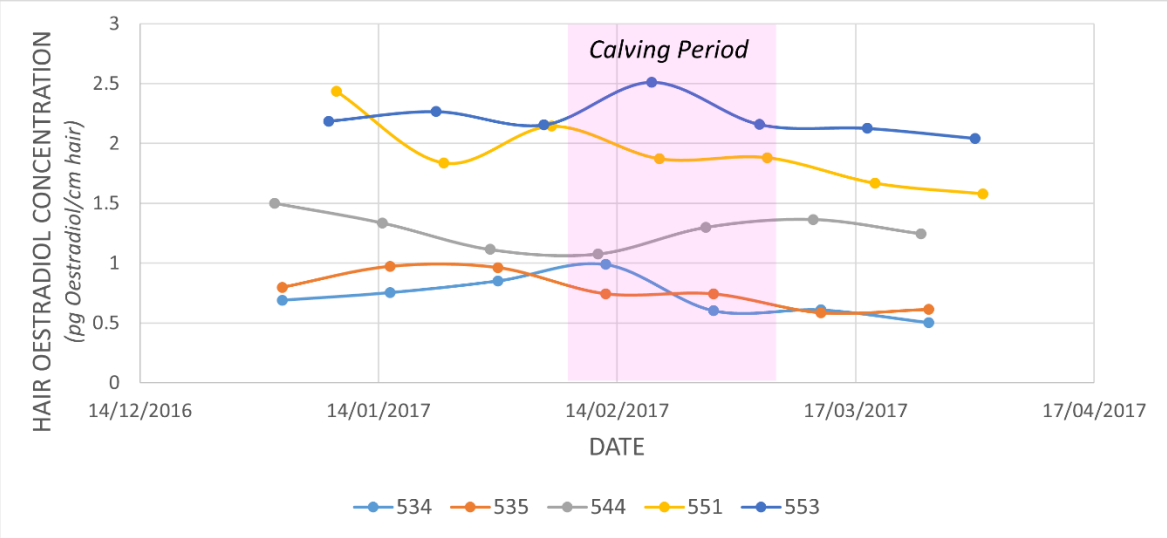


Figure 4.3 Oestradiol concentrations (n=35) in sections of hair grown before, during, and after parturition, taken from five females with calves. Two of the five samples (sample numbers 534 and 553) showed highest oestradiol concentrations in the section of hair related to when parturition was predicted to have occurred, however the other three samples did not.

Since it was not possible to detect the peak in oestradiol that occurs around parturition in our study, we were not able to use the method outlined to inform of reproductive history (i.e. whether an animal had given birth, and when). This was likely due to a mismatch in the resolution of the oestradiol peak (hours-days) and our data (14-day average). In other words, the oestradiol peak occurring at parturition is likely dampened down by the surrounding 13 days hormones also included in that section of hair, when the animal isn't giving birth

and oestradiol has returned to baseline levels. In future studies, it may be possible to detect the peak in oestradiol occurring around parturition if hair samples were cut into smaller sections, representing smaller timeframes and therefore increasing the resolution of our data (e.g. 4mm sections = 7-day average).

4.4.3 Validation of Progesterone Concentrations to Infer Reproductive History

4.4.3.1 Trends in Hair Progesterone Concentrations

Progesterone concentrations (pg/cm hair) varied along the length of the hair of all samples. However, all samples also showed a significant increase in the progesterone concentration along the growth axis of the hair. This increase was seen even when the concentration was corrected for potential increased dehydration along the length of the hair, by expression of progesterone in *pg/cm hair,* rather than by weight (pg/mg hair; the prevailing units used in the field of hair hormone analysis) (n=448; β = 0.60 ± 0.03; t = 21.06) (Fig. 4.4). To confirm that the general increase in hair progesterone was not a true reflection of the underlying physiology of the female wildebeest, a hair progesterone profile was created for a male wildebeest. This sample contained similar hair progesterone concentrations to those collected from female wildebeest and also showed an increase in progesterone concentrations along its length, further confirming this increase to not be a true reflection of progesterone concentrations within the body at the time of growth.

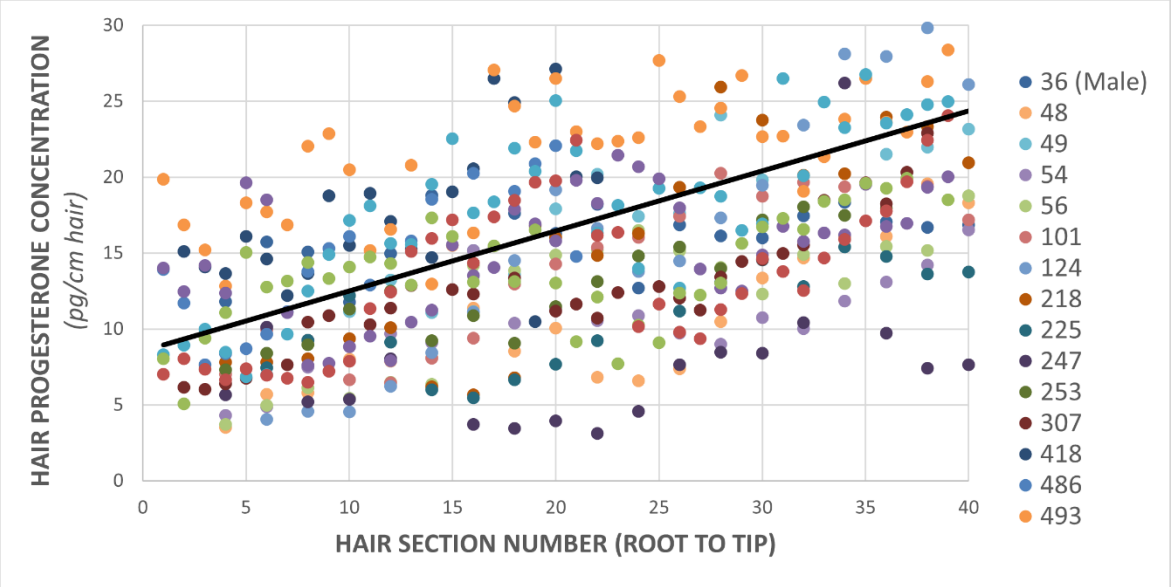


Figure 4.4 Progesterone concentrations (*pg/cm hair*) against section number (e.g. section 1 is the first 8mm section nearest the root) in wildebeest hair, showing variation within and between samples, as well as a general increase towards the tip of the hair.

4.4.3.2 Determining Drivers of the Increase in Hair Progesterone Concentrations Over Time

Yang et al (1998) did not report any trend across progesterone concentrations measured in human hair divided into three segments (top, middle and basal). Therefore, the trend seen in the wildebeest samples must be a result of something specific to: i) wildebeest hair, ii) the ELISA, iii) the environment the hair is in, or iv) a combination of the above. Since the antibody in the progesterone ELISA used in this study showed 100% cross-reactivity with the endogenous progestogen 5α-pregnane-3,20-dione, it is possible that changes in either progesterone or 5α-pregnane-3,20-dione could explain the observed increase, although there is no reason why either of these steroids should gradually increase over time. Unfortunately, no alternative progesterone ELISAs were available with lower cross-reactivity.

Since the trend seen in hair progesterone concentrations was not present in hair cortisol concentrations (Chapter 2), it can be surmised that the cause of this trend must be due to differences in the synthesis/degradation/chemical structure of cortisol and progesterone or other components of steroidogenesis. It could be hypothesised that exposure to heat and/or light in the environment

leads to the breakdown of steroid precursors or steroid intermediate products in the hair into progesterone (or 5α-pregnane-3,20-dione). As all endogenous progestogens are structurally similar, possessing a 21-carbon skeleton, it is likely they are all capable of being incorporated into hair, from the blood. Fig. 4.5 shows a portion of the steroid synthesis pathways involved in the production of progesterone and two examples of its many products (deoxycorticosterone and 17α-hydroxyprogesterone), the enzymes required for each step, and how they differ structurally. It is possible that some of these structural changes are reversible and that given enough energy some steroids such as deoxycorticosterone and 17α-hydroxyprogesterone (which do not significantly cross react in the Progesterone ELISA) could be converted back to progesterone, or a molecule the assay cannot distinguish from progesterone.

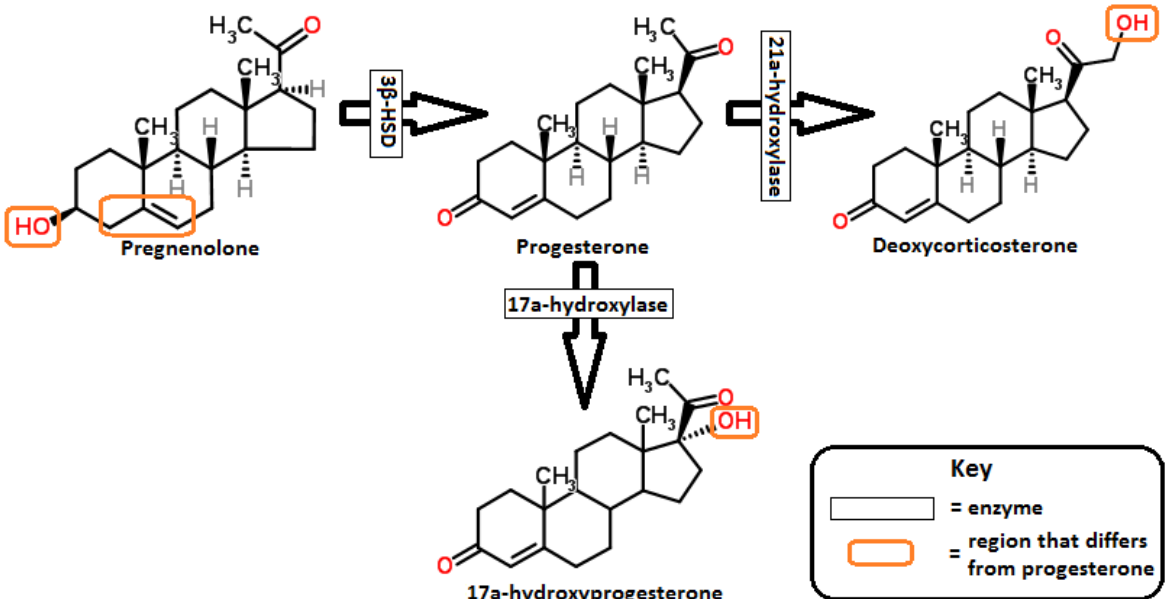


Figure 4.5 Steps involved in the formation of progesterone, and two examples of molecules derived from it, within the steroid pathway. Orange boxes indicate structural differences to progesterone. Both products of progesterone shown only differ from progesterone by the addition of a hydroxyl (OH) group.

Heat and Light Exposure

It is known that both heat and irradiation with ultraviolet light can lead to the alteration of certain biological molecules through the breakdown of chemical bonds. The wildebeest inhabit a relatively hot and dry savannah ecosystem, with temperatures that can occasionally exceed 40°C. It was confirmed through personal communication with Dr Adrian Lapthorn, an organic chemist at the University of Glasgow, that it is possible that some endogenous progestogens (e.g. 16α-hydroxyprogesterone) could degrade over time, as a result of prolonged exposure to heat and/or light.

In order to test if ultraviolet light may cause the breakdown of other steroids into progesterone, approximately 100mg of powdered wildebeest hair was placed into a weighing boat, for each of 19 different sections along a female wildebeest tail hair sample. Samples were positioned directly under an ultraviolet light for one week, and mixed daily, in order to homogenise ultraviolet exposure. To test the effects of heat, an additional set of 100mg samples were placed in an oven set to 40°C for one week and mixed daily. Although samples were only exposed to UV and heat for one week, this would be equivalent to much longer than one week in the Serengeti, as natural environmental exposure will diminish almost entirely, between sunset and sunrise. Furthermore, both the heat and light exposure in the lab were much greater than the average exposure during the day in the Serengeti (although they were still within the achievable range).

Steroids were then extracted from UV- and heat-treated samples, and hair progesterone quantified using the methods outlined above. These were compared to control samples which were kept in the dark at a stable, ambient temperature, before extraction. The results (Fig. 4.6), when analysed with a mixed effects model (with section number included as a random effect), demonstrated that both heat (n=57; β = 5.38 ± 0.48; t = 11.11) and UV (n=57; β = 1.32 ± 0.48; t = 2.73) exposure caused a highly significant increase in hair progesterone concentrations measured by ELISA, with a mean increase of 10.0 ± 4.2 % in UV-treated and 44.2 ± 11.7 % in heated samples. Therefore, it can be proposed that the cause of the observed increase in progesterone measured along wildebeest hair samples is the result of environmental factors. Since the

wildebeest occupy a near-equatorial ecosystem, where seasonal changes in heat and light are relatively small, it should be possible to predict the rate at which steroid degradation occurs and apply a linear correction to the progesterone concentrations measured in samples, based on time spent in the environment.

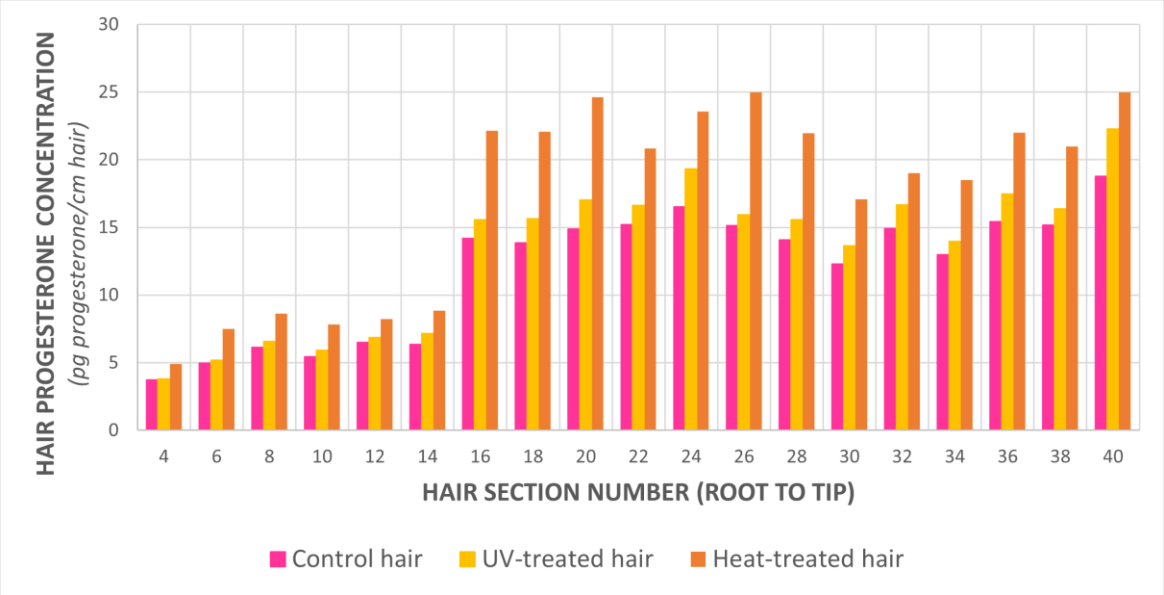


Figure 4.6 Hair progesterone concentrations along a female wildebeest tail hair sample (WH56), showing an increase in progesterone after exposure to heat and ultraviolet light for one week, when compared to the concentrations measured before exposure to heat and UV.

4.4.3.3 Detrending Progesterone Data

Since the progesterone profiles obtained from the hair samples in this study relate to the 18 months preceding sample collection (as full-length tail hair corresponds to 18 months of growth), and the wildebeest gestation period is around 240 days, all female wildebeest hair samples should contain multiple sections of hair that represent periods of anoestrus (i.e. baseline progesterone values). If the data which relates to times when the wildebeest shouldn't be pregnant (March-June) are plotted it should allow for any underlying trend to be described. Initial attempts to correct the data for such an underlying trend involved subtraction of 'baseline progesterone' (calculated from the line of best fit) from the progesterone data, to detrend for the effect of environmental exposure (Fig. 4.7).

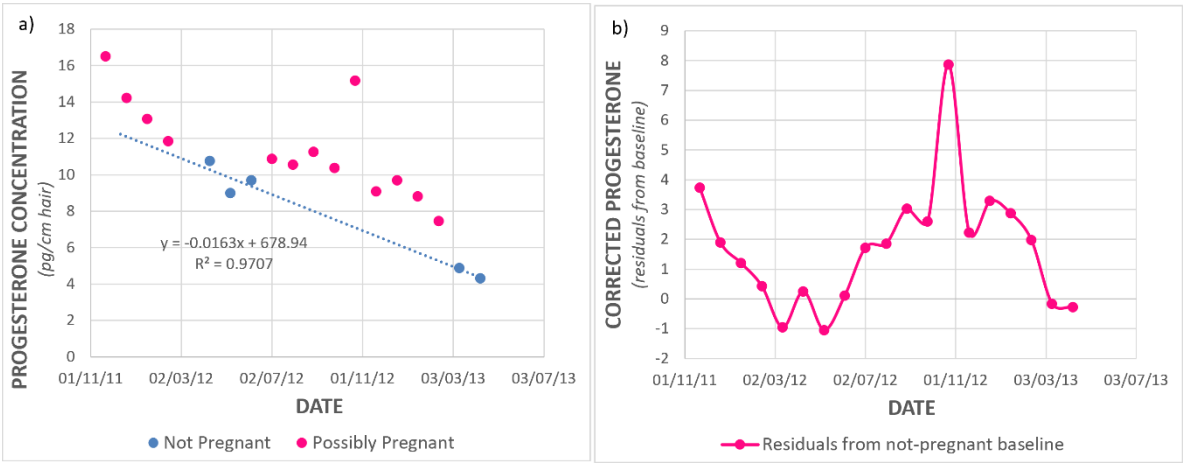


Figure 4.7 Progesterone profiles before and after correction, created from a sample taken from a female wildebeest with a calf. a) shows progesterone concentrations (*pg/cm hair*) with a line of best fit through sections relating to periods of anoestrus (Mar-Jun). The equation of this line was used to calculate the trend in baseline progesterone concentrations. b) Corrected progesterone data, which was detrended by subtraction of baseline progesterone from raw data. Corrected data shows a clear increase in progesterone at the expected time of the rut, which remains elevated until the calving season. There is also evidence of increased progesterone at the onset of the breeding season, which may be indicative of silent ovulation.

