Application of recent developments of molecular tools in the conservation of cheetahs

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This thesis is submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Canberra

2021

Abstract

Paradigm shifts in the field of conservation biology along with continuous decline in biodiversity in the past few decades have elicited the need to develop and apply robust, cheap, and easily available tools in conservation planning. Among the recent technological tools, advances in molecular techniques have presented a multitude of opportunities in the studies of biodiversity and nature conservation. The research presented in this thesis investigates the potential of two different emerging molecular techniques; DNA metabarcoding and single nucleotide polymorphisms as conservation and management tools for cheetahs *Acinonyx jubatus*, Africa's most threatened big cat. There are only 7,100 cheetahs left in the wild, reduced from over 100,000 a century ago, existing only in 9% of their historical distributional range. This range-wide decline is largely associated to habitat loss and fragmentation, prey base decimation and persecutions. The majority of the 7,100 individuals occur outside government protected areas, therefore I have used this species as a case study for managing human-carnivore conflict. In these non-protected areas, there is a high likelihood of human-carnivore conflicts with the repercussions being meted on cheetahs as they are easy to sight and kill regardless of their involvement in livestock predation. Moreover, modern cheetahs are threatened by the lack of genetic diversity linked with the population collapse more than 12,000 years ago, making them especially prone to diseases and poor reproduction. I review the current state of biodiversity loss especially in relation to carnivores and highlight different molecular techniques and approaches that have so far been used to underpin the diet and patterns of genetic variation of wild species. Next, I validate the potential of DNA metabarcoding in dietary analysis of cheetahs using captive individuals and then I apply this technique to characterise the diet of free-ranging cheetahs in Kenya to assess the level of livestock predation. Finaly, I examine the potential of cheetah-specific single nucleotide polymorphism (SNPs)

markers generated using genome-complexity reduction approach to describe evidence of population and regional substructure.

The results demonstrate that DNA metabarcoding provides a sensitive method of prey detection in cheetah scats and highlights the need to account for systematic biases to control for possible scat degradation, feeding day, meal size and prey species consumed. Also, the results showed that cheetahs in Kenya prey on a diverse range of taxa and domestic animals form a small component of their diet. Finally, the SNP data showed low values across all samples, suggesting limited genetic diversity in Kenyan cheetahs but they provided evidence for genetic differentiation between the southern population (Maasai Mara) and northern population (Laikipia). This thesis describes all of the methods used and provides a useful resource for further research that involves elusive and endangered species.

Acknowledgements

This work would not have been possible without the help of many people, whose names are simply too many to list. I will, therefore, limit my specific acknowledgements to a few key people who have been integral in the design and implementation of this study. First and foremost, I would like to thank my supervisors for providing gripping examples of how to be a creative, attentive, and diligent researcher. I sincerely appreciate the patience, time, and effort they invested to all our interactions. My supervisor, Dianne Gleeson provided advice, encouragement, and guidance throughout my candidature; most importantly she gave me a platform to challenge myself and believed in my abilities when I doubted myself. I would also like to thank Elise Furlan for supervising and supporting me from the beginning and having resolute faith in me, you are an amazing mentor. Femke Broekhuis, thank you for showing me the ropes with the cheetahs and for the many detailed research ideas that you shared with me, I am forever grateful for your continued mentorship. In a special way, I would like to thank Joseph Kamau for his supervision, guidance, innumerable personal and professional conversations that we've had and for always reminding me not to follow the crowd but rather create my own trail, if it were not for you, I would have hanged my academic boots after my undergraduate degree.

I would also like to thank the IAE community for their support and many comments/suggestions on my presentations. This feedback and ideas on my project have shaped most of the research questions I have discussed in this thesis. I wish to thank the IAE/Sci-Tech admin team, particularly Barbara Harris for helping me settle into IAE and guiding me through the administrative processes. Many thanks to the IAE and Australian Research Training Program for offering me a full scholarship to pursue what was only a pipe dream a few years ago, you have transformed an African village boy and proved to many, that the only limit is in one's imagination.

I also want to acknowledge Richard Duncan, Bernd Gruber and Andrzej Killian for their help with the statistical analyses and for having the time to explain the complex statistical models and technological concepts used in this thesis and for dampening the genomic jargons. I would like to thank the staff from the National Zoo and Aquarium in Canberra, particularly Kyle Macdonald for providing poop samples and taking care of the lovely cheetahs. The adorable cheetahs (Jura and Innis), thank you for helping your wild kind and for promoting the field of science. Many thanks to the Kenya Wildlife Trust for providing cheetah samples used in this study. A special thanks to Kenya Wildlife Service for providing us with all the permits required to conduct research in Kenya National Parks.

Special recognition goes to my wonderful wife Maureen and our precious daughter Imani, for their incredible support which has been a strong pillar in this journey-you are my love, constant companion and best friends. Maureen, you have made personal sacrifices to see this work reach a logical conclusion. The proofreading, the late nights you stayed with me for moral support and for walking with me in every way. Without a doubt, you have equally contributed to the cheetah conservation and science, and wherever this work will be read and implemented, your name shall be ingrained thereon and, therefore, I say to you: thank you.

Many thanks to the present and past HDR students who directly or indirectly contributed to this work. Thanks to all my office mates for providing a conducive working environment, great conversations and coffee breaks. Also, thanks to Jonas Bylemans and ECODNA group for helping in the lab and data processing. Many thanks to unofficial ‘battalion’ of Laura Bertola, Kiptoo Evans, Kaelo Michael, Dedan Ngatia, Hellen O’Neil, Warren Johnson and Stephen O’Brien, all your comments and suggestion during study design, sample collection, data analysis and writing have shaped this thesis.

Finally, and by no means least, I want to thank my lovely parents, my siblings, and my in-laws for their love and constant support. To the Lord my God, thank you for your grace.

You all, have been the backbone of this PhD and this thesis is as much your product as it is mine.

Ahsante sana! Thengio! Ashe Oleng! Thank You!!

Dedication

This thesis is dedicated to my loving parents, Johnson, and Gladys Thuo and my wife and daughter, Maureen, and Imani.

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Chapter 1

1.0. General introduction

Theoretical and empirical evidence suggest that we are on a trajectory for the sixth global extinction crisis (Barnosky et al., 2011; Ceballos et al., 2017; Wake and Vredenburg, 2009). Human-induced processes, such as habitat conversion, global warming and its consequences have been recognized as the leading drivers of biodiversity loss (Ceballos et al., 2015; Dirzo et al., 2014; Pimm and Raven, 2000). According to Vos et al. (2015), the current rate of species extinction is 1,000 times higher than the natural background rates of extinction and future rates are likely to be 10,000 times higher. These biodiversity cataclysms have been shown to not only be affecting the existing levels of species biodiversity but also the evolutionary processes contributing to future biological diversifications, through the loss of phylogenetic lineages and genetically-distinct populations (Mace and Purvis, 2008). Whilst species extinction is ubiquitous throughout the history of life, the rate of loss and extent to which this is now occurring is of major global concern. As such, research that addresses the biology, ecology, and conservation is invaluable in curtailing species extinction.

1.1.Large carnivores: population status, function, and conservation

Large terrestrial carnivores have historically been recorded in nearly every major habitat on earth (Hunter 2011). However, many of these species have suffered extinction and many of the extant species have either been exterminated from their historic ranges or have experienced substantial population declines (Ripple et al., 2014). Large terrestrial carnivores are usually prone to population declines because they high ecological needs. Such characteristics that include slow reproductive rates, prolonged parental care, low densities and large habitat requirements generally limit their recovery capacity (Carbone et al., 1999; Cardillo et al., 2004).

Currently, 28 large terrestrial carnivore species (those with average adult body mass ≥ 15kg) belonging to five families: Canidae, Felidae, Hyaenidae, Ursidae and Mustelidae are recognized. They are geographically distributed across six continents (Africa, Asia, Europe, North America, South America, and Australia). Of these species, 64% are threatened with extinction and 92% have declining population trends (IUCN 2019, Table 1). The current estimate of habitat contraction for 26 of the 28 species shows that they have lost on average 56.4% (minimum 12%, maximum >99%) of their historical ranges in the last one hundred years (Table 1). These range losses may result in local population extinctions, which have implications for the maintenance of ecosystem and socioeconomic services such as loss of employments (Ripple et al., 2014).

Large carnivores play a key role in maintaining a healthy ecosystem through top-down regulation of prey and mesopredator densities (Ripple et al., 2014). Removal of these large carnivores from an ecosystem can have a far-reaching trophic cascade and may trigger co-extinctions of organisms whose persistence, directly or indirectly, depend on ecological interactions provided by megafauna (Estes et al., 2011; Galetti et al., 2018). Unfortunately, many of them face grim challenges across their habitats and their survival in their natural environment is uncertain.

Large carnivores are among the most controversial species in conservation due to their predatory habits that can result domestic animal kills, property damage and in extreme cases, attacks on humans (Inskip and Zimmermann, 2009; Treves and Karanth, 2003a; Tsering et al., 2006). As the human population continues to grow, multiple studies have shown that the survival of large carnivores will solely depend on the goodwill of the local communities that share landscapes and resources with wildlife (Inskip and Zimmermann, 2009; Muriuki et al., 2017; Treves and Karanth, 2003a). To achieve a harmonious approach, a range of strategies targeting the needs of each species will be required.

1.2.Study species: the cheetah

The cheetah, *Acinonyx jubatus*, is a large felid of the subfamily Felinae within the order Carnivora (Kitchener et al., 2017). It is the only extant member of the genus *Acinonyx* (Frausman et al., 2005a). Among the cats, the cheetah is unique due to its morphological and physiological adaptation and distinctive evolutionary history (Reviewed in Meachen et al. 2018 and Van Valkenburgh et al. 2018). Based on the recent taxonomic revision of the Felidae, four cheetah subspecies, (*A.j. jubatus, A.j. soemmerringi, A.j. hecki, A.j. venaticus.*) have now been proposed. This new classification merged the East African and Southern Africa subspecies into a single subspecies *A.j. jubatus* due to lack of genetic differentiation between the two subspecies (Kitchener et al., 2017).

Cheetahs show sexual dimorphism where adult males are slightly larger than females. Male and female adults weigh between 21 and 75 kilogrammes (Andrea Bixler. 1998). Cheetahs are mainly diurnal but they are also active at night especially during periods of the full moon looking for prey rich patches, mating partners and males defending their territories (Cozzi et al., 2012).

Table 1.1. The conservation status of large terrestrial carnivore species. The species status and trend are from the IUCN Red List: CR, critically endangered; EN, endangered; NT, near threatened; LC, least concern. The table is ordered according to range loss.

Family	**Common name**	**Scientific name**	**IUCN status**	**Population trend**	**Range lost (%)**
Canidae	Red wolf	*Canis rufus*	CR	Increasing	>99
Canidae	Ethiopian wolf	*Canis simensis*	EN	Decreasing	99
Felidae	Cheetah	*Acinonyx jubatus*	VU	Decreasing	91
Canidae	African wild dog	*Lycaon pictus*	EN	Decreasing	90
Felidae	Lion	*Panthera leo*	VU	Decreasing	83
Felidae	Tiger	*Panthera tigris*	EN	Decreasing	82
Canidae	Dhole	*Cuon alpinus*	EN	Decreasing	82
Felidae	Snow leopard	*Panthera uncia*	EN	Decreasing	78
Ursidae	Andean black bear	*Tremarctos ornatus*	VU	Decreasing	75
Felidae	Clouded leopard	*Neofelis nebulosa*	VU	Decreasing	64
Ursidae	Asiatic black bear	*Ursus thibetanus*	VU	Decreasing	64
Felidae	Sunda clouded leopard	*Neofelis diar*	VU	Decreasing	51
Ursidae	Sun bear	*Helarctos malayanus*	VU	Decreasing	50
Felidae	Jaguar	*Panthera onca*	NT	Decreasing	43
Ursidae	Brown bear	*Ursus arctus*	LC	Decreasing	42
Ursidae	Sloth bear	*Melursus ursinus*	VU	Decreasing	39
Ursidae	American black bear	*Ursus americanus*	LC	Increasing	39
Felidae	Leopard	*Panthera pardus*	NT	Decreasing	35
Canidae	Maned wolf	*Chrysocyon brachyurus*	NT	Decreasing	32
Felidae	Puma	*Puma concolor*	LC	Decreasing	27
Hyaenidae	Brown hyena	*Hyaena brunnea*	NT	Stable	27
Canidae	Grey wolf	*Canis lupus*	LC	Stable	26
Hyaenidae	Spotted hyena	*Crocuta Crocuta*	LC	Decreasing	24
Hyaenidae	Striped hyena	*Hyaena hyaena*	NT	Decreasing	15
Canidae	Dingo	*Canis dingo*	VU	Decreasing	12
Felidae	Eurasian lynx	*Lynx lynx*	LC	Stable	12
Ursidae	Polar bear	*Ursus maritimus*	VU	Decreasing	N/A
Ursidae	Giant panda	*Ailuropoda melanoleuca*	EN	Decreasing	N/A

Cheetah diet is largely composed of a variety of ungulates including both wild and domestic species, their diet is a factor of 'accessible prey'- i.e. the weight ranges preferred and killed relative to their abundance. Cheetahs are generalists (Clements et al., 2014). Male cheetahs especially those in coalitions prey on larger ungulates due to their larger size and to meet the increased nutritional demands of the group (Broekhuis et al., 2018). Adult male cheetahs are sociable sometimes living in coalitions of 2-5 males while females are solitary or live with their offspring. Whilst most singleton adult males are 'floaters' and their ranges may overlap; male coalitions often establish exclusive territories and defend them from other males (Melzheimer et al., 2018). Cheetahs are induced-ovulators (Brown et al., 1996), breeding throughout the year with a gestation period of approximately 90-95 days, resulting in a litter of typically three to six cubs with an average being 3-4 per litter (Frausman et al., 2005b). Weaning occurs at six months, and they may stay with their mother for 14-22 months with littermates tending to stay together for some time after separating from their mother, after which the males and females disperse. Cheetah cubs face higher mortality than most other mammals. This has however been shown to vary between different areas (Mills and Mills, 2014), for instance, in Serengeti National Park only 10% of cubs survive the first year (Ecosystem. 2012) while in Phinda Resource Reserve, South Africa 75% of cheetah cubs seen after emergence survived to 1 year (Hunter, 1998). This may be as a result of different factors acting upon different areas such as competition from other predators or other environmental factors such as habitat loss and uncontrolled tourism (Broekhuis, 2018; Mills and Mills, 2014)

Cheetahs inhabit both open grassy plains (Frausman et al., 2005b) and thicket habitats (Bissett and Bernard, 2007). They utilise large home ranges with an estimated lifetime home range varying from 553.9 km^2 -7063.3 km^2 for females and 119.6 km^2 to 4347.6 km^2 for males (Hunter. 2005). Because of these large home-range requirements, 77% of the cheetahs are believed to occur outside formally protected areas (Durant. et al., 2017).

1.3. Cheetah distribution

The cheetah is one of the large carnivore species that has faced a major population decline through the course of time. The prehistoric records show that they were distributed throughout Asia, Africa, Europe, and North America around 12,000 but today they have been eliminated from the majority of their earlier range and are now restricted to Africa and Asia (Dobrynin et al., 2015b; Durant et al., 2016). Subsequently, they are listed as vulnerable to extinction in the IUCN red list of threatened species with Northwest African *(Acinonyx jubatus hecki)* and Asiatic *(Acinonyx jubatus venaticus)* cheetahs listed as critically endangered.

Today, the current free-ranging population of the cheetah is estimated to be about 7,100 individuals down from approximately 100,000 individuals a century ago. These adult and adolescent individuals are restricted to about 9% of their historic range and apart from a remnant population in Asia that comprise of fewer than 100 individuals, the rest occur in small, fragmented areas spread across 20 countries in Africa (Figure 1; Durant. et al., 2017; Farhadinia, 2016; IUCN/SSC, 2007; RWCP & IUCN/SSC, 2015). The largest contiguous population (> 4,000 individuals) resides in a single transboundary spanning six southern Africa countries. There is only one other population with more than 1,000 individuals while the others comprise less than 200 individuals with six populations having less than 100 (Durant. et al., 2017). All the remaining cheetah populations are threatened due to habitat loss and loss of wild prey populations, which increase their contact and likelihood of conflict with farmers and livestock (Drahansky et al., 2016). In addition to these threats, cheetahs have a relative paucity of overall genome variability attributed to two historic population bottlenecks; the earliest approximately 100,000 years ago and the latest 10,000–12,589 years ago which makes them more vulnerable to ecological and environmental changes (O'Brien et al. 2017; Dobrynin et al. 2015; Schmidt-Küntzel et al. 2017).