While this method of correction worked well for samples which contained sections of hair grown during two anoestrous periods (i.e. samples which contained sections of hair grown from March-June in two consecutive years) (Fig. 4.7), most samples contained only one year's anoestrous data. Furthermore, due to high interindividual variation in baseline progesterone concentrations a generalised correction could not be applied to all the data.

To allow detrending of the data and account for interindividual variation in baseline progesterone concentrations, all progesterone data were scaled relative to the samples it came from, by subtraction of the mean concentration and expression of the section concentration relative to the standard deviation for the sample. Scaled progesterone values were then plotted against time spent in the environment (analogous to position along the hair) (Fig. 4.8). Finally, progesterone values were detrended for the effect of time spent in the environment by measurement of corrected progesterone as the residuals to the line of best fit in Figure 4.8.

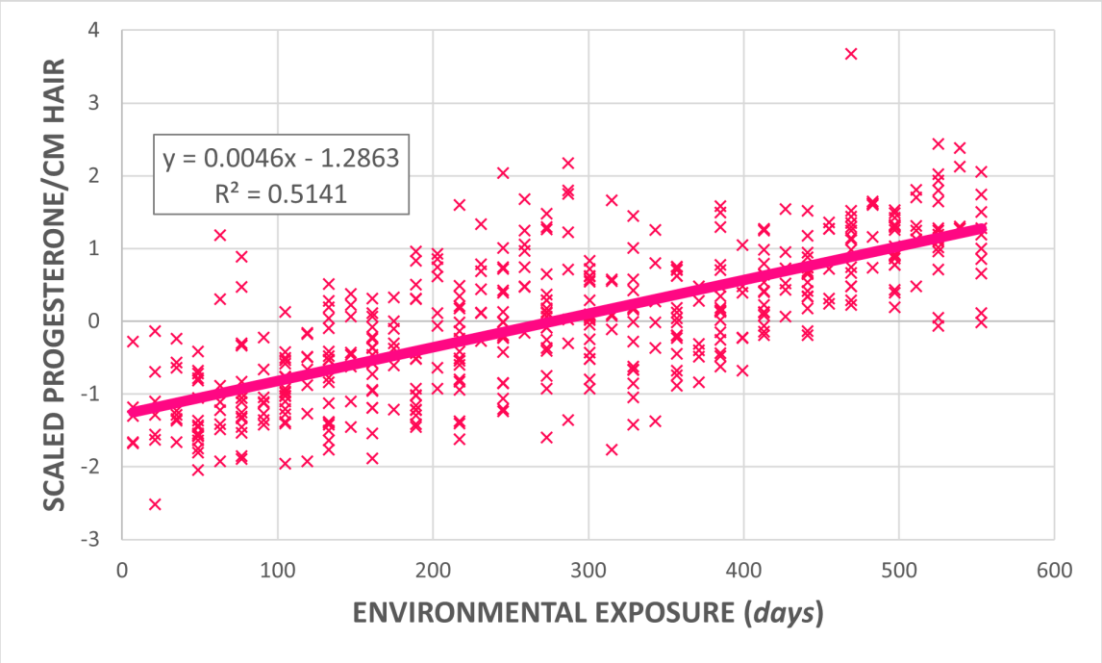


Figure 4.8 Plot of scaled progesterone concentrations (by subtracting the mean and dividing by the standard deviation for that sample) plotted against days exposed to the environment, showing an increase in progesterone as it spends longer in the environment. Residuals to the line of best fit were used as corrected progesterone values.

4.4.3.4 Inferring Reproductive Status from Corrected Progesterone Data

Fig. 4.9 shows how the mean detrended progesterone concentrations increased following the rut and remained elevated until the calving period, when they begin to decrease, eventually returning to baseline levels. The decrease from July to August (Julian day 182-243) suggests most of the animals we sampled are not becoming pregnant at the first oestrous cycle of the breeding season, which results in a temporary decrease in hair progesterone concentrations, before becoming pregnant at the following oestrus. This supports the theory that high breeding synchrony is due to wildebeest being polyoestrus (i.e. having several oestrous cycles during the rut) and that first oestrus might be silent (i.e. overt behavioral signalling is inhibited) (Watson, 1967, Clay et al., 2010). Hopcraft et al (2015b) suggest that this may be evolutionarily beneficial as it primes the bulls and elevates male competition, therefore selecting for strong, high-endurance bulls. Multiple silent ovulations also cue other females and could lead

to breeding synchrony. Statistical analysis indicated that detrended hair progesterone concentrations were significantly lower during times of expected anoestrus (i.e. the time between the calving period and the following breeding season) (n=444; β = -0.36 ± 0.08; t = -4.57) than at other times of the year.

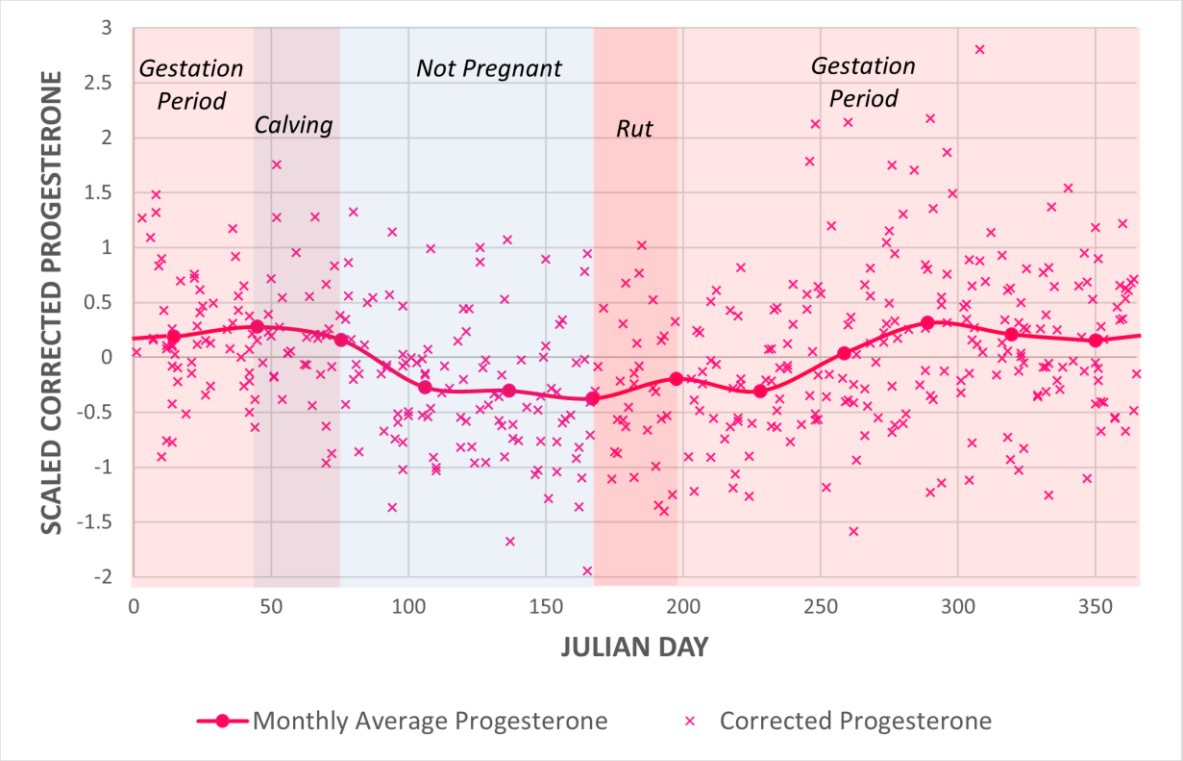


Figure 4.9 Plot of all detrended hair progesterone data (n=444), taken from 18 female wildebeest, against time of year. The line represents average detrended progesterone for each month. Progesterone increases following the rut and remains elevated during gestation, decreasing to baseline levels following calving.

Progesterone Profiles

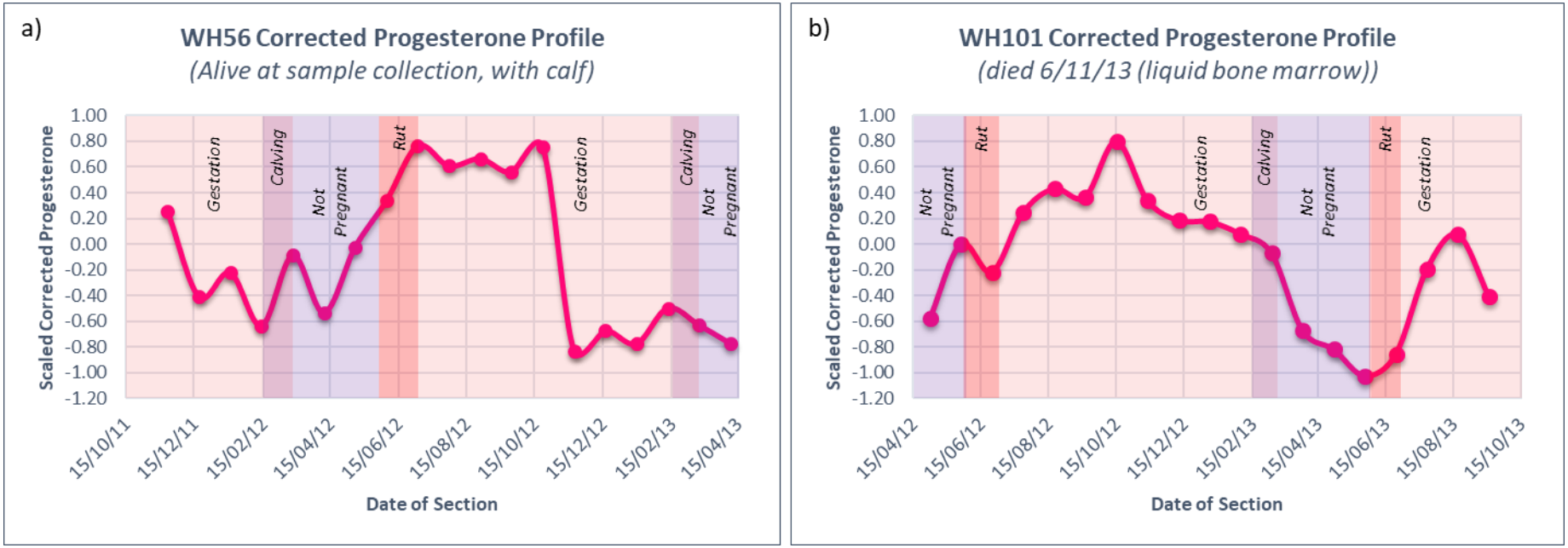


Figure 4.10 An example of two corrected progesterone profiles, showing trends associated with conception, gestation, and parturition. The pregnancy shown in sample WH56 (a) appears to have conceived early, leading to an early birth. The sample for WH101 (b) was taken from a carcass, which showed signs of starvation (liquid bone marrow), which may explain the decrease in progesterone seen in the latest section of hair. Starvation likely led the animal to miscarry, which may account for the decrease in progesterone observed in the latest hair section.

Figure 4.10 shows two examples of detrended progesterone profiles in which clear changes in hair progesterone are indicative of changes in reproductive status. The animal depicted in Fig. 4.10(a) appears to have conceived earlier than normal and has a relatively early birth. Fig. 4.10(b) shows an animal that may have miscarried (an unexpected drop in progesterone) in the latest section of hair. It is likely that this animal may have miscarried, since it died approximately two months later from starvation (liquid bone marrow). Although the profiles depicted in Fig 4.10 show distinct switches between reproductive states, not all profiles from other animals were as clear (Fig. 4.11 (Supplementary)). This is probably due to variation across a hair sample in the concentration of the molecule(s) which break down into progesterone. Therefore, the effect may not be truly linear. Additionally, some pregnant wildebeest showed a more gradual decrease in progesterone than that expected at parturition. This may be due to retention of the placenta after parturition, leading to elevated progesterone concentrations following calving, as has been observed in horses (Seamans et al., 1979).

4.4.4 **Future work**

Although this study has investigated a number of interesting avenues for determining the validity of inferring pregnancy status from hair hormone profiles, there are several ways this technique could be improved and expanded upon in the future. For instance, hair samples taken from captive or domesticated animals, whose reproductive history is known, could be used for further validation. This would provide a known behavioural timeline against which the hormones in the hair could be compared.

A potential improvement to the measurement of hormones which only increase for a short period around parturition (e.g. oestrogens, cortisol) would be to cut hair into smaller section lengths, specifically around the calving season. This would increase the temporal resolution of data and improve the probability of detection of the relatively short-term peak of such hormones.

It may also be possible to improve the accuracy of inferences of reproductive state by measurement of progestogens as a whole, rather than progesterone specifically. This may fix the trend in progesterone data, since it is most likely caused by other progestogens. Since general concentrations of progestogens are known to increase with gestation, measurement of combined progestogen concentrations could be a more reliable indicator of reproductive state, since any molecular switch between progestogens will have no effect on their overall concentrations. Alternatively, a study was able to successfully measure seven different steroid hormones, including progesterone, simultaneously, using a liquid chromatography, tandem mass spectrometry (LC–MS/MS) based method (Gao et al., 2013). Implementation of this method of quantification could allow for more specific differentiation of progesterone from its precursor and product molecules (e.g. 5α-pregnane-3,20-dione), and would potentially remove any trend seen as a result of cross-reactivity in an ELISA-based system. Additionally, mass spectrometry-based methods may allow for the quantification of alternative oestrogens and progestogens, which may be more stable, giving a clearer indication of reproductive status.

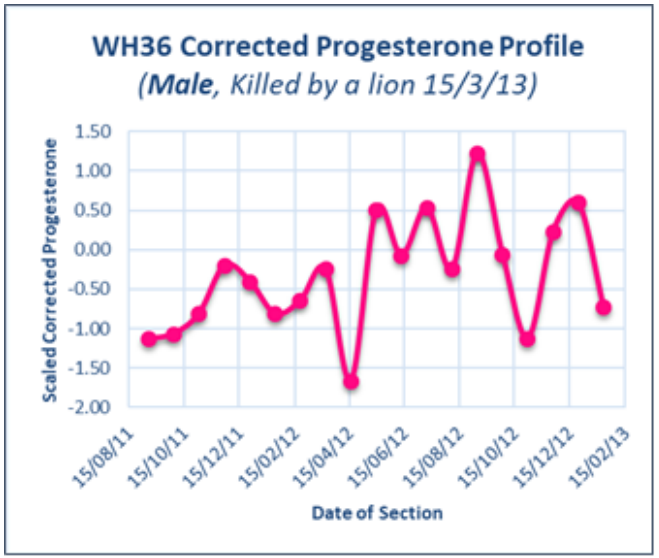
An additional source of physiological data from hair samples could be the quantification of eicosanoids. Although there doesn't appear to be any published studies to date which have attempted to measure eicosanoids in hair, they are of similar chemical size and structure to steroids. Eicosanoids regulate a variety of processes, including cell growth, immune response, and even birth and the abortion of pregnancy; if they can be measured in hair this could potentially unlock an entirely new source of physiological data.

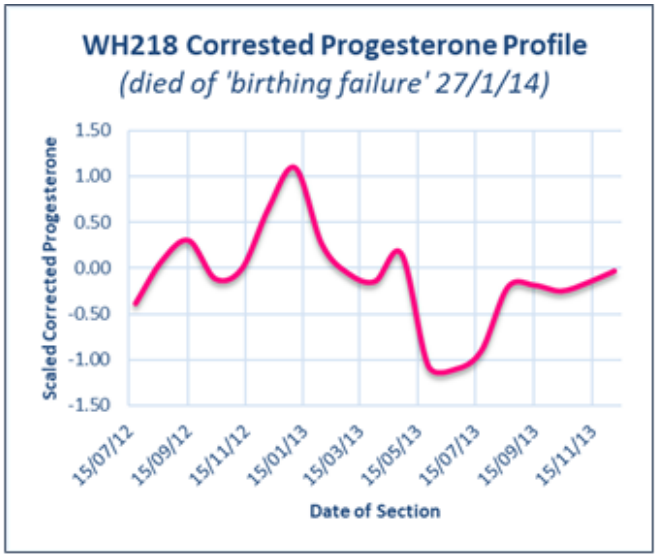
4.5 **Conclusion**

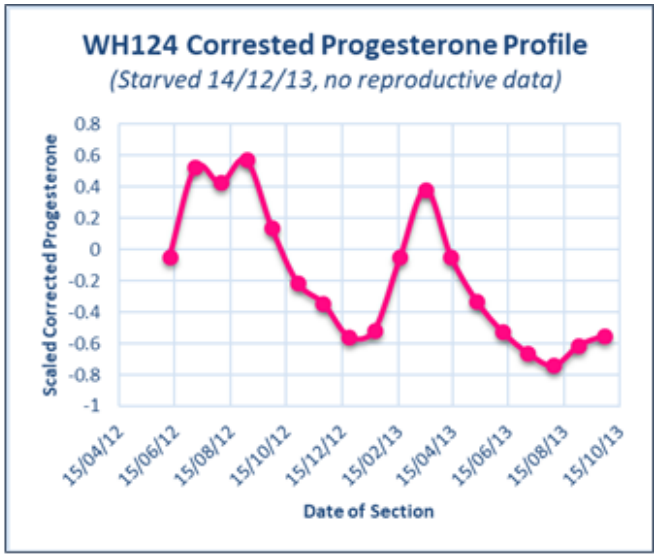
It is possible to quantify wildebeest hair concentrations of progesterone, oestrone, and oestradiol with ELISAs although the accuracy of oestrone measurements could not be guaranteed in the assay tested. Hair concentrations of progesterone varied along and between samples. There was a significant increase in hair progesterone concentrations along a sample which appeared to occur as a result of time spent in the environment. It was shown that exposure

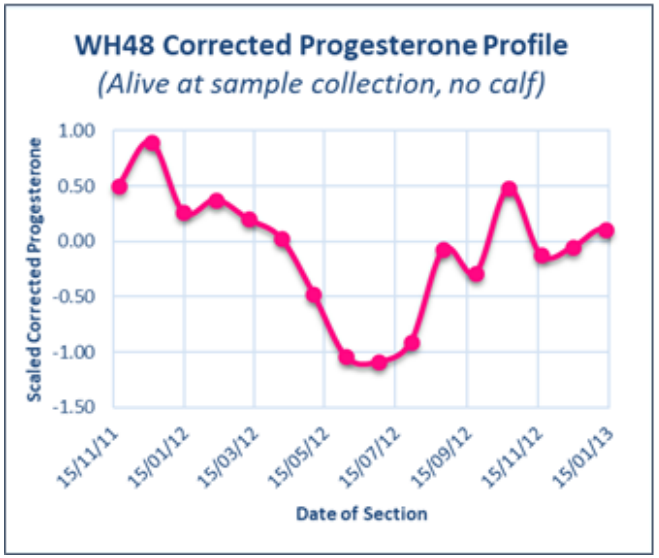
to temperatures of 40°C and high levels of UV irradiation can cause an increase in hair progesterone concentrations. Therefore, although progesterone was not stable in wildebeest hair, it may be more stable in hair collected from animals living in cooler/less sunny environments. Although wildebeest are exposed to relatively high temperatures and sunlight in the Serengeti, annual variation in heat/light is relatively low, making it possible to detrend the progesterone concentrations using the time hair had spent in the environment. Detrended hair progesterone concentrations showed a significant increase in sections of hair grown when the vast majority of females are expected to be pregnant. This shows promise for the measurement of additional steroid hormones in hair (e.g. aldosterone), which could inform of other physiological changes over time (e.g. sodium stress), even if unstable in hair.