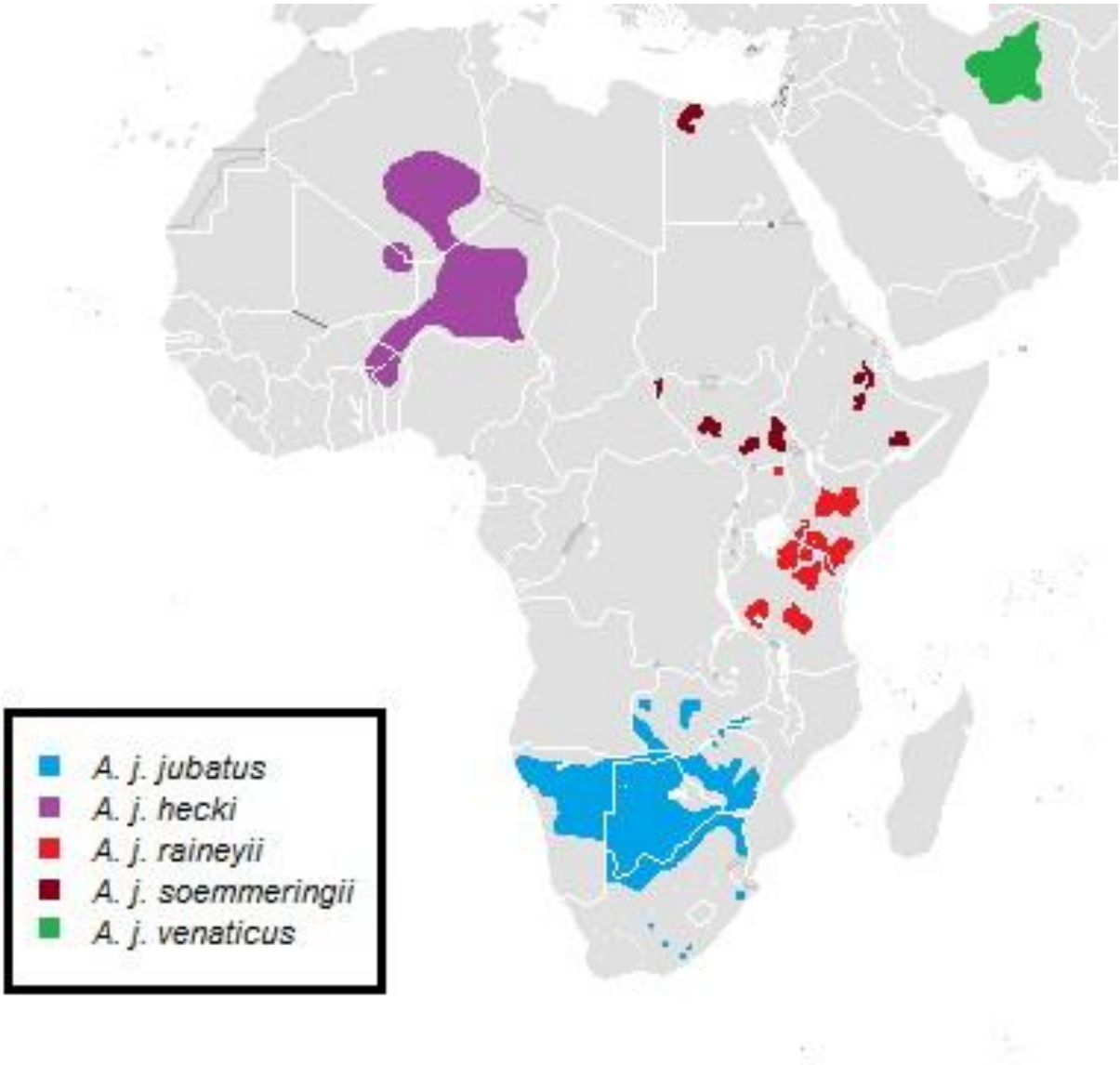


Figure 1.0. Map showing known free-ranging cheetah distribution in Africa and Asia respectively. Different shading represent different subspecies. This map was adopted from https://vividmaps.com/subspecies-of-cheetah-their-range/

1.4. Threats to the cheetah

Anthropogenic impacts on the ecosystem have been shown to be the greatest threat to the long-term survival of many species. Coupled with human population growth and climate change the persistence of species especially those that are genetically compromised is uncertain. Habitat

loss, human-wildlife conflict, decline of natural prey and illegal wildlife trade are the biggest threats to long-term survival and population growth of cheetahs (Durant. et al., 2017).

Habitat loss and fragmentation across Africa and Iran is the major problem threatening the persistence of cheetahs. In Africa, the human population has quadrupled in the last 50 years (Anthony et al., 2017) and as a result, wild land that supported thousands of cheetahs can now only support fewer numbers as these landscapes have been modified to support human interests. Subsequently, wild ungulate species that provide food for cheetahs have also been decimated by habitat loss and fragmentation. Prey loss can have a serious impact on cheetah survival, since it may push them to kill livestock more frequently where natural prey have been depleted hence intensifying the rate of retaliations by farmers whenever they lose their livestock to carnivores. As the human population continues to increase, there is a high demand for land and this will continue to impact the cheetahs, as increase in land subdivision and consequent conversion of land for agriculture and infrastructural development will decrease the available habitat for the cheetah and other wildlife species (Klaassen and Broekhuis, 2018).

Many people living alongside cheetahs in almost all their geographic range are subsistence farmers whose livelihoods depend largely on the wellbeing of their livestock. These farmers usually view cheetahs as a threat to their domestic animals and some pre-emptively kill them when facing losses of livestock or game species (Voigt et al. 2014; Figure 1.1).

Although cheetahs are regarded as a significant threat to the interests of farmers, dietary studies in Namibian farmland and Botswana have shown that they predominantly prey on abundant free-ranging game species and rarely prey on domestic animals (Boast et al. 2016; Marker et al. 2003). However, their diurnal nature and wide-ranging behaviour considerably contribute to their continued persecution for unexplained livestock losses, which could have been killed by other predators such as leopards *Panthera pardus* (Thorn et al., 2012). In southern Africa

specifically, cheetahs' conflict with game farmers is widespread and are usually seen as competitors for valuable game animals.

Figure 1.1. Cheetah attacked in response to retaliatory killing in Amboseli Kenya. Image credit: J. Parmari.

The illegal wildlife trade is the fourth most valuable global illegal activity after narcotics, counterfeit currency, and human trafficking (Haken, 2011) and has been associated with the decline of many wild species. Cheetahs have been historically illegally captured and traded for

both commercial and non-commercial purposes (Figure 1.2). Although the cheetah is currently listed as an Appendix 1 species by the Convention of International Trade in Endangered Species (CITES), meaning all the signatory members of CITES cannot trade cheetahs for commercial purposes, recent evidence shows that there has been an increase in trade in cheetahs (Nowell, 2014).

Figure 1.2. A pet cheetah in a car in Dubai. Image from Instagram and https://allthatsinteresting.com/pet-cheetahs-extinction.

Between 2005 and 2015 a total of 280 cases of wildlife trafficking of live cheetahs or their skin and other body parts were recorded (Tricorache et al., 2018). Accurate numbers of illegally traded cheetahs largely remain unknown as it is difficult to document the trade, however, records shows that only three confiscation of illegally traded cheetahs were officially recorded to CITES (Nowell, 2014). A minimum of 1,000 individual cheetahs have been estimated to

have been trafficked between 2001 and 2011 with the majority involving trade of live animals, of which mainly consisted of young cubs (Nowell, 2014).

Demand for pet cheetahs mostly comes from the Gulf states where ownership of exotic pets is considered as a status symbol, unless the trade is strictly regulated, the smuggling of cheetahs will remain profitable and will eventually decimate the wild cheetah population (Tricorache et al., 2018).

The expansion in infrastructure development has come with the associated need for more road networks. The increase in roads, especially in the areas adjoining wildlife areas, present a threat to cheetah survival (Figure 1.3). In Iran, more than 40% of the reported cheetah mortalities were caused by on road collisions mainly on roads adjacent to protected areas (Iranian Cheetah Society, 2013; Parchizadeh et al., 2018)

Figure 1.3. Cheetah roadkill. Image from Serengeti Cheetah Project

Finally, unregulated tourism has far reaching effects on cheetah survival. Cheetahs are a key attraction for wildlife tourists and subsequently attract large numbers of tourists per cheetah sighting (Roe et al., 1997). However, overcrowding at a cheetah sighting (Figure 1.4) or insensitive behaviour by the tourists or tour operators can have negative consequences such as reduction in cub recruitment (Broekhuis, 2018) and interference with hunting and feeding (Burney 1980).

Figure 1.4. Insensitive behabiour by the tourists and tour operators. Credit Frans Lanting (2011) National Geographic.

1.5.Carnivore dietary analyses: methods and implication for cheetah conservation

Diet is an important part of carnivore ecology and conservation. Precise information on diet composition helps in characterizing prey selection in regards to prey availability and in

evaluating the resource use within an ecosystem (Shehzad, 2011). In the recent past, studies of carnivore diet have been used to inform wildlife managers when predators are perceived as a threat to livestock and to provide information needed to protect rare prey species (Napolitano et al., 2008; Voigt et al., 2014). Diet is also a good indicator of a heathy ecosystem. Influx or reduction of predators can affect the trophic cascades within an ecosystem. For example, lack of coyotes (*Canis latrans*) in California increased the meso-predator populations such as foxes and house cats which ultimately increased the predation pressure on native rodents and scrub-breeding song birds (Crooks and Soule, 1999).

To date, numerous methods have been developed and applied to study the diet of predators. The most common and simple method is the direct observation of animal feeding events in the field (example in Broekhuis et al., 2018). Direct observation is the most reliable as it provide the direct information about the predator and prey including the age and sex (Shehzad, 2011). However, this method is usually labour intensive and expensive because only a few individuals can be monitored at a time. In addition, the presence of an observer could alter predation behaviour (Aguiar and Moro-Rios, 2009; Wade et al., 2005). Direct observation is sometimes impracticable for nocturnal, highly mobile and elusive animals (Klare, Kamler, and Macdonald 2011). Direct observation has reliably been used to evaluate the potential intra and interspecific competition of cheetahs in Maasai Mara, Kenya (Broekhuis et al., 2018).

Radio telemetry has widely been used to track the feeding ecology of many predators that are difficult to observe directly (Grönberg, 2011; Knopff et al., 2009; Sand et al., 2005). This method entails following the radio tagged animals and estimating the prey based on the carcass. While this approach enable the identification of age and sex of prey, it is often difficult to access the remains of prey killed in remote areas and make it impossible to identify the prey when the predator consumes the whole prey (Shehzad, 2011).

The diet of predators has been inferred from field survey, questionnaires and interview with local communities (Namgail et al., 2007). This method is may be biased towards public opinion especially when it is difficult to distinguish the predators. The survey method is mostly limited to livestock predation hence it is difficult to obtain the complete prey spectra (Shehzad, 2011).

Video monitoring is another resourceful method used to identify predator and prey (Merfield et al., 2004). This method is especially helpful as it reduces the distances to the predators hence useful when information on predatory behaviours are required. Video monitoring is however unreliable in field conditions which are usually expansive and difficult to capture predators and prey entering the field of view.

Analysis of stomach contents and protein electrophoresis followed by staining for enzyme activities have also been widely used in dietary studies of various species (Steenkamp, 2018). These methods involves killing of the animal or stomach flushing when the animal is immobilized (Wilson, 1984). When compared with microscopy of faecal samples, analysis of stomach content is more reliable especially in tracking soft-bodied prey which are usually underestimated in faeces due to their digestibility (Hyslop, 1980). Most dietary studies of carnivore that have used stomach content obtained the samples from dead carcasses (Balestrieri et al., 2011; Steenkamp, 2018). Protein electrophoresis entail analysing of homogenised stomach contents in polyacrylamide gels followed by staining for their enzymatic activities (Shehzad, 2011). The resultant bands are then compared with those of target species. Examining the stomach content using immunoassays has been used in diet studies (Fournier et al., 2008; Symondson, 2002). This approach uses specific antigen-antibody coupling interactions (Shehzad, 2011) which illuminates on an enzyme-substrate indicator system. A spectrum from less sensitive protein precipitation test to high sensitive enzyme-linked immunosorbent assays (ELISA) provide a range of immunoassays to select for diet analysis, for more details see (Berth and Delanghe, 2004; Hoyt et al., 2000; Naranjo and Hagler, 2001;

Shehzad, 2011). These three methods are majorly considered invasive because they require handling of animals and hence are not recommended for studies of large and endangered animals (Shehzad, 2011). There are more impediments when using protein electrophoresis as the method produces low resolution results due to limited species-specific bands. In addition, bands resulting from gut contents containing several prey species are usually difficult to interpret (Walrant and Loreau, 1995). The immunoassays approach is often prone to false positives due to shared antigenic determinants among prey species (Feller et al., 1997).

To deal with the above limitations, dietary analyses using non-invasively obtained samples has been presented as the most appropriate method especially for endangered species which are often sensitive to handling and elusive species (Klare et al., 2011). Animal faeces contain undigested parts and DNA fragments from food which is used to diagnose the food composition. Until recently, the diet of carnivores was mostly studied using morphological examination of hard parts and undigested materials in the faeces (Bagchi and Mishra, 2006; Wachter et al., 2012a). Undigested hair has specifically been widely used as bones and teeth obtained in the scats are generally shattered and not easy to diagnose the prey species (Pompanon et al., 2012). Analysis of prey hair in predator faeces uses hair mounting techniques, where hair are carefully obtained after washing the faeces and are mounted on the slide and then compared with reference specimen on the basis of histological examination (Mukhwana, 2015; Torres et al., 2015). Lack of diagnosable remains from soft bodied prey, dearth of comprehensive reference specimens, time and the labour required to prepare the slides and misidentification of hair due to similarity with related species has been the major limitations thus limiting the usability and reliability of this technique (Rogers, 2007; Shehzad, 2011).

Methods that do not rely on presence of hard parts in faeces have been developed to circumvent the limitations associated with morphological identification of undigested remains in faeces.

One such method is thin layer chromatography of bile acids (Quinn and Jackman, 1994). This technique relies on fatty acid signature (Sara, 1993). Fatty acids are the main components of most lipids and unlike other nutrients that are easily broken-down during digestion, fatty acids are released from ingested lipid molecules during digestion and are not degraded (Iverson et al., 2004). Because of the low numbers of fatty acids that are readily biosynthesised by animals, it is possible to discriminate between dietary and non-dietary components (Iverson et al., 2004). However, fatty acids signatures of prey species may be very similar to that of a predator hence making prey identification difficult (Piché et al., 2010). In addition, it is difficult to detect cannibalism using this approach. Other methods apply near infrared reflectance spectroscopy NIRS (Park et al., 1998) and naturally occurring stable isotopes (Lecomte et al., 2011). While this provide useful tools to study dietary patterns of animals, they are marred by numerous limitations. For example, stable nitrogen isotopes approach cannot identify a prey to species level and NIRS require specialized skills to develop a functional calibration (Shehzad, 2011).

Compared to all other dietary analysis, DNA based methods provide the most reliable technique as it does not rely on morphological characteristics that are subject to damage during animal capture, ingestion and digestion (King et al., 2008; Pompanon et al., 2012). This method relies on amplification of a specific DNA region using short, user-specified DNA primers (Deagle, 2006; Mullis and Faloona, 1987). Subsequently, the origin of the amplified DNA molecules is determined through sequencing, hybridization techniques or restriction enzyme analysis. As a result, this method is extremely sensitive even with a small amount of DNA and highly specific as only DNA that match the PCR primer is amplified. Due to the versatility of this method, it has been used to study diet of numerous vertebrate species (Biffi et al., 2017; Casper et al., 2007; Deagle et al., 2010, 2005; Jr, 2007; Lopes et al., 2015; McInnes et al., 2017a, 2017b; Mumma et al., 2016; Pompanon et al., 2012; Shehzad et al., 2012; Srivathsan et al., 2015). The DNA-based dietary analyses utilizes samples ranging from stomach/gut content

(King et al., 2010; Vestheim and Jarman, 2008), regurgitates (Taberlet and Fumagalli, 1996) and faeces - including degraded samples (Bohmann et al., 2018; Deagle et al., 2009, 2005; Mumma et al., 2016; Mengyin Xiong et al., 2017).

A major advantage of using DNA based approaches is that they allow for the identification of specific prey items in the diet using species-specific primers as well as simultaneous identification of a broad range of prey items using primers that bind to DNA region conserved to the target prey species (Deagle, 2006). However, these approaches have some limitations. For example, identification of prey item using species-specific primers require *a priori* knowledge of the potential prey species which is often difficult for wild and elusive species and is also not suitable for studying the diet of predators with large potential prey range. On the other hand, identification of multiple prey items in a sample/samples may be impeded by the design of group-specific primers as the conserved sequences that act as priming sites for prey may be too close to the homologous sequence of the predator (Shehzad. et al., 2012).

The field of DNA based dietary analyses have developed over the years, mostly with the aim of increasing the detection success of prey items. These advances include the use of restriction enzymes that digest the target fragment of the predator DNA, leaving the DNA from all prey intact (Suzuki et al., 2008, 2006) and use of modified oligonucleotides to suppress specific dominant sequences and restrain their amplification (Chow et al, 2011; Vestheim and Jarman, 2008). Among these advances, the use of blocking oligonucleotides is the simplest, cheapest, and most reliable. This technique involves hybridization of blocking oligonucleotides in the form of a primer, with the complementary sequence hence making inaccessible for the polymerase (Shehzad, 2011) while simultaneously amplifying the rare sequence present in the template using universal primers (Vestheim and Jarman, 2008). The combination of blocking oligonucleotides and universal primers has been effectively used to characterise the diet of predators and other animals (Deagle et al, 2009, 2010; Vestheim and Jarman, 2008).

The recent advances in DNA based dietary analyses are revolutionizing how we study the feeding ecology of carnivores. Specifically, DNA metabarcoding has been used to illuminate the extent of human-carnivore conflicts which is one of the main threats facing wild carnivores globally (Shehzad et al., 2015). Accurate information on the nature and degree of livestock depredation and property damage is crucial in formulation of long-term conflict mitigation strategies that contribute to peaceful coexistence between human and carnivores that live in sympatry and share the same resources.

The cheetah *Acinonyx jubatus* is a species with large home-ranges requirements and about 77% of their range falls outside formally protected areas (Durant. et al., 2017). Here, they come into contact with people and are believed to be a threat to small livestock (sheep and goats), calves and important game species (Dickman et al. 2018; Woodroffe et al. 2007), although it has been shown that they typically prefer the most abundant small to medium-sized wild ungulates over domestic animals (Clements et al., 2014; Hayward et al., 2006). Some farmers pre-emptively kill cheetahs when they experience losses of livestock or game species (Voigt et al., 2014) regardless of the lack of direct evidence that cheetahs are involved in depredation (see Introduction of Chapter 2). Accurate determination of the proportion of domestic species that contribute to cheetahs' overall diet and factors that have the potential to influence the likelihood of their involvement in conflicts is a key component to the conservation efforts.

1.6. Application of genomics to the conservation of threatened and endangered species

In response to the increasing biodiversity crisis, genomics has opened exciting possibilities in the field of conservation biology by enabling whole genomic analyses of threatened species that up until recently were limited to model organisms (Steiner et al., 2013). Unlike previously used genetic markers such as microsatellites, genomics relies upon the detection of genome-wide polymorphisms among individuals, populations, and species in the form of single nucleotide polymorphisms (SNPs) or copy number variants. This has considerably increased

accuracy and precision in estimating recent demographic events, genetic variation, and population structure (Allendorf et al., 2010).