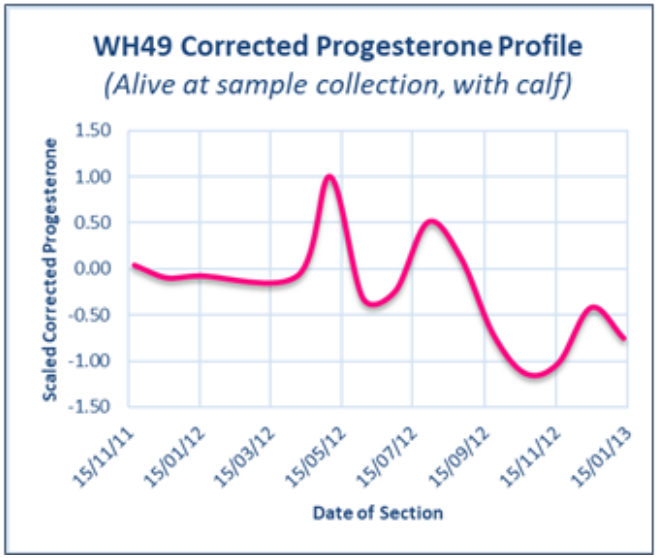
4.6 **Supplementary Material**

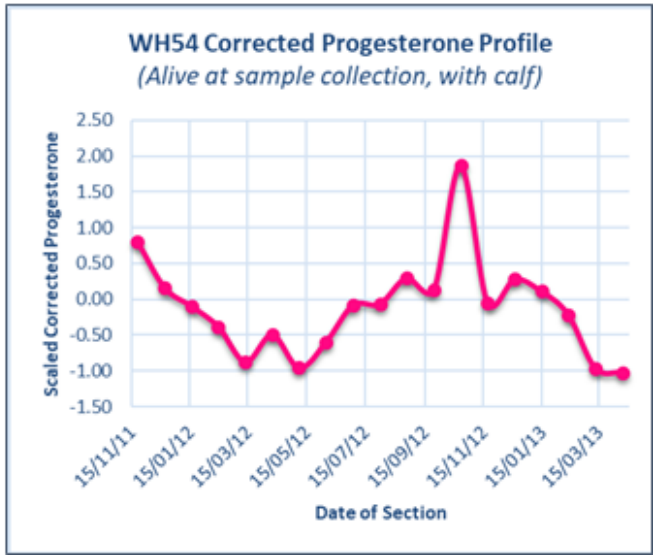

WH36 Corrected Progesterone Profile
(Male, Killed by a lion 15/3/13)
Scaled Corrected Progesterone
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
15/08/11
15/10/11
15/12/11
15/02/12
15/04/12
15/06/12
15/08/12
15/10/12
15/12/12
15/02/13
Date of Section

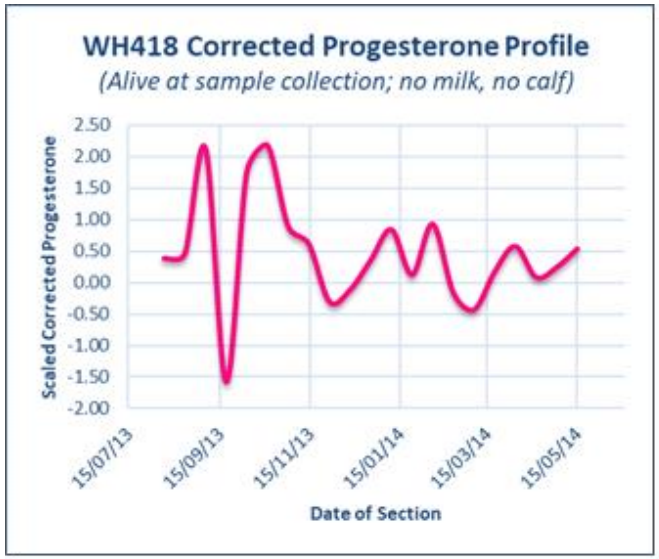

WH218 Corrested Progesterone Profile
(died of 'birthing failure' 27/1/14)
Scaled Corrected Progesterone
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
15/07/12
15/09/12
15/11/12
15/01/13
15/03/13
15/05/13
15/07/13
15/09/13
15/11/13
Date of Section

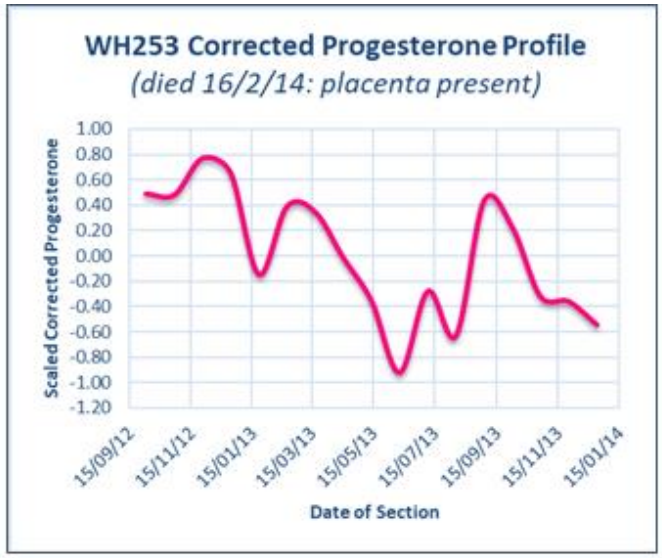

WH124 Corrested Progesterone Profile
(Starved 14/12/13, no reproductive data)
Scaled Corrected Progesterone
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
15/04/12
15/06/12
15/08/12
15/10/12
15/12/12
15/02/13
15/04/13
15/06/13
15/08/13
15/10/13
Date of Section

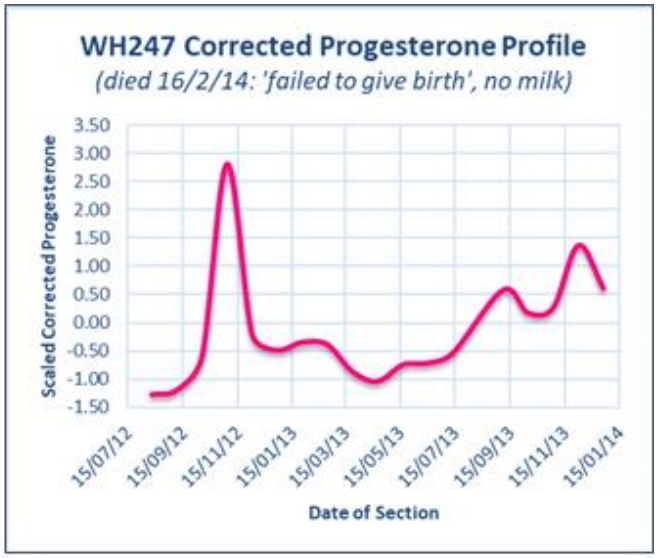

WH48 Corrected Progesterone Profile
(Alive at sample collection, no calf)
Scaled Corrected Progesterone
1.00
0.50
0.00
-0.50
-1.00
-1.50
15/11/11
15/01/12
15/03/12
15/05/12
15/07/12
15/09/12
15/11/12
15/01/13
Date of Section

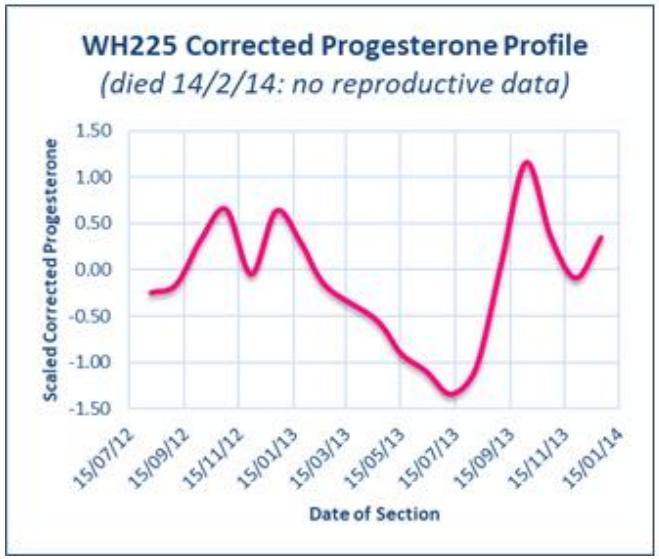

WH49 Corrected Progesterone Profile
(Alive at sample collection, with calf)
Scaled Corrected Progesterone
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
15/11/11
15/01/12
15/03/12
15/05/12
15/07/12
15/09/12
15/11/12
15/01/13
Date of Section

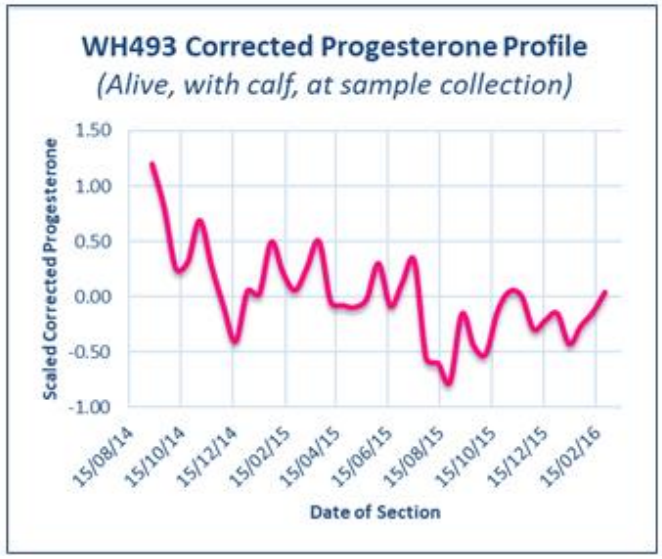

WH54 Corrected Progesterone Profile
(Alive at sample collection, with calf)
Scaled Corrected Progesterone
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
15/11/11
15/01/12
15/03/12
15/05/12
15/07/12
15/09/12
15/11/12
15/01/13
15/03/13
Date of Section

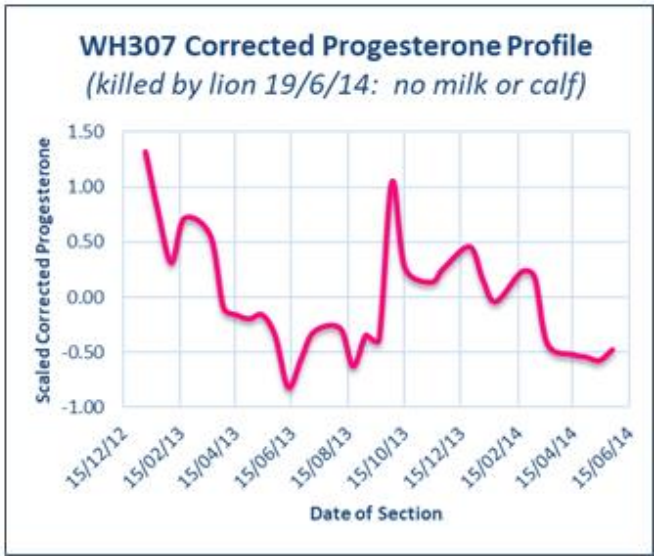

WH418 Corrected Progesterone Profile
(Alive at sample collection; no milk, no calf)
Scaled Corrected Progesterone
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00
15/07/13
15/09/13
15/11/13
15/01/14
15/03/14
15/05/14
Date of Section

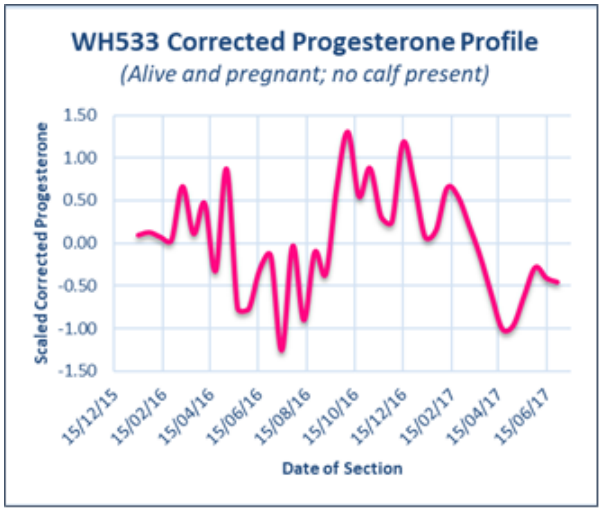

WH253 Corrected Progesterone Profile
(died 16/2/14: placenta present)
Scaled Corrected Progesterone
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
-1.20
15/09/12
15/11/12
15/01/13
15/03/13
15/05/13
15/07/13
15/09/13
15/11/13
15/01/14
Date of Section

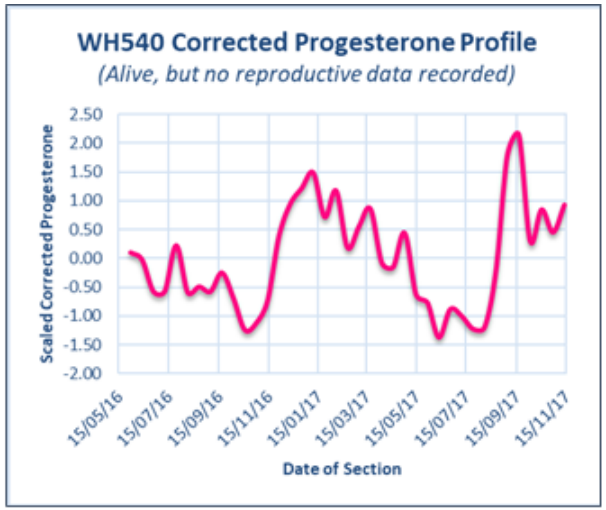

WH247 Corrected Progesterone Profile
(died 16/2/14: 'failed to give birth', no milk)
Scaled Corrected Progesterone
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
15/07/12
15/09/12
15/11/12
15/01/13
15/03/13
15/05/13
15/07/13
15/09/13
15/11/13
15/01/14
Date of Section

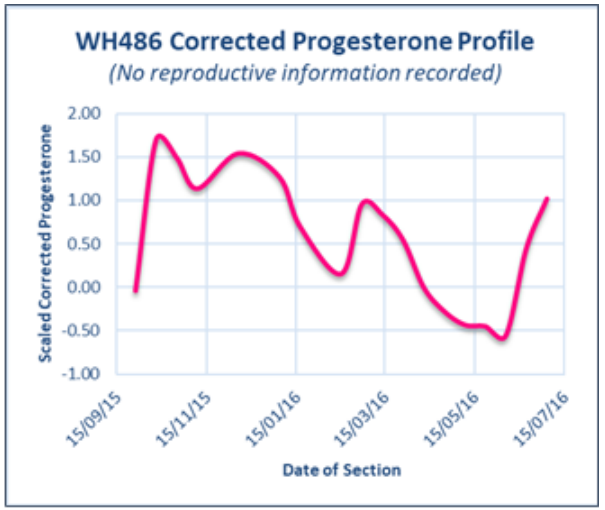

WH225 Corrected Progesterone Profile
(died 14/2/14: no reproductive data)
Scaled Corrected Progesterone
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
15/07/12
15/09/12
15/11/12
15/01/13
15/03/13
15/05/13
15/07/13
15/09/13
15/11/13
15/01/14
Date of Section

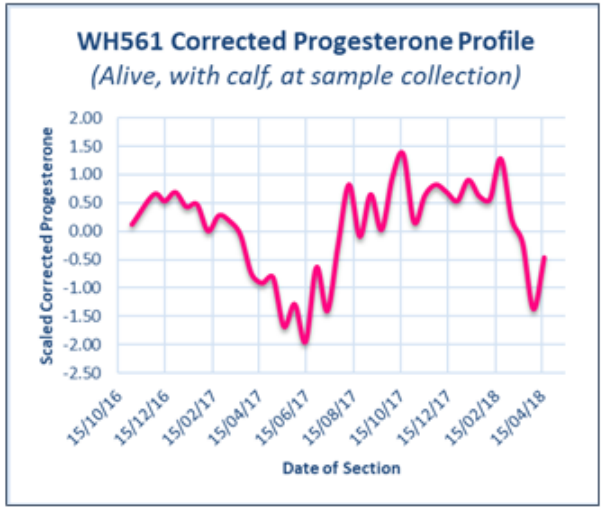

WH493 Corrected Progesterone Profile
(Alive, with calf, at sample collection)
Scaled Corrected Progesterone
1.50
1.00
0.50
0.00
-0.50
-1.00
15/08/14
15/10/14
15/12/14
15/02/15
15/04/15
15/06/15
15/08/15
15/10/15
15/12/15
15/02/16
Date of Section


WH307 Corrected Progesterone Profile
(killed by lion 19/6/14: no milk or calf)
Scaled Corrected Progesterone
1.50
1.00
0.50
0.00
-0.50
-1.00
15/12/12
15/02/13
15/04/13
15/06/13
15/08/13
15/10/13
15/12/13
15/02/14
15/04/14
15/06/14
Date of Section









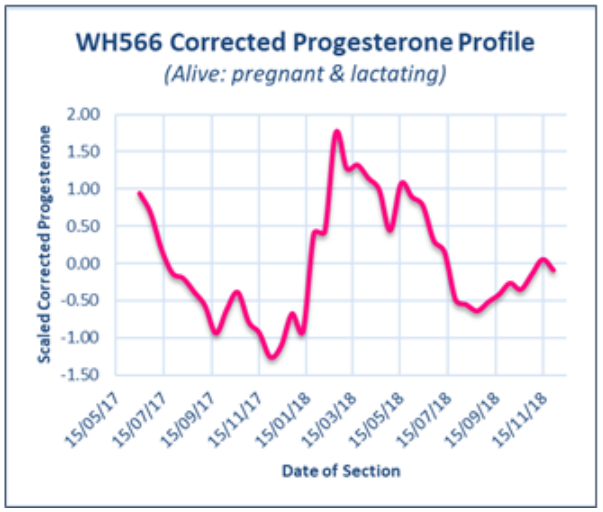


Figure 4.11 (Supplementary) **Additional corrected progesterone profiles, produced from longitudinally sectioned wildebeest hair samples. Information in brackets indicates whether the animal was alive or dead at sample collection, as well as any reproductive data recorded at the time of sample collection.**

5 Hair Aldosterone Concentrations Indicate Higher Sodium Stress in Migrant than Resident Wildebeest

5.1 Abstract

Sodium is an essential mineral, responsible for the maintenance of stable blood pressure and water balance in the body. Herbivores strongly prefer food with high sodium, especially when sodium is limited in the environment. Therefore, sodium likely plays an important role in animal movement. A major issue in population ecology is the lack of robust methods for the collection of physiological time-series data within individuals. A practical method for the collection of time series physiological data relating to the regulation of sodium would allow investigation of the underlying physiological condition of individuals that may lead to population level change.

This chapter implements the method described in Chapter 2 for the collection of longitudinal data pertaining to hormone concentrations to assess aldosterone, an indicator of sodium stress. Hair samples (n=27) were collected from resident and migrant GPS-collared wildebeest (*Connochaetes taurinus*) in the Serengeti-Mara ecosystem. The aldosterone data were compared with a variety of environmental factors extracted from telemetry data, as well as season, to determine potential drivers of variation in sodium homeostasis experienced by wildebeest as they traverse their environment.

Hair Aldosterone Concentrations (HAC) were measured in hair samples taken from wild animals for the first time and were shown to vary within and between individual hair samples. HAC increased along the growth axis of the hair, however it was possible to detrend the data using the time spent in the environment. Overall, HAC was higher in migratory wildebeest than in wildebeest resident to sodium-rich areas of the ecosystem, suggesting migratory animals may experience greater sodium stress. HAC was also correlated with

season (i.e. proximity to time of maximum cumulative rainfall) and NDVI (i.e. vegetation greenness), which suggests that wildebeest are most sodium-deprived at the end of the dry season, when they occupy the most sodium-poor area of the ecosystem. Significant interactions also indicated higher seasonal variation in HAC in migrants (compared to residents), while NDVI had a greater effect on HAC in residents than in migrants.

This study validates a relatively straight-forward and robust method for the collection of chronic physiological time series data indicative of sodium homeostasis. Lower HAC in resident wildebeest indicates reduced sodium stress, which may be one of the factors explaining why movement strategy appears to be fixed throughout an animal's life (i.e. why residents do not migrate).

5.2 Introduction

Physiology is rarely incorporated into our understanding of animal movement, due to the difficulties in obtaining physiological data from free-living animals; especially in migratory animals, whose home ranges are vast (Jachowski and Singh, 2015). Animal movement decisions are affected by processes that act over a variety of spatial and temporal scales; for instance, split-second decisions accumulate to inform annual movement patterns (Torney et al., 2018). Although advances in tracking technologies have led to an unprecedented growth in the collection of movement data (Seidel et al., 2018), discrepancies in scale between observations and questions of ecological interest make it difficult to determine the drivers of migration using telemetry data alone (Torney et al., 2018). Movement patterns are driven by interactions between animals and their environment, which vary across species and ecosystems (Johnson et al., 1992, Shaw, 2016). Factors that may contribute to animal movement decisions, include: resource abundance; habitat selection; landscape permeability; animal memory; evolved, innate responses; and the physiological state of the animal (Andersen, 1991, Stamps and Krishnan, 2001, Nathan et al., 2008, Avgar et al., 2013). There is good reason to suspect that dietary sodium may be an additional

factor affecting animal movement: sodium is a critical animal nutrient; herbivores strongly prefer food with high sodium, especially when sodium is limited in the environment (Botkin et al., 1973, Weeks and Kirkpatrick, 1978, Holdø et al., 2002); and sodium has been shown to directly limit population densities (Aumann and Emlen, 1965). Thus, we should expect sodium to play a central role in animal movement. However, the role of sodium in driving migration has not been well explored. Previous chapters have demonstrated the ability to obtain long-term physiological data related to stress and reproductive status by the measurement of the relative concentrations of specific steroid hormones (cortisol and progesterone, respectively) along a hair sample. By measuring alternative steroid hormones in hair, it may be possible to shed light on the endogenous regulation of sodium, and therefore the role played by sodium in driving movement.

Aldosterone is the primary steroid hormone of the Renin-Angiotensin-Aldosterone System (RAAS), the hormone system responsible for electrolyte and fluid homeostasis, specifically through the regulation of essential inorganic ions (e.g. Na^+, Cl^-, K^+) and water. This is achieved through activation of mineralocorticoid receptors in the distal tubules and collecting ducts of the renal nephron and the epithelia of the distal colon (Sjaastad et al., 2010). These upregulate and activate Na^+/K^+ pumps in the cell membrane, which stimulate Na^+ (and indirectly, water) (re)absorption and K^+ secretion, causing an increase in both the volume of extracellular fluid and arterial blood pressure (Booth et al., 2002). This triggers the retention of sodium in the body when levels are low, to inhibit the complete depletion of sodium (Blazer-Yost et al., 1999). The adrenal zona glomerulosa is the principal source of circulating and locally available aldosterone. The most important factors which affect the synthesis and secretion of aldosterone are: the concentration of renin (an enzyme produced in the kidneys, which is secreted when arterial blood pressure falls or when kidney activity increases) and the concentrations of K^+ and Na^+ in the extracellular fluid (sources of K^+ and Na^+ in the body are entirely exogenous and depend entirely on concentrations in the food and water available to the animal). Aldosterone synthesis is highly sensitive to increased extracellular K^+ concentrations (a 2-3% increase in K^+ incurs a 30% increase in plasma aldosterone concentrations) and moderately stimulated by decreased extracellular Na^+ concentrations (Sjaastad

et al., 2010). The drop in blood pressure associated with low aldosterone concentrations also leads to constriction of the capillaries, which minimises the breakdown of aldosterone which occurs in the liver and increases the relative concentration of aldosterone.

A positive relationship between plasma aldosterone concentrations and chronic Na^+ depletion has been documented in a variety of species, including: sheep (Denton et al., 1959, Blair-West et al., 1962), dogs (Farrell and Rosnagle, 1956, Davis et al., 1961), and rats (Singer and Stack-Dunne, 1955, Halevy et al., 1986). Therefore, high aldosterone concentrations can be indicative that an animal is actively conserving sodium, potentially due to low environmental sodium availability, while low aldosterone concentrations may suggest sufficient environmental sodium. Aldosterone has been measured in human hair (Gaudl et al., 2016), however no studies have measured aldosterone in any sample media obtained from wild animals, or in hair samples obtained from any non-human species. However, if longitudinal aldosterone measures can be obtained from free-living animals, it may be possible to determine 'sodium-status' in these animals as they move through their environment. This would improve our understanding of how species respond to temporal changes in their environment, and allow for better informed, and therefore more effective, conservation strategies to be developed and implemented. A study species which experiences gradual, predictable changes in nutrient availability would be ideal to determine the efficacy of this technique.

5.2.1 **Wildebeest Migration**

One of the clearest examples of where migration allows a species to escape local regulation is the wildebeest migration of the Serengeti-Mara ecosystem, one of the largest terrestrial migrations in the world (Fryxell et al., 1988). The combination of wildebeest biology, their adaptive migratory behaviour, and the unique geomorphological features of the landscape to which the wildebeest are particularly well suited explains the vast abundance of wildebeest in the ecosystem (Hopcraft et al., 2015b). As described in Chapter 1, the 650km migratory route occurs in a clockwise fashion and can be divided into three

general areas: south, west, and north (as identified by orange arrows on Figure 5.2). The migration begins each year in the nutrient-rich shortgrass plains in the southernmost region of the ecosystem, where migrants remain throughout the wet season. Around May, as this area begins to dry, migrants move north and west into the Western Corridor of the ecosystem. This area contains two rivers (Mbalageti and Grumeti) that drain westerly towards Lake Victoria. This produces heterogeneous alluvial floodplains between the neighbouring valleys, composed of large, nutrient-rich grass meadows mixed with open woodland and separated by closed canopy riverine forests. By August, the continued drying of the ecosystem pushes the migration further north, into the Maasai Mara (the northernmost region of the migratory route). Although this area is composed primarily of leached and eroded granite soils, dominated by mixed *Acacia* and *Commiphora* woodlands with nutrient poor grasses below the canopy, it is the only permanent source of water large enough to support the migratory population throughout the dry season. This is generally where migrants spend the remainder of the dry season, before they return to the shortgrass plains in the south at the onset of the wet season, around December. A strong north-south seasonal rainfall gradient, coupled with the soil fertility gradient (high in the south and west; low in the north), creates a natural pattern of high-quality grazing that is seasonally available and which is the primary driver of the migration at the population level (Boone et al., 2006, Holdo et al., 2009). However, the specific driver(s) of the swing into the Western Corridor as the southern plains dry, are more difficult to determine. The concentration of sodium, calcium, and phosphorus in the vegetation have each been hypothesised to play a role in driving the migration, however sodium is the only mineral in sufficient concentrations in the Western Corridor to maintain homeostasis (i.e. both calcium and phosphorus are high in the south but low in the west and north) (Hopcraft, 2010). Since sodium is regulated by the steroid hormone aldosterone, it may be possible to assess if sodium availability is a significant determinant of movement by associating variation in hair aldosterone concentrations with environmental conditions encountered by animals as they move.

5.2.2 **Sodium Availability**

The distribution of sodium in the grasses of the Serengeti varies across the ecosystem (Fig. 5.1). The highest sodium concentrations available to the wildebeest occur in the southern plains and Western Corridor, while the grasses in the northern woodlands are especially sodium-poor and do not meet the minimum requirements to sustain the wildebeest for extended periods of time (Hopcraft, 2010). The high sodium concentration on the shortgrass plains in the south are only available for a few months during the wet season, when the grasses are green and drinkable surface water is available. The short grass plains are too dry to host high densities of wildebeest during all other times of the year. The annual rainfall in the west is higher than the south and occurs over a longer period of time.

Sodium in the west occurs in heterogeneous patches and as a result it remains available in the grasses early into the dry season (Hopcraft, 2010). Since lactating wildebeest require almost double the sodium of females in the early stages of pregnancy this adds a large nutritional demand for animals with calves (Murray, 1995). Migratory wildebeest lactate from February until September, with peak lactation occurring from February to May, when rainfall and forage are most abundant (Kreulen, 1975). However, from May to September, lactating females are faced with rapidly drying conditions while simultaneously lactating, becoming impregnated with the following year's calf, and migrating. Hopcraft (2010) suggests that, since the Western Corridor is the closest reliable source of sodium once the southern plains have dried, sodium availability in this area may be one of the factors that causes the migration move westward instead of simply going directly north to their dry season refuge near the Mara River.

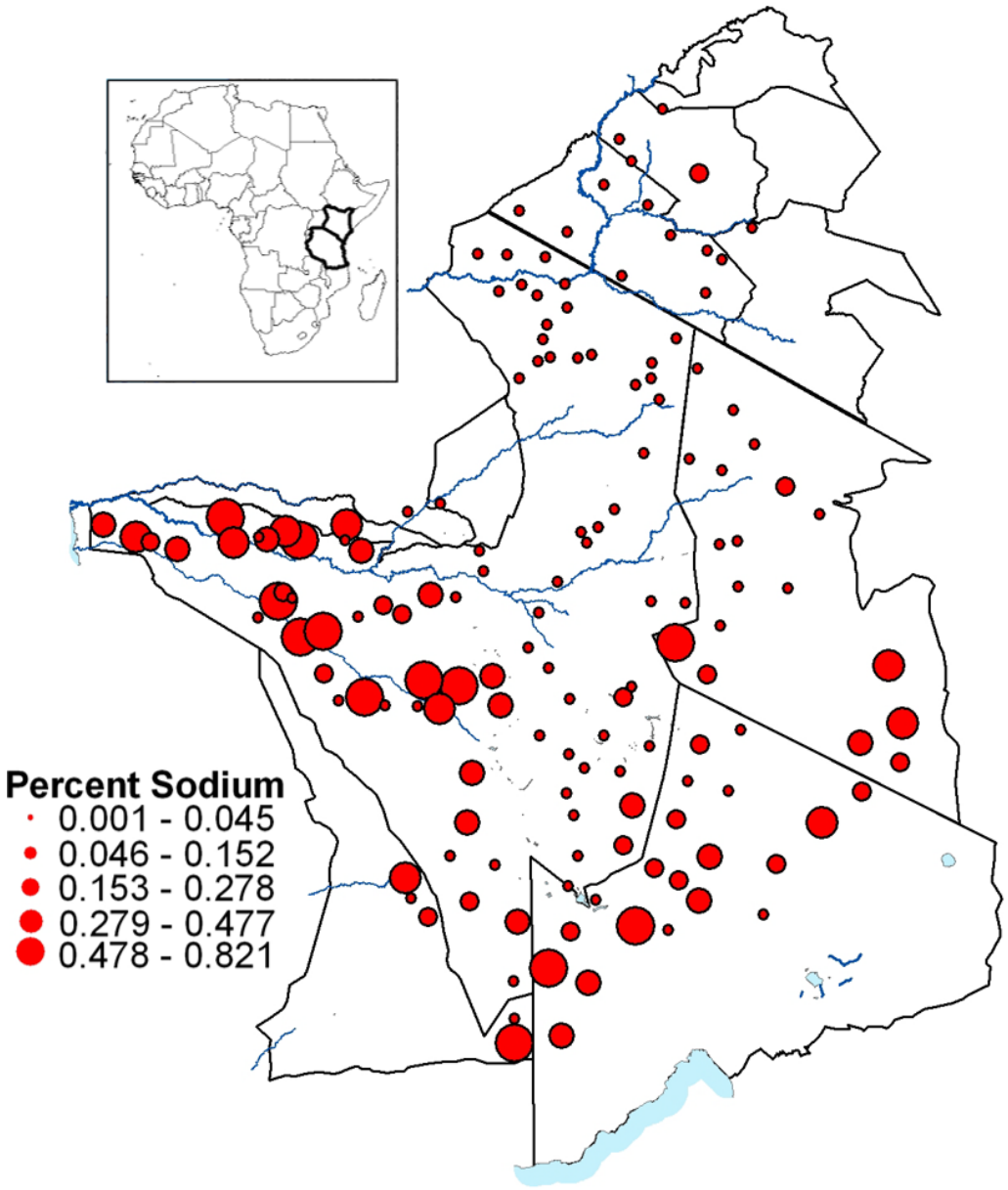


Figure 5.1 **The distribution of sodium in grasses varies across the Serengeti-Mara ecosystem. Only the shortgrass plains (South and East parts of the ecosystem) and localized patches in the Western Corridor contain sufficient sodium to meet their nutrient requirements (Kreulen, 1975).**

5.2.3 **Resident Subpopulations**

Although the vast majority of wildebeest within the ecosystem are migratory (~1.3 million), they also share the ecosystem with ~75,000 resident wildebeest, divided into four subpopulations, that tend to remain in the same areas year-round, or perform a much smaller, local migration each year (Hopcraft et al., 2015b). These resident subpopulations occur in the Western Corridor, Maasai Mara, Loliondo, and the Ngorongoro crater (Fig. 5.2). Resident wildebeest only occupy areas with annual rainfall that exceeds 900mm; permanent access to water; and fine-scale local heterogeneity of forage quality and quantity. There are only a few locations in the ecosystem that meet this requirement, which may explain why there are far fewer residents than migrants (Hopcraft, 2010). Of the four populations of resident wildebeest, the Western Corridor has the most sodium-rich areas and therefore makes a good comparison of aldosterone responses between the resident and migratory life-history strategies. We therefore focused on sampling both migratory wildebeest and Western Corridor residents.