Gene flow among populations contributes to maintaining genetic diversity, which is fundamental to ensure species sustainability and reduce the risk of extinction (Frankham, 2005). Owing to current widespread habitat fragmentation, biologists seek to estimate the spatial scale of gene flow to examine historical and contemporary population connectivity (Steiner et al., 2013). These analyses can ultimately be used to guide the development of boundaries and wildlife corridors between the remaining habitats and populations. Application of genomic data has improved resolution in gene flow studies. For example, vonHoldt et al (2011) used SNPs from across the genome of wolf-like canids and found an unidentified admixture pattern which was previously not noted using mitochondrial DNA (Vila et al., 1999). They showed that in highly mobile carnivores, ecology might have an important role in restricting gene flow among populations.

Genomic data continues to generate more reliable information which has far-reaching conservation implications. Results obtained from a recent study of elephants revealed a deep divergence between African savanna elephants (*Loxodonta africana*) and forest elephants (*Loxodonta cyclotis*), which had previously been thought to be the same species based on shared mitochondrial DNA haplotypes (Rohland et al., 2010). These taxonomic inferences will assist in addressing ancient geographic structure, range, and differences in life history traits without generalisation as each species is unique.

Inbreeding depression has been shown to increase the risk of extinction, with data from mammal populations suggest that it often significantly affects birth weight, survival, reproduction and resistance to disease, predation and environmental stress (Keller and Waller, 2002). Evidence suggests that both inbreeding and inbreeding depression are more pervasive than previously realised (Keller and Waller, 2002). Although inbreeding is known to reduce

evolutionary adaptive potential, little information is known on the basic underlying mechanisms that produce inbreeding depression or the number of loci that contribute to inbreeding. The use of genomics has enabled, loci contributing to inbreeding depression to be identified by sequencing the whole genomes of parents and offspring or by examining gene-expression profiles (Chelo et al., 2014; Paige, 2010). For example, a study on inbred Scandinavian wolves (*Canis lupus*) at relatively low resolution provided evidence of increased linkage disequilibrium compared to outbred populations (Hagenblad et al., 2009).

Genomic analyses are being used to better estimate inbreeding coefficients especially among wildlife species that lack pedigree information. Previously, these estimates have relied on neutral genetic markers such as microsatellites thereby resulting in high sampling variances which reduce accuracy (Lynch and Ritland, 1999). High-density genomic data has been suggested to decrease this large variance. Li et al. (2011) obtained consistent values while comparing methods for estimating individual inbreeding coefficients and pairwise relatedness based on genome-wide SNPs and genealogies separately. They concluded that genomic data provide useful information such as inbreeding depression and predicting fitness in cases of complex or absent pedigrees (Steiner et al., 2013).

Diseases are recognised increasingly as playing important roles in natural systems (Altizer et al., 2003). Since they trigger sudden epidemics in naturally occurring populations, diseases can present a major concern in conservation biology. As a result, probing of the genetic basis of disease susceptibility and resistance in wildlife and endangered species is ongoing. Several studies have shown that depletion of genetic diversity within populations may increase their vulnerability to diseases (Altizer et al., 2003; De Castro and Bolker, 2005; Keller and Waller, 2002; Figure 1.5).

It has also been suggested that loss of genetic variation at genes responsible for resistance to parasites and diseases may render populations more susceptible to infections (Radwan et al.,

2010). With the current ability to identify and characterise adaptive loci through genomic approaches, this argument may finally be resolved. The Major Histocompatibility Complex (MHC) gene complex consist of major fitness related genes that codes for proteins presenting pathogen-derived antigens to T-cells, thus initiating the adaptive immune response (Nursalam, 2016, 2013).

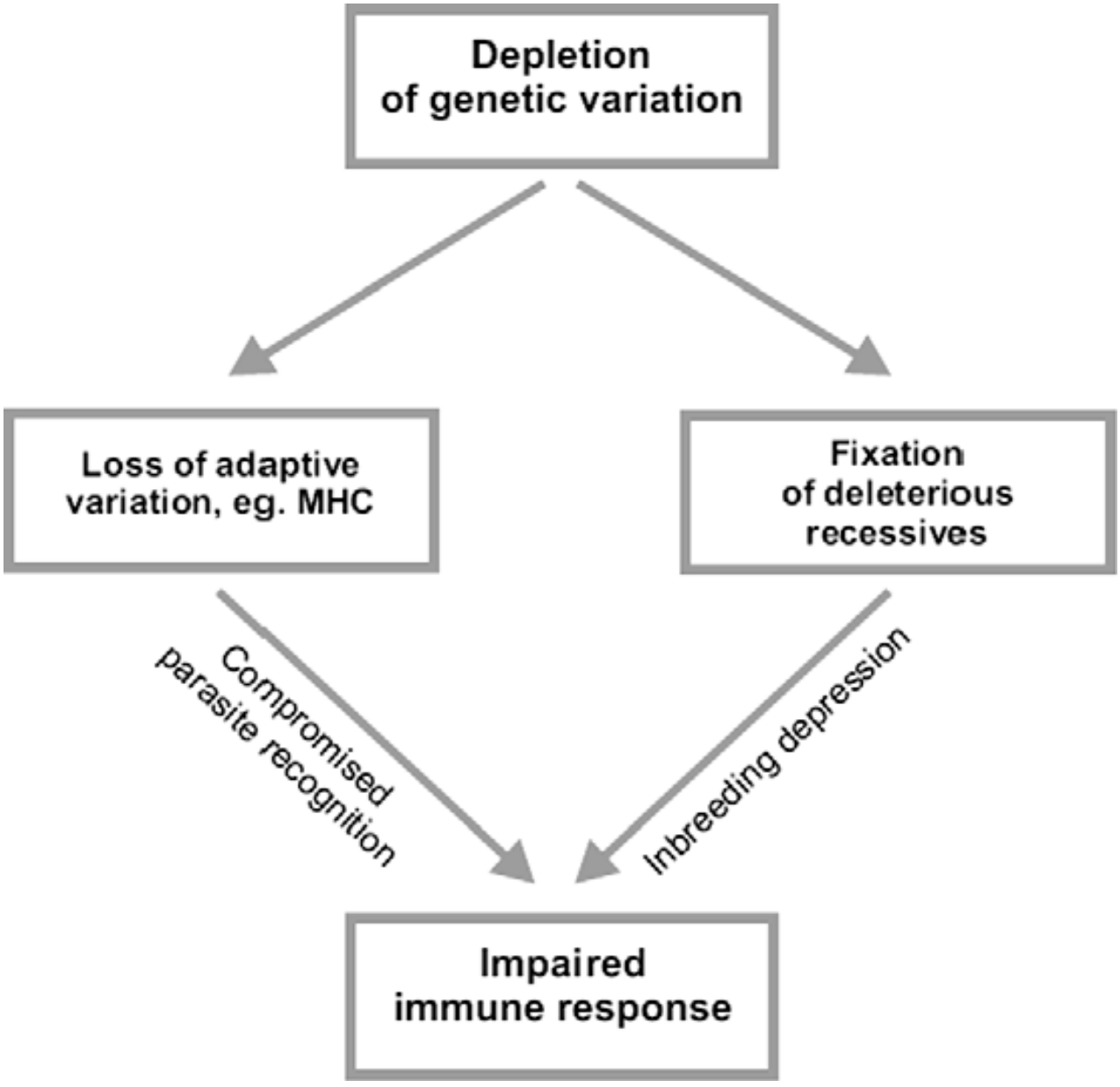


Figure 1.5. Conceptual diagram showing how depletion of genetic variation may impair immune responses via different paths (Radwan et al., 2010)

Therefore, retention of genetic variation in these genes is an essential element of effective conservation programs. A recent genetic study on wild born cheetahs suggests that their immune system is not as genetically invariant as first thought, and they may not be as vulnerable to disease (Castro-Prieto et al., 2011). This is contrary to earlier studies that used methods with low resolution to quantify MHC diversity and/or small sample sizes. Current advances in genomics are making it possible to explore more immunity related genes such as Toll-Like Receptors (TLR) genes. TLRs are an important innate immune gene family; as they are the first receptors to interact with invading microorganisms, including viral, bacterial, fungal and parasitic pathogens (Jin and Lee, 2008). The unusually low level of TLRs found in Tasmanian devils (*Sarcophilus harrisii*) (Cui et al., 2015) might be contributing to their susceptibility to the Devil Facial Tumour Disease, a fatal contagious cancer that is pushing the species to extinction in the wild. It has been recommended that all of the existing TLRs alleles be maintained in the captive insurance population (Cui et al., 2015). This conclusion derived from a genomic approach could similarly be replicated in other wild species once their genomes have been evaluated.

1.7.Molecular marker choice

To date, genetic levels and processes in wild species have been investigated using a wide array of molecular markers including the products of mitochondrial genes, restriction fragment length polymorphism (RFLPs), random amplified polymorphic DNAs (RAPDs) and nuclear genes (Arif et al., 2011; Society, 2016). However, as additional markers become available, the ability to detect population dynamic with greater accuracy and precision has increased as well. In the past two decades, microsatellite and single nucleotide polymorphisms (SNPs) markers

have become increasingly popular in population genetics and molecular ecology (Abdul-Muneer, 2014; Morin et al., 2004). Both types of markers possess numerous attributes that make them ideal for answering important questions of conservation concern.

Analysis of mitochondrial DNA (mtDNA) has previously been a powerful tool in wildlife genetic studies due to its maternal mode of inheritance, the rapid rate of mutation and lack of recombination (Castro Antönia and Ramon, 1998). Its maternal and haploid nature of inheritance results in a four-fold lower effective population size relative to nuclear markers, providing a greater sensitivity to population bottlenecks (Castro Antönia and Ramon, 1998). In eukaryotes, mtDNA encodes 37 genes comprised of 13 protein genes and 24 RNA and has been used widely in wildlife studies. Examples include studies of population genetics, species identification, and wildlife forensics (Hui et al. 2014; Nwafili and Gao 2016; Park et al. 2009; Zhang et al. 2017). Despite the utility of mtDNA as a molecular tool, its maternal mode of inheritance results in a female-biased description of population dynamics.

Microsatellites consist of short tandemly repeated (STR) nucleotide sequences, generally, 2-6 base pairs long, exhibit codominant inheritance and are selectively neutral (Ellegren, 2004; Jan and Fumagalli, 2016; Ogden and Linacre, 2015; Tamaki and Jeffreys, 2005) The hypervariability and high mutation rate of microsatellites make them one of the most widely used molecular markers for species identification, traceability, paternity and population studies (Fernández et al., 2013). Despite being highly polymorphic, informative and interspersed throughout the entire genome (Tian et al., 2008), STRs are time-consuming and expensive to analyse (Doeschl-Wilson et al., 2008). These drawbacks have led to the development and increased use of SNP markers which are more powerful and give a high level of reproducibility. As the acronym suggest, SNP (Single Nucleotide Polymorphism) markers are single base change in a DNA sequences, with a usual alternative of two possible nucleotides at a given position. In principle, at each position of a sequence stretch, any of the four possible nucleotide

bases can be present and as such, a base change to be considered as a SNP, the least frequent allele should have a frequency of $\geq 1\%$ (Doeschl-Wilson et al., 2008). SNPs are usually bi-allelic in practice and are located either in the coding or non-coding regions of a genome. They are relatively conserved in the genome and are less subjected to evolutionary changes compared to other markers over time, these makes SNPs an ideal molecular marker for various types of genetic studies (Aitken et al., 2004). A study comparing the utility of SNPs in population history revealed a strikingly different population structure across the range of the western pond turtle (*Emys marmorata*) than what was previously inferred from single markers, they further noted that while using SNPs smaller sample size is required (Spinks et al., 2014). As compared to other molecular markers SNPs have simpler mutational dynamics and therefore experience a reduced rate of homoplasy, providing more comprehensive outcome in population genetics and forensic analysis (Hutchinson, 2005). While SNPs offer great advantages over other conventional markers (Table 1.2) these markers can experience technological and analytical problems, in particular ascertainment bias (Morin et al., 2004). However, these crucial issues could potentially be addressed by technological improvement and the development of new analytical methods.

Table 1.2. Comparison of the characteristics of different molecular markers. mtDNA= mitochondrial DNA, Msats=microsatellites, SNPs=single nucleotide polymorphism.

Marker	Mutation rate	Resolution	Reproducibility	Cost	Abundance	Ability to isolate from degraded DNA?
mtDNA	High	Low	High	Low		yes
Msats	High	Moderate	Moderate	High	Low 1/15000bp	yes
SNPs	Moderate	High	High	Moderate	More1/300-1000bp	yes

1.8. Conservation genetics of the cheetah: past, present and prospect for the future

The cheetah is a species with low levels of genetic diversity (Dobrynin et al., 2015a; Stephen J O'Brien et al., 1983). Due to their unique evolutionary history and adaptations, the cheetah is today considered a symbol of threats facing wildlife. The levels of genetic diversity in cheetahs became of concern between 1950-1980 when breeding of captive cheetahs was attempted and resulted in low fecundity and high cub mortality (Marker, 1989; O'Brien. et al., 1985). This prompted an inquest to investigate the biological basis of such poor breeding success in captivity (O'Brien. et al., 1985). Cheetah males showed an extreme reduction in sperm count and high rates of malformed spermatozoa (Crosier et al., 2007; O'Brien. et al., 1985, 1983). Plausible explanation for the impaired reproduction was the lack of overall genome variability among the sampled individuals. When compared with other cats and mammals, cheetahs showed 90-99% overall paucity in genome diversity using nuclear allozymes, 2DE skin fibroblast proteins, and RFLP diversity in the major histocompatibility

complex genes (O'Brien. et al. 1983, 1985; Yuhki 1990) and most recently using the whole genome data (Figure 7; Dobrynin et al., 2015)

Remarkably, cheetahs accept skin allografts from unrelated cheetah donors while adequately rejecting xenograft skin from the domestic cat, which is a possible consequence of compromised immunocompetence (O'Brien. et al., 1985). This exceptional reduction of genetic variation has been attributed to two historic population bottlenecks; the earliest approximately 100,000 years ago (coincident with the postulated cheetah migration from North America to Africa) and the most recent 10,000–12,589 years ago, coincident with the Pleistocene mammal extinction (Dobrynin et al., 2015a; Menotti-Raymond and O'Brien, 1993).

Genetic diversity underpins other levels of biodiversity including functional traits, species and ecosystems (Bruford et al., 2017). Thus, its depletion limits the ability of species to adapt to rapid environmental changes and disease outbreaks (Hughes et al., 2008). Such has been observed in cheetahs when a devastating outbreak of feline corona virus (FeCV) occurred in a cheetah breeding facility killing over 50% cheetahs within 3 years (O'Brien. et al., 1985). FeCV a close relative of human SARS corona virus has a morbidity of less than 10% and 1% mortality in cats' facilities hence the high levels of mortalities observed in cheetahs is a clear indication of genetic uniformity which compromises the immune system.

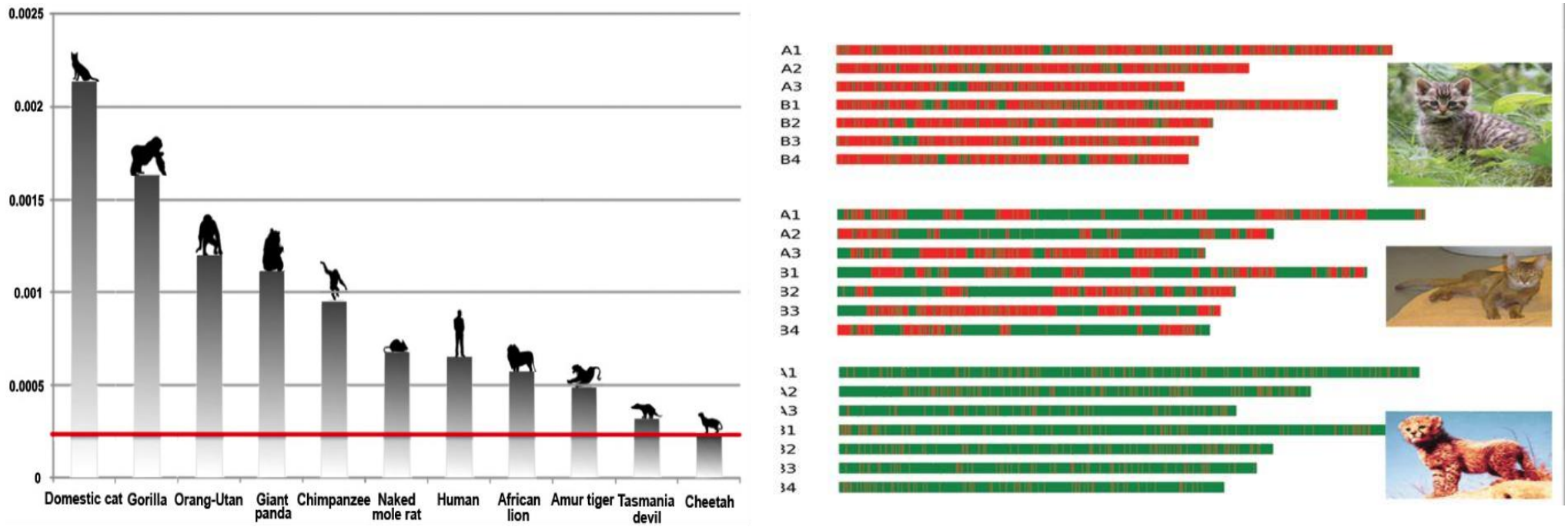


Figure 1.6. To the left: the estimates of genome diversity of cheetahs relative to other mammals. To the right: Homozygosity stretches in cheetahs in comparison with the genome of Boris, an outbred feral domestic cat living in St. Petersburg (top) and Cinnamon, a highly inbred Abyssinian cat. Highly variable regions (>40 single nucleotide variants (SNVs)/100 kbp) are coloured red while highly homozygous regions (≤40 SNVs/100 kbp) are coloured green. The first seven chromosome homologues of the genomes of the two domestic cats and cheetah are shown for direct comparison (Dobrynin et al., 2015a)

To date, genetic homogeneity in cheetahs has been confirmed using multiple genetic measures (reviewed in Schmidt-Küntzel et al. 2018) and although this species has managed to persist for thousands of years, this does not guarantee their survival in the future, as lack of genetic diversity compromises their ability to adapt and survive the ongoing climate change (Schmidt-Küntzel et al., 2018). As we look into the future of cheetah conservation, there is no doubt that their genetic diversity will compound the already identified threats of the remaining fragmented populations if careful conservation actions are not implemented. For cheetahs to escape the extinction vortex a participatory approach addressing both the needs of the cheetah and of the people who live within the cheetah range is required (Marker et al., 2018).