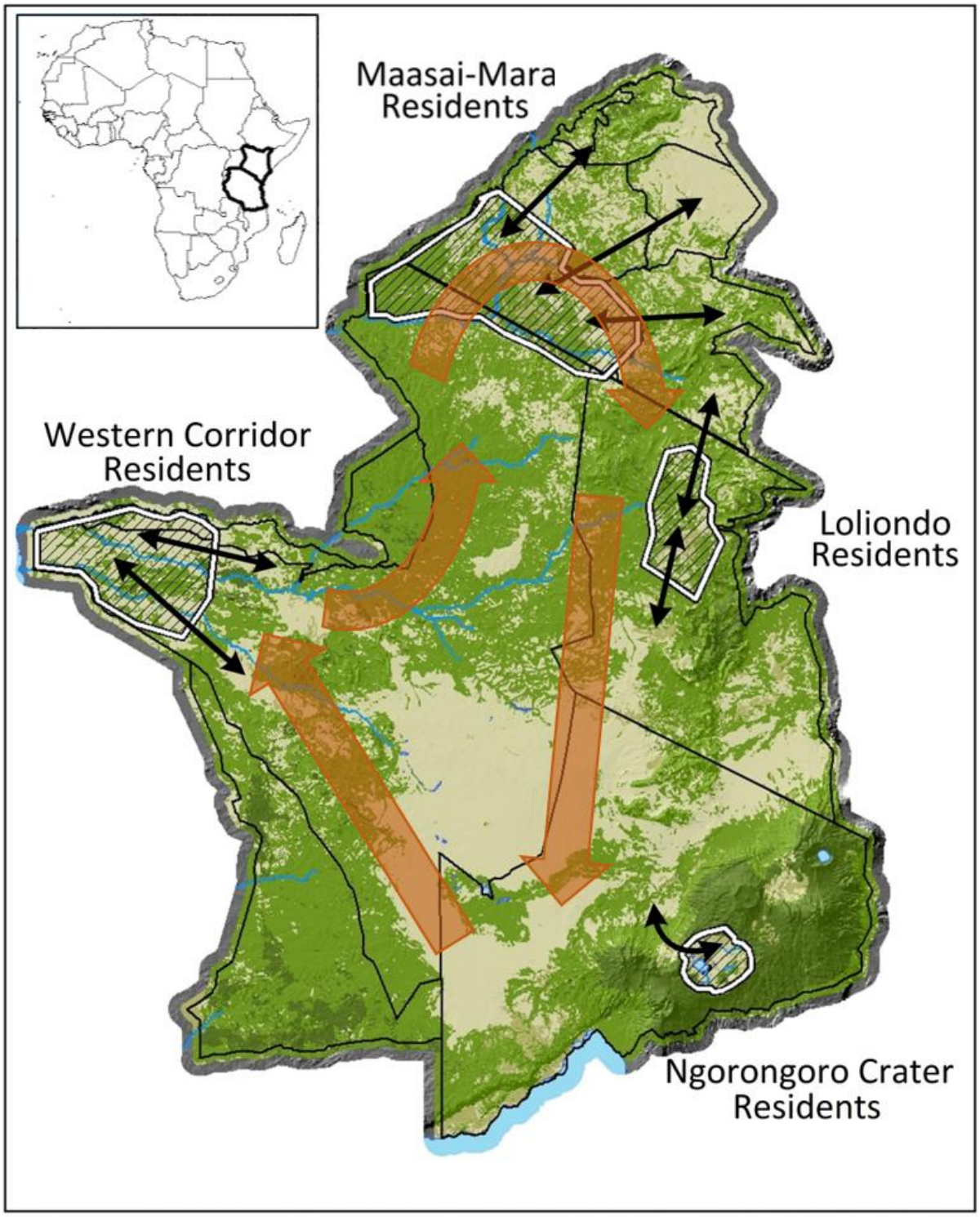


Figure 5.2 Map of resident wildebeest subpopulations in the Serengeti-Mara ecosystem, adapted from (Hopcraft et al., 2015b). White hatched areas show approximate dry season range for each subpopulation, with arrows showing direction of movement during the wet season. Light, medium, and dark shading correspond to grassland, woodland, and dense forests, respectively. Bold orange arrows indicate approximate annual route of migratory wildebeest.

Genetic evidence suggests that the resident subpopulations were once part of a larger interbreeding pool, which included the migrant population (Georgiadis, 1995). However, current spatiotemporal distributions and the synchronicity of breeding in the migratory herd means that this is no longer possible, despite seasonal sympatry occurring between migrant wildebeest and all four resident subpopulations.

Although experimentation would be required to fully determine the relationship between sodium availability and movement strategy, a comparison between aldosterone profiles of resident and migrant wildebeest may shed some light on this. Additionally, the alignment of spatiotemporal aldosterone data with environmental factors extracted from GPS locations of collared animals would be valuable in determining physiological changes over time, as wildebeest move through a variety of landscapes.

5.2.4 **Aims and Predictions**

The aims of this study are to: determine if aldosterone in wildebeest tail hair can be detected and quantified; measure the stability of aldosterone in hair as the hair grows; create temporal aldosterone profiles and assess whether hair aldosterone concentrations vary in relation to environmental sodium, season, rainfall, and grass greenness; and determine if there are significant differences in seasonal aldosterone profiles in wildebeest using different movement strategies.

We predict that aldosterone will be measurable in wildebeest tail hair and that it will show a strong seasonal trend, being lowest in sections of hair that correspond to the end of the wet season (when wildebeest have spent the longest period in areas of high-sodium grazing) and highest at the end of the dry season (when wildebeest have spent the longest time in areas of low-sodium vegetation). We predict local sodium content, green vegetation abundance (NDVI), and rainfall will be negatively correlated with aldosterone concentrations. We also expect aldosterone concentrations to be higher, and

show more seasonal variation, in migrants, relative to animals that are resident to the sodium-rich Western Corridor region of the ecosystem.

5.3 Materials & Methods

Hair samples (n = 27) used in this study were collected from the tail of 24 free-ranging female wildebeest equipped with GPS-collars, as described in Chapter 3. Samples came from migratory wildebeest (n = 20) and wildebeest resident to the western corridor (n = 4). Three migrant wildebeest were sampled twice, with 18 months between sampling events (to allow for the growth of a new full-length hair sample). All hair samples were washed, sectioned (n = 813), and steroids extracted following the methods outlined in Chapters 2 and 3.

5.3.1 Validation of ELISA

Aldosterone concentrations were measured using the commercially available Cayman aldosterone Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI). The reported cross-reactivity of the antibody is as follows: aldosterone (100%); 3β,5β-tetrahydroaldosterone (41%); 3α,5β-tetrahydroaldosterone (0.5%); corticosterone (0.1%); progesterone (0.04%); pregnenolone (0.02%); androstenedione (0.02%); 11-deoxycorticosterone (0.02%); testosterone (0.01%); 17-hydroxypregnenolone (<0.01%); 17-hydroxyprogesterone (<0.01%); 11-deoxycortisol (<0.01%); Dehydroepiandrosterone (DHEA) (<0.01%); cortisol (<0.01%); cortisone (<0.01%); DHEA sulfate (<0.01%); 5α-dihydrotestosterone (<0.01%); oestrone (<0.01%); dexamethasone (0.01%); β-oestradiol (<0.01%); oestriol (<0.01%); oestrone sulfate (<0.01%).

A serial dilution of wildebeest tail hair extract was assayed and tested for parallelism between samples and standards, to validate the use of this ELISA for quantifying aldosterone in our samples, across a range of concentrations (as described in Chapter 2.3.4). For assay, all samples were reconstituted in 400ul

ELISA assay buffer (included with kit) and measured in duplicate. All samples from the same individual were included in the same assay, to minimise error associated with interassay variation. Assay Zap software (Biosoft, Cambridge, UK) was used to interpolate aldosterone concentration from absorbance values. Intra- and inter-assay coefficients of variation were reported at 8.53% and 11.10%, respectively. Aldosterone concentrations as determined from Assay Zap were standardized using the dilution factor, initial mass of hair used, and hair degradation rate calculated in Chapter 2, and are expressed in pg/cm of hair.

5.3.2 Stability of Aldosterone Over Time

Cortisol is stable in wildebeest tail hair (Chapter 1), however, hair progesterone concentrations are correlated with the length of time spent in the ecosystem (Chapter 3) (analogous to position along the length of the hair sample). To determine the stability of aldosterone in hair, time spent in the ecosystem, as well as the 'age' of the hair (i.e. the number of days between the growth of the hair section and the extraction of aldosterone from the sample) was included in the analysis.

5.3.3 Environmental Covariates

5.3.3.1 Season

Rainfall is highest in the ecosystem from February to May, therefore the end of May should be when the ecosystem has experienced maximum cumulative rainfall. Likewise, the end of the dry season which occurs approximately 6 months later should be the time of year with the lowest cumulative rainfall. To allow for the effect of season to be plotted linearly, proximity to the end of the wet season was calculated as the number of days between the date each section of hair was grown and the end of the nearest wet season (i.e. May 31st). In other words, low numbers relate to dates close to peak cumulative rainfall (the end of the wet season), while high numbers relate to dates nearest minimum cumulative rainfall (the end of the dry season).

5.3.3.2 Proximity to High Sodium

A GIS layer was created of sodium concentration using data collected by Hopcraft (2010). High spatial variation in sodium values relative to spatial scale made sodium interpolation difficult, therefore proximity to areas of high sodium was used as an alternative metric. All sodium values in the upper 75th percentile of sodium (i.e. 0.153% of dried plant matter) were identified. A distance raster was then created for the entire ecosystem, with a 1km pixel size (Fig. 5.3). Proximity to the nearest high-sodium area of the ecosystem was then extracted for each GPS location and averaged for each section of hair.

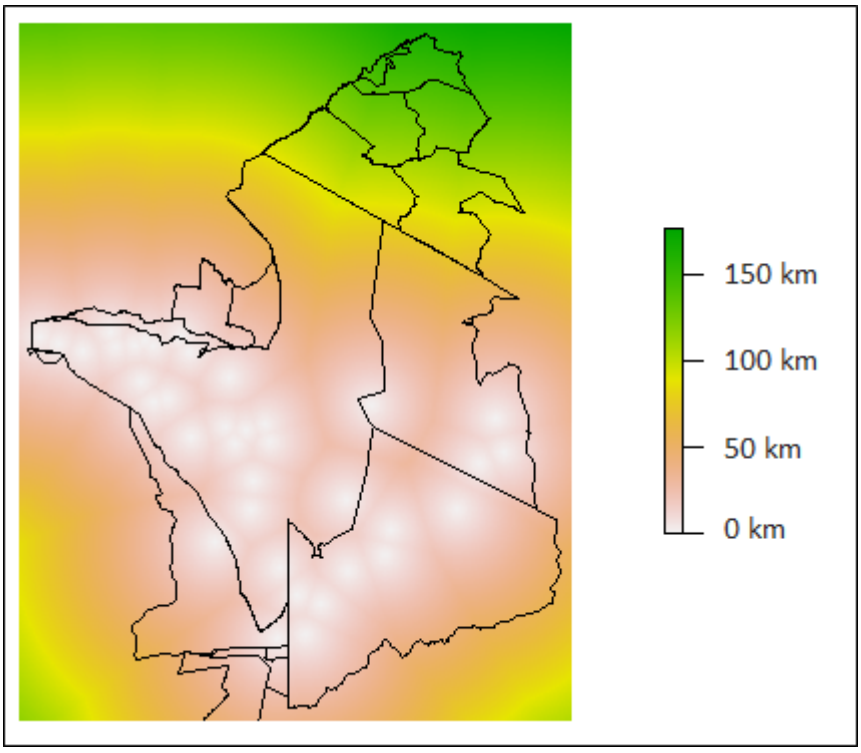


Figure 5.3 A map of proximity to the nearest area of high sodium (>75th percentile) across the Serengeti-Mara ecosystem, created from data collected by Hopcraft (2010) (represented in Fig. 5.1).

5.3.3.3 Rainfall

Rainfall data was obtained from the satellite based African Rainfall Climatology, version 2 (ARC2) daily rainfall estimates, with a 0.1° spatial resolution. ARC2

interpolates daily rainfall using 3-hourly geostationary infrared data from meteorological satellites (e.g. cloud and vapour density), and quality-controlled Global Telecommunication System (GTS) gauge observations reporting 24-hour rainfall accumulations (Novella and Thiaw, 2013). Daily and previous 14-days cumulative rainfall were calculated for each GPS location. Mean daily rainfall and cumulative 14-day rainfall were then calculated for each hair section, by taking the average rainfall value for all GPS locations that occur within the specific two weeks each section of hair was grown.

5.3.3.4 NDVI

The Normalized Difference Vegetation Index (NDVI, where high values indicate live, green vegetation) and the rate of change of NDVI (ΔNDVI) were calculated using the methods outlined in Chapter 3.

5.3.4 Statistical Analysis

Statistical analyses were performed using R version 3.5.1 (R Core Team, 2018). Aldosterone concentrations were log transformed prior to analysis to conform to assumptions of normality, determined using the Shapiro-Wilks test. All variables were scaled by subtraction of the mean and division by their standard deviation, to facilitate model convergence and allow for a direct comparison between the effect of covariates.

Linear mixed effects models were fitted to the data to determine whether the time exposed to the ecosystem or the ‘age’ of the hair (i.e. time between hair growth and hormone extraction) had an effect on hair aldosterone concentrations. Due to high correlation between these covariates (singe ‘age’ is effectively ‘time spent in the environment’ plus the time between sample collection and hormone extraction), they could not be included in the same model. Separate models were fitted to determine whether time spent in the environment or age of sample had an effect on aldosterone, using AIC to

determine the optimal model. Time exposed to the ecosystem was a significant predictor of aldosterone (ΔAIC = 6.862) so was included in all statistical models.

To test the effects of the explanatory variables outlined above on hair aldosterone concentrations, linear mixed effects models were fitted to the data (using R package lme4 (Bates et al., 2014)). AIC was used to determine the top model amongst a suite of candidate models (Table 5.2 (Supplementary)). Animal ID was included as a random effect, to control for pseudo-replication.

5.4 Results

5.4.1 Validation of Aldosterone ELISA

The results from the test for parallelism between a serial dilution of wildebeest tail hair extract show that aldosterone is present in wildebeest tail hair and can be measured accurately across a range of concentrations, using the Cayman aldosterone ELISA kit ($r^2 = 0.904$, $p < 0.01$) (Fig. 5.4). These results were also used to determine the optimal mass of hair to be extracted, as well as the optimal volume of assay buffer with which to reconstitute samples for assay, to optimise their position relative to the standard curve.

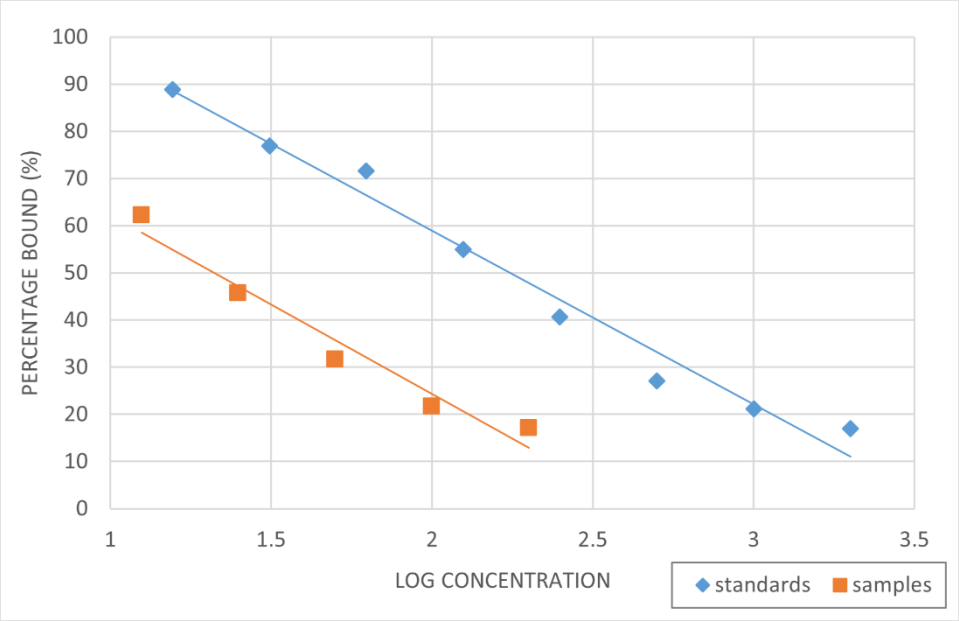


Figure 5.4 Test for parallelism between a serial dilution of wildebeest tail hair extract and standards included in the Cayman aldosterone ELISA kit.

5.4.2 **Stability of Aldosterone in Hair**

Hair aldosterone concentrations were positively correlated with time spent in the ecosystem (analogous to position along the hair sample) (n = 813; β = 0.28 ± 0.02; t = 12.45) (Fig. 5.5). This shows that the longer the hair spends in the environment, the greater the concentration of aldosterone. Time spent in the ecosystem was therefore included as an explanatory variable in all further analysis.

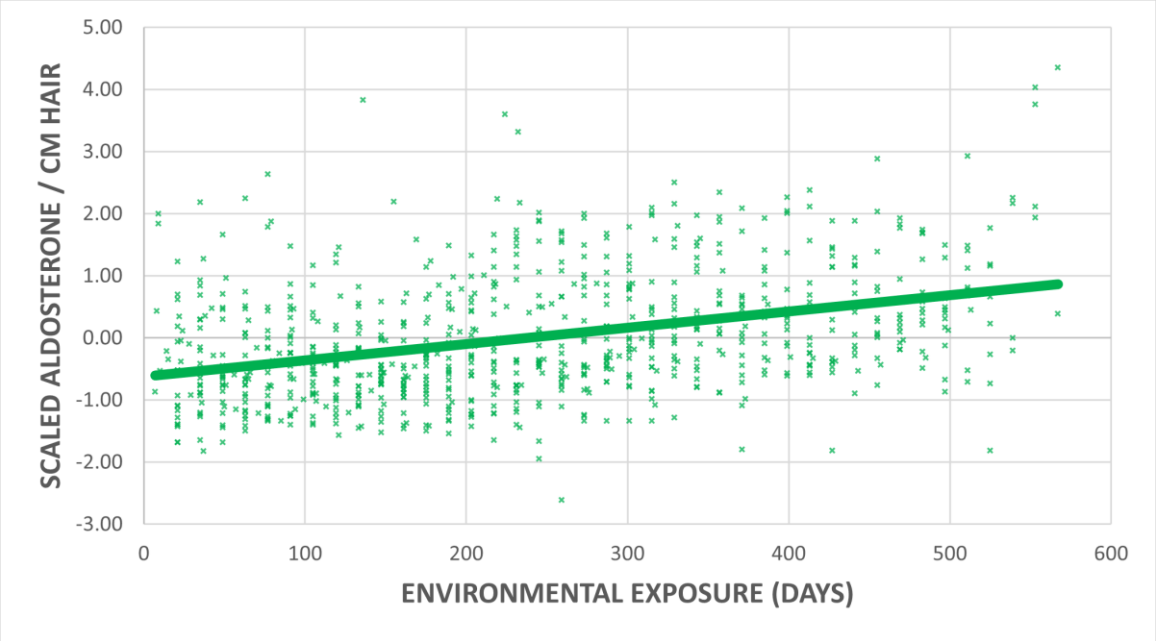


Figure 5.5 The effect of environmental exposure (analogous to position along the length of the hair) on scaled aldosterone concentrations in wildebeest tail hair.

5.4.3 **Aldosterone Profiles**

Aldosterone was measured in 813 sections of hair, and aldosterone profiles generated for 27 hair samples, taken from 24 individual wildebeest. Hair aldosterone concentrations varied both temporally and spatially within individuals, as shown for an example individual in Figure 5.6, as well as between individuals (Figure 5.10 (Supplementary)).