1.9. Knowledge gap and thesis aims

Kenya is a critical part of the global cheetah population. Together with Tanzania, Ethiopia, South Sudan, Somalia and Uganda they form the second-largest contiguous population after Southern African cheetah (Durant et al., 2016). Today, cheetahs are confirmed to occur in about 23% of their historic range across Kenya and a recent survey showed that over 80% of their resident range falls outside protected areas (reviewed in Marker et al. 2018) where their risk of conflicts with humans is high. Although cheetahs are protected under national law in Kenya, their numbers continue to decline due to the threats mentioned above (Durant. et al., 2017). Considering the declining trend, increase in fragmentation and the global importance of Kenyan cheetah populations there is urgent conservation needs especially regarding their feeding ecology to understand the proportion of domestic animals that makes up their diet and determine the factors that influences their tendency to kill livestock as well as understanding the patterns of genetic diversity in order formulate sustainable human-cheetah co-existence strategies.

Currently, dietary knowledge of the cheetah population in Kenya is based on direct observations and visual inspection of undigested matter in faecal samples. These data are

known to not always be reliable as rare, small, soft-bodied species and unobserved feeding events or prey killed at night can be missed. In addition, nothing has so far been documented about the genetic composition of cheetahs in Kenya. Genetic information is important to wildlife managers because it provides basis for population monitoring and conservation.

This research aims to address the above gaps and where possible recommend solutions or conservation implication for cheetahs in Kenya. Overall, this research utilizes the recent development of molecular tools at different scales and study areas to address three specific research objectives:

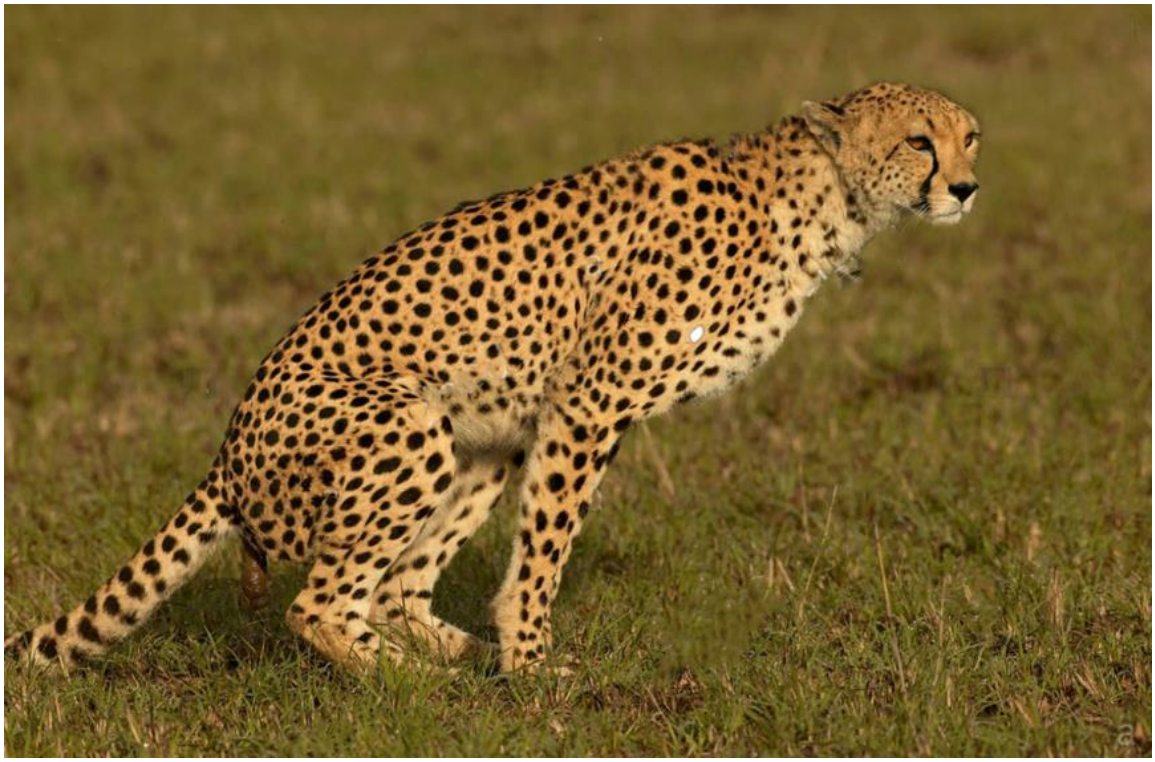
1. Evaluate the efficacy of faecal DNA metabarcoding in dietary analysis of cheetahs.
2. Determine the vertebrate prey spectra and levels of livestock depredation by cheetahs in Kenya using faecal DNA metabarcoding.
3. Assess the genetic diversity and population structure of cheetah population in Kenya using a set of cheetah-specific SNPs.

Chapter 2

Food from faeces: evaluating the efficacy of scat DNA metabarcoding in dietary analyses

*The following research is published in PLOS ONE and, can be cited as:

Thuo D, Furlan E, Broekhuis F, Kamau J, Macdonald K, Gleeson DM (2019) Food from faeces: Evaluating the efficacy of scat DNA metabarcoding in dietary analyses. PLoS ONE 14(12): e0225805. https://doi.org/10.1371/journal.pone.0225805



Abstract

Scat DNA metabarcoding is increasingly being used to track the feeding ecology of elusive wildlife species. This approach has greatly increased the resolution and detection success of prey items contained in scats when compared with other classical methods. However, there have been few studies that have systematically tested the applicability and reliability of this approach to study the diet of large felids species in the wild. Here we assessed the effectiveness of this approach in the cheetah *Acinonyx jubatus.* We tested how scat degradation, meal size, prey species consumed and feeding day (the day a particular prey was consumed) influenced prey DNA detection success in captive cheetahs. We demonstrated that it is possible to obtain diet information from 60-day old scats using genetic approaches, but the efficiency decreased over time. Probability of species-identification was highest for food items consumed one day prior to scat collection and the probability of being able to identify the species consumed increased with the proportion of the prey consumed. Detection success varied among prey species but not by individual cheetah. Identification of prey species using DNA detection methods from a single consumption event worked for samples collected between 8 and 72 hours post-feeding. Our approach confirms the utility of genetic approaches to identify prey species in scats and highlight the need to account for the systematic bias in results to control for possible scat degradation, feeding day, meal size and prey species consumed especially in the wild-collected scats.

Keywords: Cheetah, *Acinonyx jubatus*, scat DNA metabarcoding, diet, prey, felids.

2.0. Introduction

Development of accurate methods to study the diet of terrestrial carnivores has been an active area of research and continues to attract increasing interest in conservation studies. Feeding patterns are a fundamental part of carnivore ecology and conservation (Wachter et al., 2012b). Therefore, accurate inferences of breadth and diversity of feeding behaviour in the wild is required to understand their impacts on the ecosystem to develop reliable management programs of rare prey species and to predict potential human-wildlife conflicts (Broekhuis et al., 2018; Ghoddousi et al., 2016; Wittmer et al., 2014; Włodzimierz et al., 2002). However, it is often challenging to accurately infer carnivore diets because most terrestrial carnivores exist in relatively low numbers and are generally elusive and wide-ranging (Long et al., 2007; Ripple et al., 2014) and often opportunistic, thus making observational studies of diet logistically difficult, financially expensive and almost impossible under natural conditions (Klare et al., 2011).

DNA-based diet analyses of non-invasively collected samples, e.g. scat DNA metabarcoding (sDNA metabarcoding) has been presented as a reliable alternative method (De Barba et al., 2014; Pompanon et al., 2012; Shehzad et al., 2012; Mengyin Xiong et al., 2017). This technique analyses DNA contained in scats collected from the wild using high-throughput sequencing using small, highly variable universal primers (barcodes) (Deagle et al., 2009; Piñol et al., 2014) to identify prey species. Relative to conventional dietary studies that typically rely on morphological identification of undigested remains in scats (Pompanon et al., 2012), sDNA metabarcoding has been shown to have higher sensitivity, greater taxonomic resolution and to be relatively cost-efficient (Galan et al., 2017; Stein et al., 2014). In order to determine the reliability of sDNA metabarcoding, several controlled experimental studies have been conducted to examine the potential strengths and weaknesses. These studies have mainly scrutinized the specificity and sensitivity of PCR assays (Esnaola et al., 2018; Riaz et al., 2011),

library preparation and sequencing technologies (Carøe et al., 2018; Divoll et al., 2018; Forin-Wiart et al., 2018), impact of environmental factors on scats (McInnes et al., 2017a), biological and physiological status of the defecator (McInnes et al., 2017a; Oehm et al., 2011). Few sDNA studies have empirically tested the effectiveness of sDNA metabarcoding in large felids, (Shehzad et al., 2012), and therefore drawing general conclusions from different taxa may introduce bias in result interpretation.