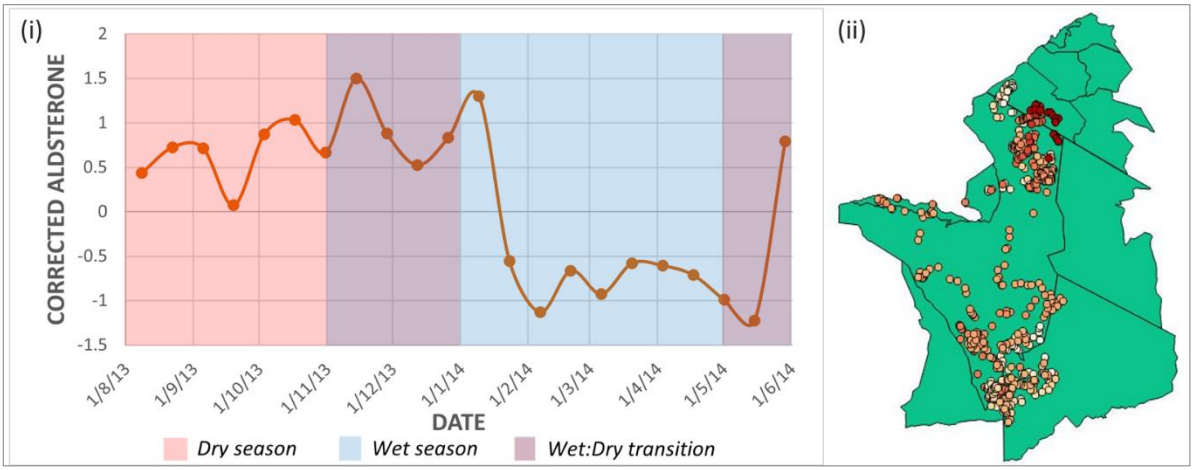


Figure 5.6 Aldosterone concentrations in hair sample WH418 (taken from wildebeest W28 during immobilization) plotted (i) over time, and (ii) with the corresponding locations the animal visited during that time, superimposed onto a map of the Serengeti-Mara Ecosystem. Coloured bands in (i) indicating wet and dry season were produced using qualitative data. Standardized aldosterone concentrations in figure (ii) were plotted using a colour gradient, with darker points representing higher standardized hair aldosterone concentrations.

5.4.4 **Movement Strategy**

The results from the optimal linear mixed effects model (Table 5.1) reveal that movement strategy was a significant predictor of hair aldosterone concentrations. Overall, aldosterone concentrations were significantly lower in hair samples obtained from Western Corridor residents (n=4) than migrants (n=20) (n=813 hair sections from n=24 wildebeest; β = -1.03 ± 0.40; t = -2.54) (Fig. 5.7).

Table 5.1 Output from the AIC-optimised linear mixed-effects model, indicating that there are important environmental determinants of aldosterone concentrations in wildebeest tail hair. All covariates were normalized to allow direct comparison of effect size.

Covariate	Coefficient Estimate	Standard Error	T-statistic
(Intercept)	0.22	0.16	1.41
Prox. Wet:Dry Season	0.27	0.02	11.96
NDVI	- 0.04	0.02	- 1.65
Days Exposed to Environment	0.28	0.02	12.45
Migrant/Resident	- 1.03	0.40	- 2.54
*NDVI * Migrant/Resident*	- 0.18	0.06	- 2.83
*Prox. Wet:Dry Season * Migrant/Resident*	-0.21	0.06	-3.64

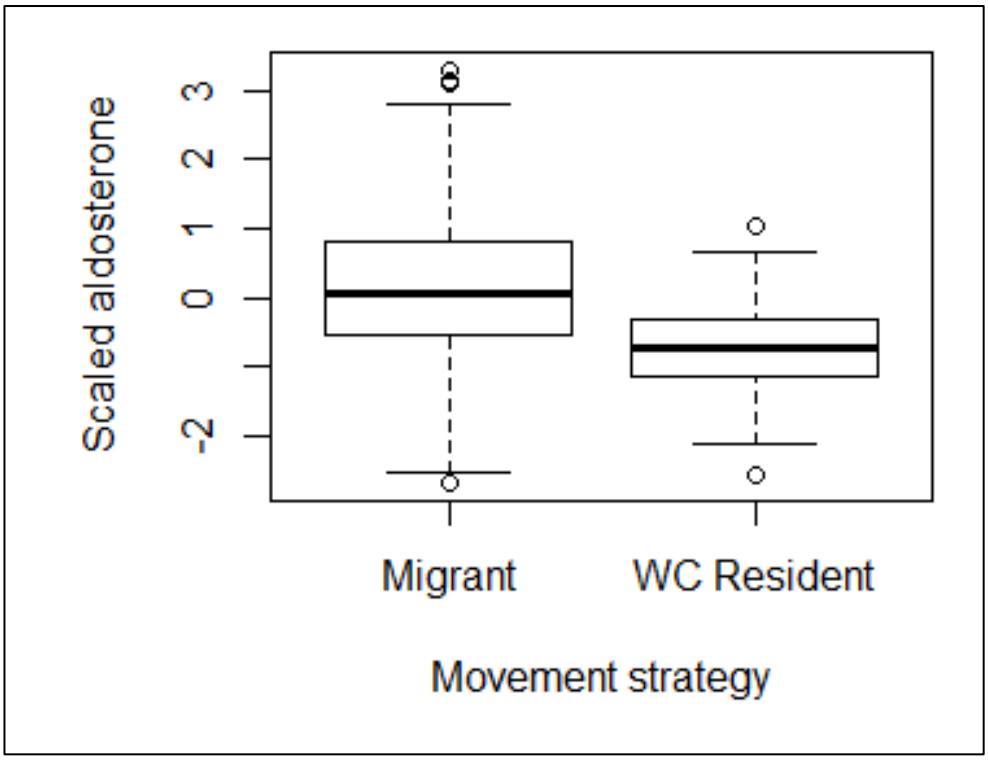


Figure 5.7 Boxplot showing scaled hair aldosterone concentrations were higher in migratory wildebeest compared to residents of the Western Corridor region of the Serengeti-Mara ecosystem.

5.4.5 Environmental Covariates

5.4.5.1 Season

Season (measured as the proximity to the end of the wet season), was a significant predictor of hair aldosterone concentrations in the optimal model (Table 5.1). Aldosterone concentrations were positively correlated with proximity to the end of the wet season (β = 0.27 ± 0.02; t = 11.96). An interaction between season and the movement strategy of the animal also remained in the optimal model (β = -0.21 ± 0.06; t = -3.64), as shown in Fig. 5.8.

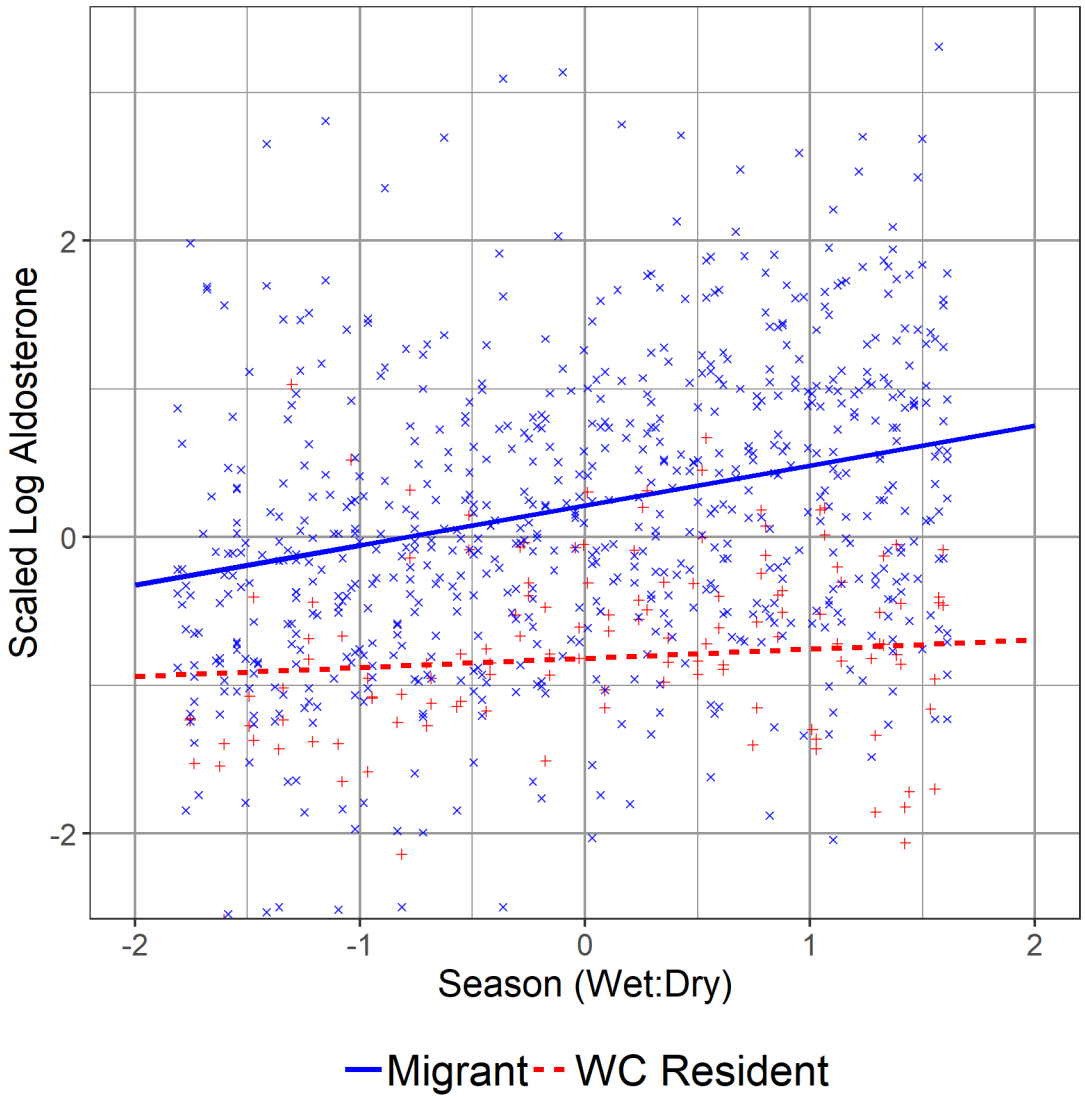


Figure 5.8 Plot showing hair aldosterone concentrations were positively correlated with season (proximity to the end of the wet season).

5.4.5.2 Proximity to High Sodium

Proximity to high sodium was included in preliminary analysis however dropping this variable improved the model (as measured through AIC), therefore it was not retained in the optimal model (Table 5.1).

5.4.5.3 Rainfall

Days rainfall and cumulative 14-day rainfall were both included in preliminary analysis, however, neither variable explained variation in hair aldosterone concentrations.

5.4.5.4 NDVI

NDVI was negatively related with hair aldosterone concentrations (β = -0.04 ± 0.02; t = -1.65); the interaction between movement strategy and NDVI also remained in the most parsimonious model (β = -0.18 ± 0.06; t = -2.83). The negative relationship between aldosterone concentrations and NDVI was larger in resident wildebeest, compared to migrants (Fig. 5.9). ΔNDVI was also included in preliminary analysis but was dropped from the final model.

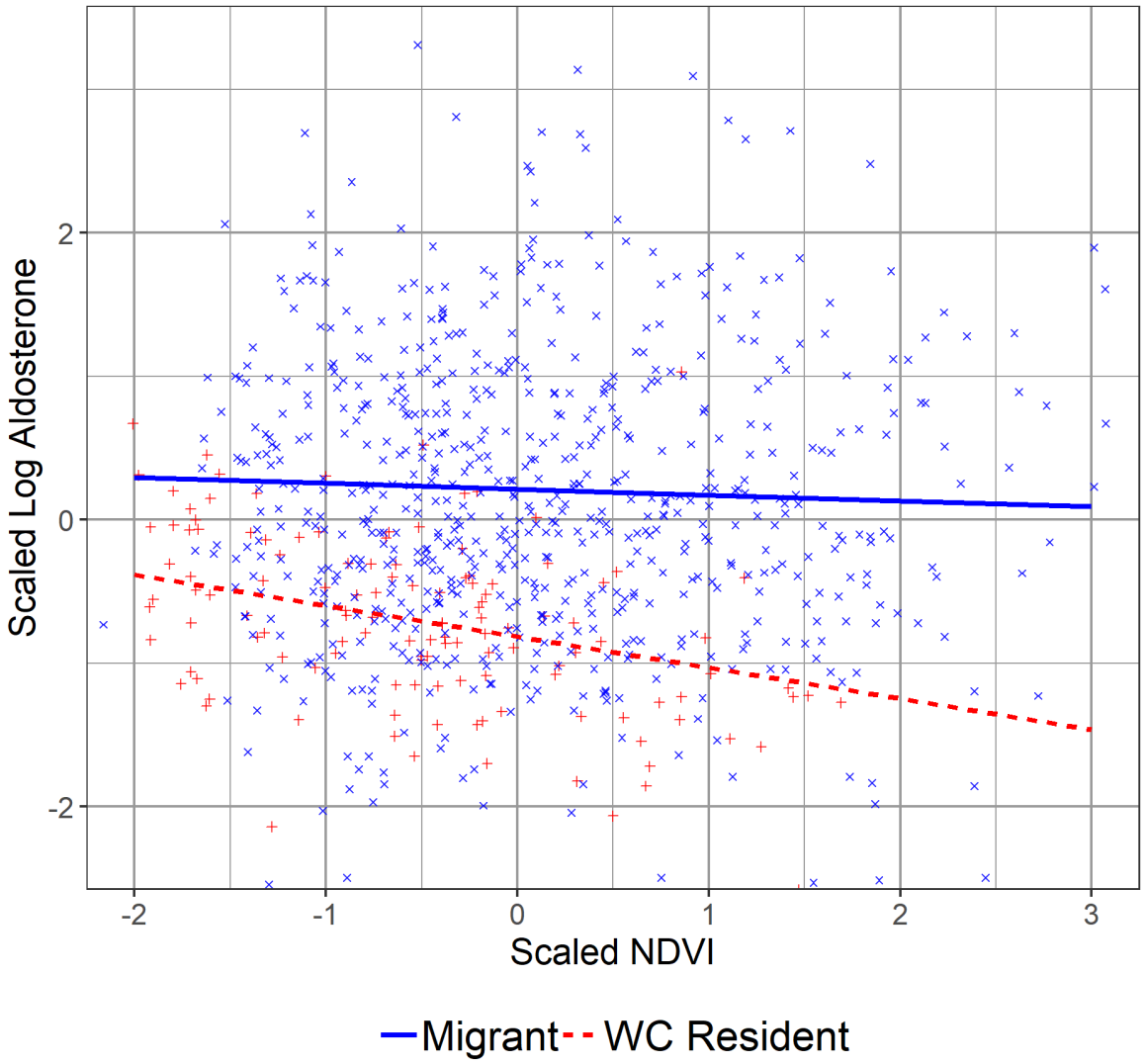


Figure 5.9 The effect of NDVI on hair aldosterone concentrations in Western Corridor resident and migratory wildebeest. Both populations indicate a general decrease in aldosterone concentrations as NDVI increases, however the effect is greater in the resident subpopulation.

5.5 **Discussion**

This study is the first to document the quantification of aldosterone in hair samples taken from wild animals. The results indicate that hair aldosterone concentrations can be used to inform of an animal's underlying sodium regulation, even in samples taken from animals occupying an ecosystem that experiences high temperatures and exposure to ultraviolet light. It is therefore

likely that aldosterone can be measured in hair samples obtained from other species, especially animals occupying colder environments. The results also indicated that hair aldosterone concentrations may be of value when studying animal movement, since aldosterone is higher, and shows greater seasonal variation, in migratory wildebeest, compared to residents.

5.5.1 **Stability of Aldosterone in Hair**

Aldosterone concentrations in wildebeest tail hair samples increased by a factor of 1.14 for every 30 days spent in the environment. This small change over time may be a result of the breakdown of structurally similar steroids in the hair into aldosterone (or 3β,5β-tetrahydroaldosterone, since the ELISA also has 41% cross-reactivity with this molecule), as it is exposed to the relatively high heat and ultraviolet light of the Serengeti. Due to the linear and consistent nature of the observed change in aldosterone concentrations within and between samples it is possible to mathematically correct aldosterone concentrations to account for this artefactual increase. Corrected hair aldosterone concentrations were therefore used to visualise data in plots and figures, while environmental exposure was included as an explanatory variable in statistical models attempting to analyse the effects of other variables on aldosterone concentrations.

5.5.2 **Movement Strategy**

More than 95% of the wildebeest population in Serengeti are migratory and so the question why some wildebeest have adopted a resident life history strategy is intriguing. A comparison between wildebeest employing different movement strategies indicated that aldosterone concentrations were lower in wildebeest resident to the Western Corridor region of the ecosystem, relative to migrants. The Western Corridor is relatively sodium rich (Hopcraft, 2010); therefore, this result not only validates the use of hair aldosterone concentrations to assess the physiological response to environmental differences in sodium, but also suggests

that electrolyte and fluid homeostasis may play an important role in prompting the specific movement strategy employed by individual wildebeest. Specific migratory movements can be divided into three general categories: alimental (to increase food and/or water availability); climatic (to avoid unfavourable conditions), and gametic movement (to reproduce) (Heape and Marshall, 1931). Although most migratory species exhibit all three types of movement, each specific movement in a certain direction tends to primarily be driven by one of these (Shaw, 2016). For example, our results indicate that sodium limitation may play a role in wildebeest movement, suggesting that the migration of wildebeest into the Western Corridor at the onset of the dry season is not just climatically driven, but also alimental. This aligns with other studies on drivers of migration in species showing partial migration (i.e. some individuals migrate while others do not), with resource limitations having been shown to be the drivers of partial migration in tortoises (Bastille-Rousseau et al., 2017), fish (Tamario et al., 2019), and even many insect species that exhibit migratory dichotomies (Menz et al., 2019).

It is generally understood that resident populations can only exist in areas that have enough resources to support animals all year round. To date, four populations of resident wildebeest have been identified in the Serengeti-Mara ecosystem which together contain at most 75,000 wildebeest (5% of the size of the migratory population), presumably due to environmental constraints. However, recent GPS data suggests that some residents join the migration for a year and then revert to being residents (Hopcraft *unpubl. data*). The recruitment in the resident wildebeest populations has not been extensively studied but it is thought that wildebeest calves adopt the movement strategy of their mother. Two behaviours expressed in wildebeest calves are crucial for this to occur: imprinting and a strong “follower” instinct (Estes, 1966). Maternal wildebeest and their calf will imprint on each other within minutes of birth, and are able to recognise each other’s scent and calls (Estes and Estes, 1979). Wildebeest calves are precocial, being able to stand within 6 minutes and walk within half an hour of birth (Sinclair et al., 2000). They will follow their mother for at least nine months, until they are fully weaned. However, specific factors that determine whether a population has a resident versus a migrant movement

strategy are not fully understood (i.e. either it is hereditarily predetermined, or it is a plastic behaviour in response to variable environmental conditions).

The results of this study illustrate that resident wildebeest have lower hair aldosterone concentrations than migrants, which suggests that the sodium profile of the residents and migrants differ. Work in rats has shown that the administration of aldosterone (i.e. high aldosterone) can produce a 'sodium appetite' (i.e. an increased consumption of sodium-containing solutions), but this is only observed in individuals who have previously experienced periods of sodium deficiency (Weisinger and Woods, 1971). This may be of relevance to Serengeti wildebeest, as migrant wildebeest calves will follow their mothers into the nutrient-poor northern reaches of the ecosystem in the dry season, where sodium is lowest but where they can access water. During this period, it is likely calves experience sodium stress which thereafter may lead to higher aldosterone concentrations. However, resident calves (which will stay with their mothers in the sodium-rich Western Corridor) may never experience comparable sodium deficiencies to produce a sodium appetite. Differences in the sodium appetite between resident and migrant wildebeest may contribute to the large-scale movement of animals as the migrant portion of the population may actively seek new areas of sodium rich grasses. Variation in individual response within the migrant population could partially explain why some wildebeest specifically swing into the Western Corridor during their migration, while others use a more direct north-south route.

Although these results suggest a number of interesting trends in resident and migrant wildebeest physiology, the sample size for our resident population was low (n = 4). Therefore, results relating to differences in movement strategy should be interpreted with caution. Although not possible in this study, future studies investigating the differences between resident and migrant wildebeest physiology would benefit greatly from an increase in the sampling of resident animals, to increase the statistical power of the analysis.

5.5.3 Environmental Drivers of Aldosterone

There is some evidence to suggest that the abundance of environmental sodium in the grass may be associated with lower physiological requirements to regulate endogenous sodium, as reflected by hair aldosterone concentrations. The sodium concentration in the vegetation the animals access varies across the landscape. The lowest grass sodium content occurs in the north (Fig. 5.3), which tends to be occupied by the migrant wildebeest during the dry season, when aldosterone concentrations in the hair are greatest (Fig. 5.8). Likewise, aldosterone concentrations are lowest at the end of the wet season, once wildebeest have spent the most time in the sodium-rich areas in the south. This strong seasonal trend in aldosterone concentrations was greatest in migratory compared to resident wildebeest, which corroborates the above interpretation.

Given the observed differences between hair aldosterone concentration and the resident-migrant movement strategy, it may be expected that we should see a clear relationship between aldosterone and proximity to high grass sodium, particularly within the migratory population. However, there was no evidence that proximity to grass sodium correlates with tail hair aldosterone in our data, despite what we would expect from the extensive literature about the relationship between sodium and aldosterone. This lack of correlation may be the result of differences in the resolution of our metrics for sodium and aldosterone data. Sodium data were derived from a static GIS layer (i.e. a one-off measure of grass sodium at 148 sites across the entire ecosystems), while hair aldosterone concentrations are an integrative chronic measure over a 14-day period, which is dependent upon the animal's cumulative sodium consumption over an extended period of time, combined with its relative level of hydration. This may explain why aldosterone levels correlate with season, but not sodium concentration, since season relates to more gradual, long term changes in the overall availability of environmental factors such as sodium concentration, while the sodium concentration experienced by the animal at that time, does not. For example, although aldosterone concentrations begin to decrease following the end of the dry season, this decrease occurs over an extended period of time, meaning that relative aldosterone concentrations are still relatively high as wildebeest return to the southern plains at the onset of

the wet season; an area which has a relatively high concentration of environmental sodium. Therefore, hair aldosterone concentrations may more accurately reflect cumulative environmental sodium availability (e.g. a lag effect, operating over several months).

While aldosterone regulates sodium in the body (high aldosterone results in increased conservation of sodium in the body), aldosterone and renal sodium handling are also closely linked with body water regulation. Thus, when sodium concentrations are low in the body, aldosterone production increases to compensate, and water is retained. Migratory wildebeest may experience greater variation in both vegetation sodium content and exposure to dehydration. An additional interpretation of the seasonal patterns in aldosterone concentrations observed in our data is that the high aldosterone concentrations seen at the end of the dry season relate to chronic levels of active water conservation by wildebeest, resulting in increased aldosterone. To test this, we included two measures of rainfall in our analysis: daily rainfall and a 14-day cumulative rainfall index. Neither measure was found to correlate with aldosterone concentrations. This does not provide conclusive support for the hypothesis that dehydration is a source of chronic activation of the Renin-Angiotensin-Aldosterone System (RAAS). The poor correlation could be due to either (i) differences in the resolution of our metric (i.e. an integrated aldosterone metric over 14 day period does not accurately capture daily access to drinkable water) or (ii) it may be that wildebeest rarely experience periods of severe and prolonged dehydration that would be required to see changes in hair aldosterone, or (iii) hydration is correlated with another explanatory covariate, such as grass greenness (NDVI). Wildebeest tend to drink every 1-2 days if they have access to water (however they can survive for several days without drinking, particularly if the water content in the grass is high). During the dry season they tend to have adequate access to drinking water from the Mara River. Therefore, dehydration may only become a problem in severely dry years, when grass is unavailable within daily walking distance of the river (at the end of the dry season), or when the river stops flowing.

The negative correlation between grass greenness index (NDVI) and aldosterone supports the proposition that green vegetation is associated with lower hair aldosterone concentrations. This relationship was more pronounced in resident

wildebeest, compared to migrants. This may suggest that by migrating individuals may experience more consistent access to a broader range of vegetation that would not be available to resident populations (i.e. they are consistently able to move to the greenest areas and most nutrient rich areas). Additionally, correlation between NDVI and proximity to high sodium (0.40) suggests that although sodium did not remain in the optimal model, NDVI may be explaining some of the variation that is driven by sodium content.

5.5.4 **Future Work**

It may be possible to improve model fit in future studies by measuring sodium in the landscape in a more dynamic manner, both in space and time. In other words, aldosterone concentrations could correlate with mean sodium content, not just at the current GPS position but across all previous locations as well (cumulative lag effects). Furthermore, very little is known about how the mean sodium content in Serengeti grasses changes seasonally across the landscape. A spatially interpolated and seasonally dynamic layer of grass sodium that accurately estimates the grass sodium in space and time may improve the correlation between the aldosterone concentration in the hair and the estimated sodium content in the landscape. Additionally, future studies could shed more light on the role played by sodium in wildebeest physiology by measuring circulatory sodium concentrations directly in darted wildebeest, to determine whether wildebeest are sodium limited, as our results suggest.

Future studies could also gain valuable information about several other aspects of the population by using the time-series data of aldosterone. For example, future work could explore the following aspects:

1. Since aldosterone correlates strongly with seasonal variation, aldosterone could be used to estimate and validate hair growth rate in other species (i.e. a natural biomarker of the start and end of the dry season).
2. Differences in the aldosterone profiles between resident and migratory wildebeest hair samples suggests this technique could be used to

determine the movement strategy of animal's whose history is unknown (e.g. carcasses, museum specimens).

3. A comparison between modern and historic samples obtained from museums and private collections may inform how sodium homeostasis has changed as a function of increasingly variable climates.
4. Finally, the aldosterone time-series for an individual could be combined with other covariates from the hair (such as, stable sulphur, strontium, or nitrogen isotope ratios) in a multivariate ordination analysis (such as principal component analysis), to estimate the geolocation of an animal through time.

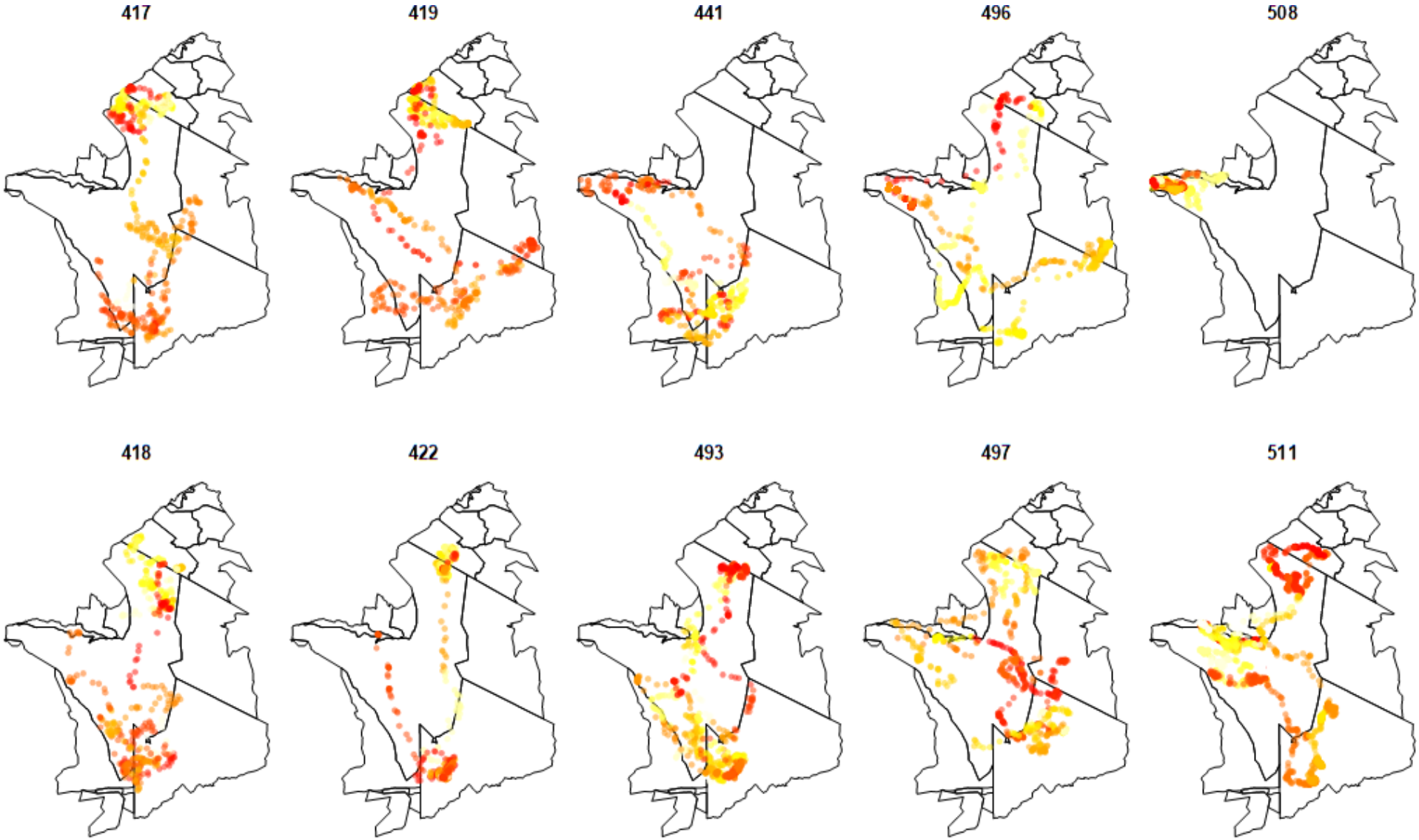
5.6 **Conclusion**

This study validates a relatively straight-forward and robust method for the collection of chronic physiological time series data indicative of sodium homeostasis, relating to 18 months of an animal's life. Lower aldosterone concentrations in resident wildebeest suggests reduced sodium stress. Sodium appetite, induced by extended periods of sodium deprivation, may be one of the factors explaining why movement strategy appears to be fixed throughout an animal's life (i.e. why resident animals raised in a sodium-rich environment rarely migrate, while migrant animals raised in a heterogenous sodium landscape with extended periods of low sodium intake rarely become resident).

5.7 Supporting Information

Table 5.2 (Supplementary) Comparison of models explaining variation in hair aldosterone concentrations in 813 sections of hair from 24 female wildebeest in the Serengeti-Mara ecosystem. Sample ID was also included as a random effect. Models ranked by AIC, with the optimal model in bold.

Model	DF	AIC	ΔAIC	Log Likelihood
*Season + NDVI + Migrant + Migrant*NDVI + Migrant*Season + Env.Exp.*	9	1503.68	-	-742.84
*Season + Sodium + NDVI + Migrant + Migrant*NDVI + Migrant*Season + Env.Exp.*	10	1506.44	2.76	-743.22
*Season + Migrant + Migrant*Season + Env.Exp.*	7	1506.78	3.10	-746.39
*Season + Sodium + Migrant + Migrant*Season + Env.Exp.*	8	1507.25	3.57	-745.62
*Season + Sodium + NDVI + Migrant + Migrant*Sodium + Migrant*NDVI + Migrant*Season + Env.Exp.*	11	1509.04	5.36	-743.52
Season + Migrant + Env.Exp.	6	1510.23	6.55	-749.12
*Season + Sodium + NDVI + Age + Dead.Alive + Migrant + Migrant*Sodium + Migrant*NDVI + Migrant*Season + Env.Exp.*	13	1519.08	15.4	-746.54
*Season + Sodium + NDVI + Age + Dead.Alive + ΔNDVI + DayRainfall + Prev14dayRainfall + Migrant + Migrant*Sodium + Migrant*NDVI + Migrant*Season + Migrant*ΔNDVI + Migrant*DayRainfall + Migrant*Prev14dayRainfall + Env.Exp.*	19	1543.05	39.37	-752.53
Migrant + Env.Exp.	5	1644.44	140.76	-817.22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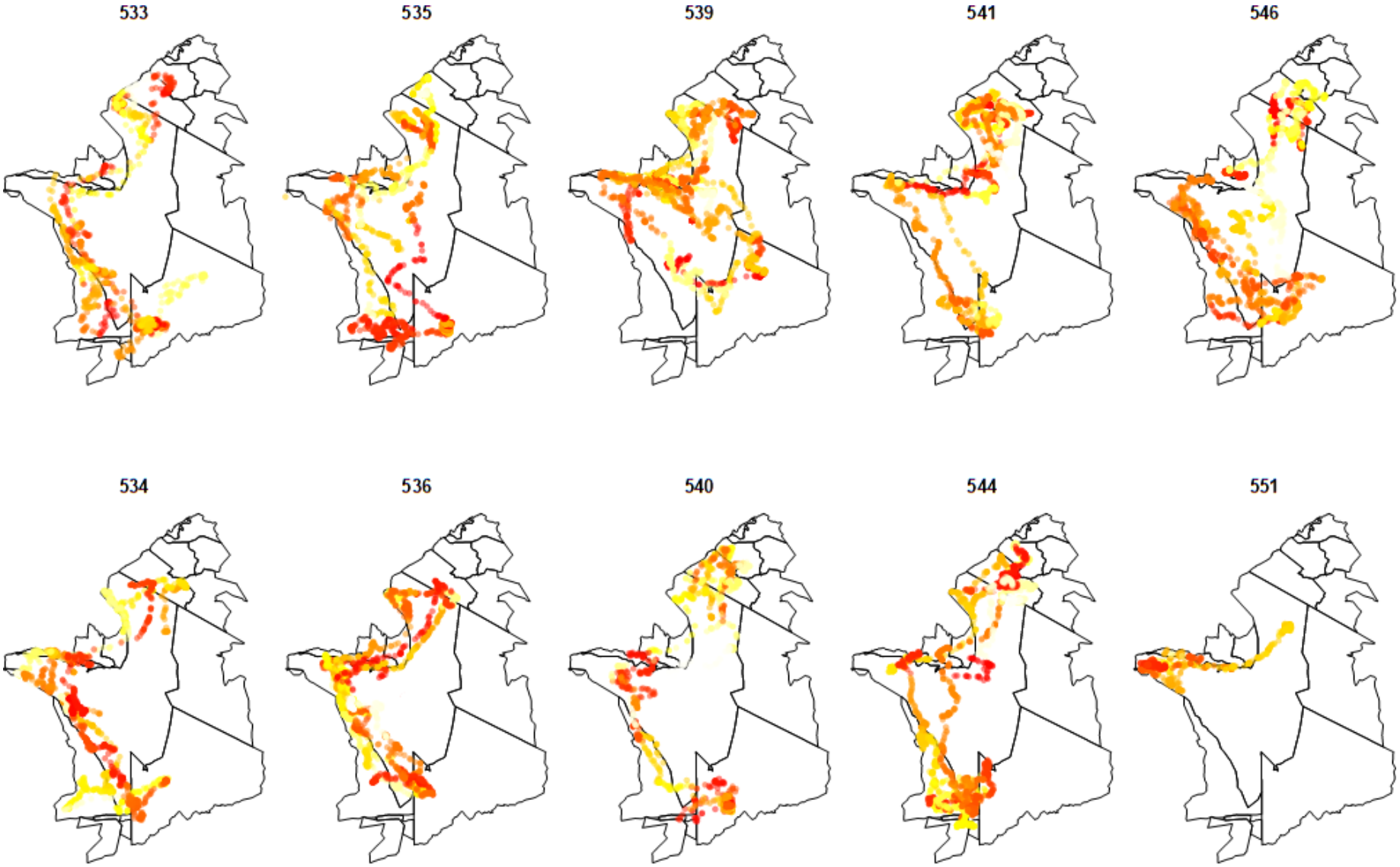
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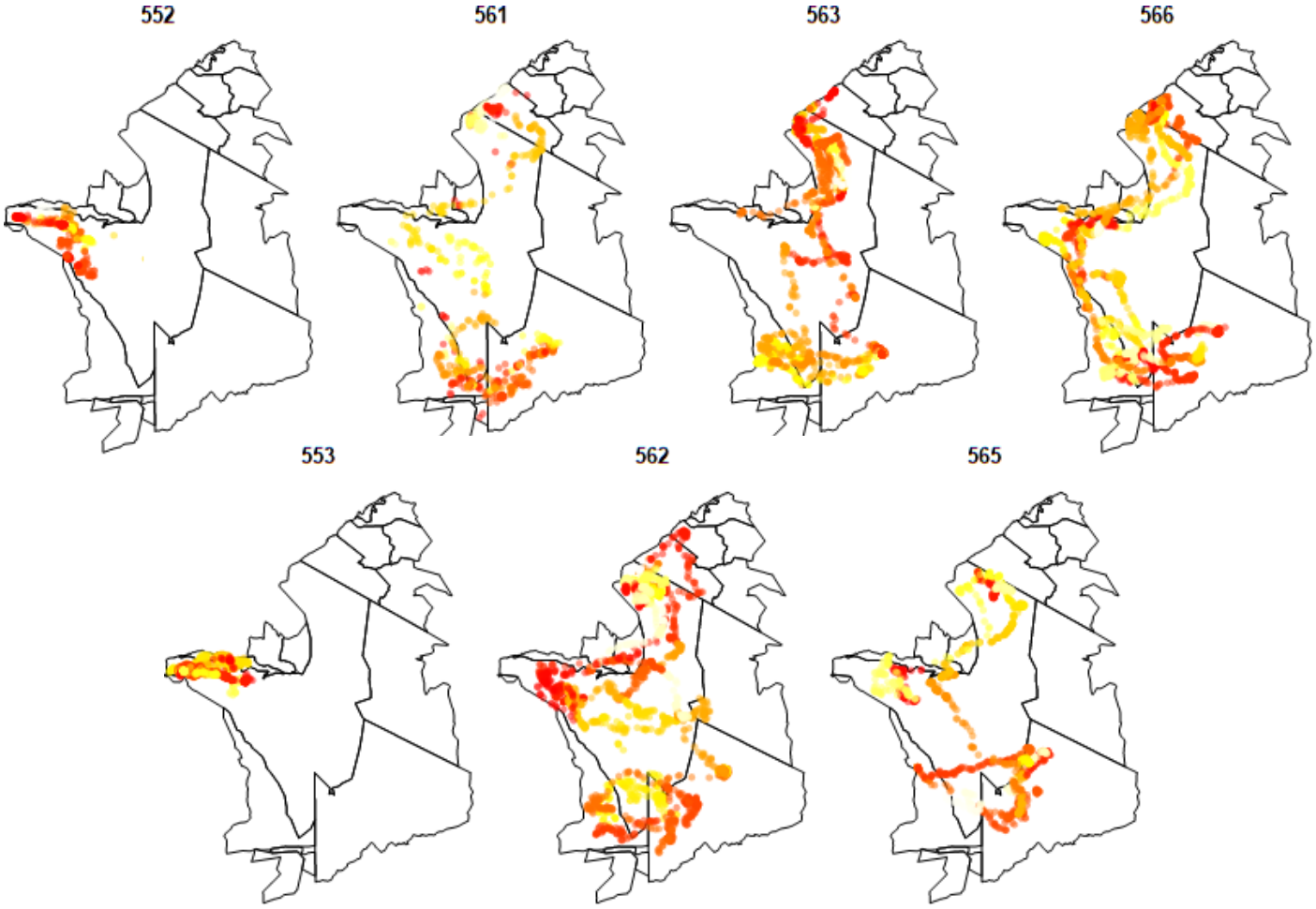

Figure 5.10 (Supplementary) Corrected hair aldosterone concentrations plotted relative to the corresponding locations the animal visited during that time, superimposed onto a map of the Serengeti-Mara Ecosystem. Darker points represent higher standardized hair aldosterone concentrations (i.e. highest aldosterone concentrations within a sample are illustrated by dark red points; lowest aldosterone is represented by light yellow points).

6 General Discussion

This thesis validated a method for the collection of time-series physiological data from steroid hormone concentrations in longitudinal sections of wildebeest tail hair. Further, cortisol and aldosterone profiles produced from GPS-collared animals were aligned with telemetry data, to determine the relationship between animal physiology and the environmental context in which animals live. This study indicated both environmental and anthropogenic sources of physiological stress in wildebeest, as well as seasonal trends in progesterone and aldosterone, indicative of changes in reproductive state and forage quality, respectively. Therefore, the relatively novel method outlined in this study is a valuable source of physiological data, which is important in building a more complete picture of animals in the wild and how they affect, and are affected by, their environment.

Chapter 2 validated the general method for the temporal alignment of physiological profiles by calculating wildebeest tail hair growth rate (4mm/week) and demonstrated that cortisol is stable in wildebeest tail hair over time. A caveat here was that hair composition was shown to change along the length of a sample, and therefore a method of standardising hair cortisol concentrations (*pg/cm hair*, as opposed to the traditional units of *pg/mg hair*) was required. This validated our method for the collection of time-series cortisol data from sequentially sectioned hair.

Chapter 3 sought to identify drivers of hair cortisol concentrations, both environmental (NDVI: a remotely-sensed metric of vegetative productivity) and anthropogenic (proximity to villages), and suggested that chronic stress is increased when wildebeest are in locations where food availability is low and where villages are nearby. Hair cortisol concentrations (analogous to 'stress levels') were also higher in samples taken from animals found dead, compared to samples obtained from live animals, which could indicate a link between chronic stress and fitness. It was also discovered that the effect of NDVI and on cortisol differed between samples taken from dead and living animals, which

suggested that chronically stressed animals (i.e. carcass samples) respond to environmental challenges differently to animals that were alive at the time of sampling.

Chapter 4 determined the validity of a number of hormones related to reproduction for the creation of profiles of reproductive history (i.e. if/when an animal has been pregnant and calved). Hair progesterone (and/or a molecule insufficiently distinct from progesterone, in terms of chemical structure) was shown to be unstable in the environment over extended periods of time. However, this instability appeared to be linearly related to time in the environment and could therefore be corrected, thereby allowing the assessment of reproductive profiles. Overall, corrected hair progesterone concentrations were found to increase at the rut (when the vast majority of females become pregnant) and remained elevated until the calving period, when they returned to basal progesterone levels.

Finally, Chapter 5 validated the use of aldosterone concentrations measured in consecutively sectioned hair samples taken from GPS-collared animals to produce spatio-temporal profiles of sodium stress. Like progesterone, hair aldosterone concentrations increased along the growth axis of the hair, and it was possible to detrend the data using the position along the hair sample (analogous to time spent in the environment). Samples taken from migratory wildebeest exhibited higher aldosterone concentrations than wildebeest that were resident in sodium-rich areas of the ecosystem, which would suggest that migratory animals may experience greater sodium stress. Aldosterone concentrations were also correlated with season and vegetation greenness. This result would suggest that wildebeest are most sodium-deprived at the end of the dry season. This physiological inference is supported by movement data as this is when they occupy the most sodium-poor area of the ecosystem. Significant interactions also indicated higher seasonal variation in aldosterone concentrations in migrants (compared to residents), while NDVI had a greater effect on aldosterone in residents than in migrants. This suggests that movement strategy may have an effect on the regulation of salt and water balance in the body, which in turn may play a role in the general lack of plasticity in the movement strategy of individual wildebeest (i.e. migrants experience high

sodium stress as calves, which leads them to develop a ‘sodium appetite’, actively seeking new areas of sodium rich grasses, while resident calves do not experience sufficiently high sodium stress to elicit a sodium appetite).

Overall, this thesis has demonstrated a relatively straightforward and robust method for the measurement of an individual animal’s physiology over long time periods, which can be linked with spatial information obtained from GPS locations. This opens exciting new avenues for ecological research for the collection of long-term physiological data in mammals, however, there are a number of metrics which must be measured before this method can be applied to a new species and ecosystem.

6.1 **Method Transferability**

There are five main factors to consider when validating the use of the technique outlined in this thesis on an alternative study species. First, the area of the body where the longest hairs grow must be determined. Long hairs allow for the creation of physiological profiles relating to long periods of time. Additionally, longer hairs spend a longer proportion of time in the anagen (growth) phase, relative to the catagen (transition) and telogen (resting) phases (Alonso and Fuchs, 2006). This minimises any error associated with changes in hair growth phase. For instance, any hair which has stopped growing but has not yet fallen out will misalign, temporally, with hairs in the anagen phase. This introduces error when calculating the corresponding date of growth for each section of hair, but it is minimised in longer hair. Additionally, since hair growth rate varies across different areas of the body (LeBeau et al., 2011), hair should be collected from precisely the same area on all sampled animals (e.g. the tip of the tail).

Once the area of the body to be sampled has been determined, the second factor to be established is the growth rate. Ideally, hair growth rate would be measured directly for each animal, by resampling the same area of an animal every few weeks and recording the length of regrown hair (Burnik Šturm et al., 2015). However, this isn’t always possible in free-living animals. If the study species experiences dramatic, predictable changes in their environment (e.g.

strong seasonal changes) or their physiological state (e.g. impregnation, parturition), it may be possible to use hormone concentrations measured in sectioned hair samples to fit a hair growth rate, retroactively. For instance, if a female is with a calf, the approximate age of the calf can be used to calculate approximate dates for parturition and conception, which can in turn be aligned with trends in reproductive hormones, observed along the hair sample. Measures of hair growth rate for the study species (or a related species) in the primary literature should be used as a guide, in this instance.

The third and fourth factors to consider are the mass of sample to be collected and the length of the sections of hair per sample, (and therefore, the temporal resolution of the physiological data obtained). These two factors are directly linked, as the section length is limited by the minimum sample mass necessary to obtain a hormone concentration. For instance, due to the relatively high mass of hair collected using our sampling technique, we were able to section our hair into lengths of 8mm, to produce sections relating to two weeks of hair growth (growth rate = 4mm/week). This resolution was optimal for studying changes in wildebeest physiology over the temporal range of a full-length sample (18 months), while allowing for all sections produced from a sample to be assayed on a single plate (minimising the error associated with inter-assay variation). However, if there were only half the number of hairs in each sample, we would only be able to produce 16mm sections, with a resolution of four weeks, instead of two. It is therefore optimal to collect as large a volume of hair as possible. Minimum required mass of hair sample was calculated for a number of species during our research (section 2.4.6), which may be used as a guide. Hair section length should be selected in order to optimise the temporal resolution of the data, to answer questions of ecological importance to the study, while minimising error.

The final factor to consider when using this technique in a new species is the stability of the hormone of interest. This can be determined by investigating whether position along the growth axis of a hair sample (analogous to time spent in the environment) has any effect on the hair hormone concentration (while controlling for potentially correlating temporal factors, e.g. time of year). If the

position along the hair is a significant predictor of hormone concentrations, it may be possible to correct for this trend (as demonstrated in Chapters 4 and 5).

This method can be further validated in other species by comparing between hair hormone profiles created from samples taken from the same individual a few months apart. By resampling from an area of the body immediately adjacent to the area originally sampled, two full-length hair samples can be collected, which partially overlap (with regards to time of growth). These hormone profiles can then be used to align hair samples and determine mean hair growth rate, intra-individual variation in hair growth rate, and the stability of the hormone(s) of interest, over time (as demonstrated in Chapter 2). For example, the method outlined above was used to validate the collection of temporal hormone profiles using zebra tail hair, as an additional part of this study. Both cortisol and progesterone were measured in zebra tail hair, showing variation along and between samples. Additionally, zebra hair growth rate (calculated by optimising the temporal alignment of cortisol profiles) was similar to rates measured in other equid species (Burnik Šturm et al., 2015). Although only a preliminary study, this shows promise for the implementation of this technique with alternative study species.

6.2 **Recent Studies of Note**

In the past few years, the number of studies utilizing hair analysis for the collection of physiological data has grown a great deal. Several studies have successfully measured cortisol in an ever-growing list of species, including coyotes (Schell et al., 2017), camels (Shah et al., 2018), goats (Endo et al., 2018) and lemurs (Rakotoniaina et al., 2017). Additionally, the variety of hormones that have successfully been measured in hair continues to grow, including testosterone (measured in coyote hair) (Schell et al., 2017), and progesterone (measured in brown bear hair) (Cattet et al., 2017). Several studies have also focused on the validation of hair analysis techniques, such as by comparing hormone concentrations in hair taken from different areas of the body (Fourie et al., 2016) or through the comparison of hair hormone concentrations to those measured in blood (Koren et al., 2019). Several studies

have also implemented a similar technique to the one outlined in this thesis, for the segmental analysis of hair samples, to produce time series data of stable isotope ratios which can inform of diet shifts and starvation, using known life history events to validate measures of hair growth rate (Rysava et al., 2016, Kaczensky et al., 2017, Burnik Šturm et al., 2017). Recent studies have also successfully measured steroid hormones in other keratinized media such as feathers, scales, beaks, and nails (Will et al., 2014, Baxter-Gilbert et al., 2014, Hamilton et al., 2018, Voegel et al., 2018). If the growth rate of these samples can be measured, these media could be an additional source of longitudinal physiological data, relating to alternative timespans. This opens the application of this method to not just mammals, but effectively any species which produces keratinized tissues such as those outlined above, including birds, fish, and reptiles.

6.3 Future Work

The results of this thesis open a number of potential avenues for future studies in eco-physiology beyond the implementation of this technique to study other species. For instance, the sequential analysis of hair samples obtained from taxidermied animals, as demonstrated in Chapter 2, indicates the potential to obtain physiological data on historic animal populations, including populations and species that are now extinct. Additionally, a comparison between physiological profiles obtained from historic and modern animals would allow for the study of changes in physiology over extended periods of time (e.g. decades to centuries). This could be a highly valuable source of data for studies interested in studying the long-term effects of human disturbance and even climate change on wild animal populations.

Another area of research where the implementation of the technique outlined in this thesis may be of value is animal welfare. For example, a comparison between hair cortisol profiles obtained from free-living and captive animals would allow for a better understanding of the long-term physiological effects of captivity, with regards to chronic stress. Welfare studies have used hair cortisol concentrations to indicate of poor welfare, when compared to a control group

(i.e. a study on dogs farmed for meat in South Korea found meat dogs were significantly more stressed than pet dogs (Maxwell et al., 2019)) (Carroll et al., 2018, Sharma et al., 2019); this field could benefit further from the implementation of sequential hair analysis to determine onset and duration of stressors, etc. Further, the analysis of samples collected from captive animals, where physiological data is collected (e.g. diaries of reproductive history), could also be used to cross-validate the use of this thesis' method in other species.

A comparison between physiological profiles produced in this study and ones created from samples obtained from captive wildebeest, living in different climates, may aid in understanding the role of environmental exposure on hair hormone concentrations. For instance, if heat and exposure to ultraviolet light are responsible for the increase in progesterone concentrations observed along wildebeest hair samples, this trend should be less severe, or absent, in samples taken from animals living in cooler climates.

Future studies could also benefit from the creation of physiological profiles relating to different temporal resolutions, by adjusting the length of hair sections produced from a sample. For instance, the relative peak in concentration of cortisol and oestrogens which occurs at parturition (Hill et al., 2004) could not be detected in wildebeest hair samples segmented into 8mm sections (corresponding to two weeks' growth) (Chapters 3 and 4, respectively). However, the temporal resolution of physiological data could be increased, by cutting hair into smaller sections (e.g. 4mm sections, relating to 1 week's growth), to determine whether the underlying trends in hormone concentrations that are indicative of parturition can be detected at a finer scale.

Although this thesis focused exclusively on the quantification of steroid hormones extracted from hair by Enzyme-Linked ImmunoSorbent Assay (ELISA), a few studies have successfully developed a method for the quantification of a suite of steroid hormones in hair using liquid chromatography tandem mass spectrometry (LC–MS/MS) (Gaudl et al., 2016). Although immunoassays benefit from being relatively cheap, fast, and easy to perform, there are many benefits to the use of LC-MS/MS for steroid analysis. It is more specific than ELISA, since it does not rely on antibodies for quantification, eliminating the error associated with cross-reactivity (i.e. antibodies in ELISA kits tend to also bind substances

that are structurally similar, but different, to the target molecule). Another major benefit of LC-MS/MS is that it is possible to measure the concentration of multiple hormones at one time, from a single sample (Gao et al., 2013). This method therefore saves time (since an ELISA can only measure one hormone per assay) but is also a valuable source of additional physiological data. For instance, mass spectrometry has the potential to simultaneously quantify all of the steroid hormones studied in this thesis, as well as others, such as testosterone (Gaudl et al., 2016). Testosterone concentrations have been shown to correlate with social rank, aggression, and risk taking (in both males and females) (Beehner et al., 2005, Mehta et al., 2015); highlighting additional physiological metrics, of interest to ecological studies, which could be obtained from hair samples. Additionally, the ability to quantify multiple steroid hormones simultaneously would allow for the relationships between hormones, as well as their cumulative effects on physiology and behaviour, to be studied in far greater detail. This is highlighted by our results indicating a link between cortisol and aldosterone concentrations in resident and migrant wildebeest.

Furthermore, combining data relating to multiple hormones with other covariates from the hair (such as, stable nitrogen isotope ratios), as well as GPS data, would greatly increase our understanding of how animals interact with their environment. These variables could also be of value in a multivariate ordination analysis (such as principal component analysis), to anticipate animal re-distributions and their ecological consequences.

Finally, the incorporation of the physiological indicators developed in this thesis into animal movement models would be a valuable next step to better understand the behavioural decisions of these animals. Migratory wildebeest vastly outnumber resident wildebeest populations in the Serengeti-Mara ecosystem, as moving large distances frees animals from the restrictions imposed by local forage abundance (Fryxell et al., 1988, Hopcraft et al., 2014). As a result, the migratory wildebeest population both affects a vast array of ecological processes in the ecosystem, such as plant and animal biodiversity, while also being particularly vulnerable to anthropogenic factors such as habitat loss and fragmentation (Anderson et al., 2007, Bolger et al., 2008). It is

therefore of great importance to improve our understanding of how and why wildebeest migrate.

6.4 **Conclusion**

The overall results of this thesis validate a method for the collection of data relating to the chronic concentration of cortisol, progesterone, and aldosterone from segmental analysis of hair samples. These findings also show promise for the transferability of this method to other taxa and settings, opening an array of potential avenues for future research relevant to movement ecology, conservation management, and animal welfare. Of particular interest is the ability to implement this method for the collection of historic physiological data from hair samples collected from taxidermied animals (as demonstrated in Chapter 2). This not only indicates the robustness and transferability of our method, but also shows great potential for the study of animal physiology over extended periods of time (hundreds of years), using museum samples to collect data previously believed to be lost to time.

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