Prey DNA detectability in scat varies depending on both the prey species eaten and the predator species (Alberdi et al., 2018). Thus, species-specific studies are needed to understand how biological, technical and environmental factors could affect the prey DNA signature recovered from a scat sample to inform optimal study design. Studies of captive animals with known diets allow sDNA methods to be trialed with the aim of maximizing prey detectability and identifying optimal designs for field studies (Marker. et al., 2003; Wachter et al., 2012b).

The cheetah *Acinonyx jubatus* is Africa's most endangered large cat with the majority of remaining wild populations existing outside protected areas and hence prone to negative human interactions (Durant. et al., 2017; Marker. et al., 2003). Cheetahs have large home ranges, are cryptic (Houser et al., 2009; Marnewick and Somers, 2015) and usually conceal their kills to minimize losses to other predators (Mills et al., 2004). Consequently, monitoring of cheetah dietary habits using direct observation or carcasses can be time-consuming and expensive. Although cheetahs consume more pure muscle than bone and skin (Van Valkenburgh, 2006), prey items can be identified in cheetah scat samples (Boast et al., 2016; Craig et al., 2017), suggesting that sDNA metabarcoding has potential for wild cheetah dietary studies. Cheetah scats can persist in the field under dry environmental conditions for weeks and can easily be located at marking trees or using professionally-trained scent detection dogs (Schmidt-küntzel et al., 2018). However, obtaining freshly deposited cheetah scats in the wild is difficult, and it is not known how aging affects the ability to detect prey in cheetah scats.

The aim of this study was to analyse scats obtained from the captive cheetahs fed a known diet to address two questions (i) what is the length of time after consumption that prey DNA is detectable in fresh scats as a function of prey species and proportion of prey consumed, and (ii) how does the detection probability change over time in scats left outside to degrade. We discuss how these findings can be used to inform sDNA metabarcoding studies of wild cheetah diets.

2.1. Materials and methods

2.2.1. Feeding trials

We conducted a controlled feeding trial with two adult male cheetahs (Jura and Innis) between 2nd November and 20th November 2017. The cheetahs are brothers born in 2013 and housed individually in outdoor enclosures at the National Zoo and Aquarium in Canberra, Australia. During the study period, Jura and Innis were fed six prey species; horse (*Equus caballus*), rabbit (*Oryctolagus cuniculus*), deer (*Cervus spp*), quail (*Coturnix Coturnix*), chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) in different proportions on different days (Table 2.1). Each day the selected prey items were weighed, placed in a bowl and fed to the individual cheetah. The cheetahs were fed once a day between 9 am and 11 am with total daily food intake varied based on cheetah body condition scores (Bus, 2018; Fuller et al., 2007). Jura weighed 53.9kg and was fed 1700g of food daily while Innis, weighed 50.4kg and was fed 1800g of food daily. To investigate the window of prey DNA detection in fresh scats (i.e. the number of days after consumption of a prey item that the prey was detectable in scats), Innis was fed once on quail hereafter referred to as spike diet, on day two of the experiment.

Tabe 2.1 List of Prey species(and proportions) fed to cheetahs each day during captive feeding experiment.

Day, month and year	Cheetah ID	Prey species 1	Prey Species2	Prey species 3
03.11.2017	Jura	Deer (0.47)	Chicken (0.18)	Rabbit (0.35)
04.11.2017	Jura	Deer (0.47)	Chicken (0.29)	Rabbit (0.24)
05.11.2017	Jura	Deer (0.82)	Chicken (0.18)	-
06.11.2017	Innis	Deer (0.56)	Horse (0.27)	Chicken (0.17)
	Jura	Deer (0.82)	Chicken (0.18)	-
07.11.2017	Innis	Horse (0.61)	Turkey (0.06)	Chicken (0.33)
	Jura	Deer (0.82)	Chicken (0.18)	-
08.11.2017	Innis	Deer (0.56)	Rabbit (0.6)	Quail (0.38)
	Jura	Deer (0.88)	Chicken (0.6)	Rabbit (0.6)
09.11.2017	Innis	Horse (0.11)	Rabbit (0.6)	Chicken (0.83)
	Jura	Deer (0.88)	Horse (0.12)	-
10.11.2017	Innis	Rabbit (0.17)	Chicken (0.83)	-
	Jura	Deer (0.88)	Chicken (0.12)	-
11.11.2017	Innis	Horse (0.33)	Chicken (0.67)	-
	Jura	Deer (0.88)	Chicken (0.12)	-
12.11.2017	Innis	Deer (0.89)	Chicken (0.11)	-
	Jura	Deer (0.88)	Chicken (0.12)	-
13.11.2017	Innis	Deer (1.0)	-	-
	Jura	Deer (0.88)	Chicken (0.12)	-
14.11.2017	Innis	Rabbit (0.22)	Chicken (0.78)	-
	Jura	Deer (0.88)	Chicken (0.12)	-
15.11.2017	Innis	Rabbit (0.22)	chicken (0.78)	-
	Jura	Deer (0.88)	Chicken (0.12)	-
16.11.2017	Jura	Deer (0.88)	Chicken (0.12)	-
17.11.2017	Jura	Deer (0.88)	Chicken (0.12)	-
18.11.2017	Jura	Deer (0.88)	Chicken (0.12)	-
19.11.2017	Jura	Deer (0.88)	Chicken (0.12)	-

2.2.2. Scat sampling

During the feeding experiment, scat samples from both cheetahs were collected daily except for days when the cheetah did not defecate. We collected a total of 16 and 10 fresh scats from Jura and Innis, respectively. All fresh scats were placed in separate greaseproof paper bags and transported to the University of Canberra. For each scat, ~5 grams of material were subsampled on the day of deposit and stored at -20 ºC. The remaining scats were then placed outside in an open field about 10 metres apart and exposed to natural weather to simulate wild conditions. Scats were individually labelled, and their location marked using 10" metal garden stakes. Each scat was then subsampled by removing ~5 grams of material on days 3, 5, 12, 15, 20, 27, 48 and 60 after being placed in the open. Not all scats survived to day 60 as some were eaten or removed, most likely by birds, foxes or insects. For subsampling, each scat was cut cross-sectionally using single-use sterilized surgical blade (Livingstone International, Australia) and material was taken from the upper, middle and lower surface of the cross-section.

In total, 203 subsamples were collected for DNA extraction. Daily weather data (temperature, rainfall and relative humidity) throughout the experiment was obtained from the nearest weather station (approximately 11 kilometres) to the open field site (Canberra Airport Station; Bureau of Meteorology, Australia 2018).

2.2.3. Primers

We amplified the scat DNA using a previously published universal vertebrate primer set (Riaz et al., 2011). The primer set was selected based on taxonomical coverage and discrimination power. This set of primers has been demonstrated to have high-resolution capacity to identify the genus and species across a wide range of vertebrate taxa (Riaz et al., 2011). This primer pair amplifies an ~100 bp fragment of the V5 loop of mitochondrial 12S rRNA gene (Table 2.2).

Table 2.2. Details of the primer sequences used in the study.

Primer name	Primer sequence (5´ - 3)	Product size	References
12SV5F	TAGAACAGGCTCCTCTAG	~100bp	Riaz et al. (2011)
12SV5R	TTAGATACCCCACTATGC	~100bp	Riaz et al. (2011)

2.2.4. DNA extraction and PCR amplification

Approximately 0.1-0.2g of the material was removed from each scat subsample and DNA was extracted using the Invitrogen ChargeSwitch® Forensic DNA Purification Kit (Invitrogen™ Life Technologies, USA) following the manufacturer's instructions and using overnight digestion at 55ºC rocking at 850rpm in a thermomixer. Samples were extracted in batches of 23 including a negative control in which no sample was added. In order to assess the amplification efficiency and inhibition, all extracts were diluted to 1/10 and 1/100 and used along with undiluted aliquot during qualitative PCR (qPCR) amplification. All qPCR reactions were carried out in 25µl consisting of final concentration of: 0.20 µl of AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA), 2.5µl of GeneAmp 10× Gold Buffer (Applied Biosystems, USA), 2µl of MgCl2 (25 mmol/L; Applied Biosystems, USA), 0.2µl UltraPure BSA (50 mg/ml; Invitrogen), 0.65 µl of GeneAmp dNTP Blend (10 mmol/L; Applied Biosystems, USA), 0.6 µl SYBR Green I Nucleic Acid Gel Stain (5X; Invitrogen), 1µl of forward and reverse primer (10 µmol/L), and 3µl of template DNA and made to volume with DEPC-treated water (Invitrogen™ Life Technologies, USA). Each qPCR was run using a Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, USA) under the following conditions: initial activation at 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min and a final extension of 10 min at 72°C and a melting curve with a stepwise increase of 0.1°C/5 s from 60 to 95°C completed the reaction. The PCR set-ups were conducted in a dedicated trace DNA laboratory at the University of Canberra to

minimise the risk of contamination. The DNA dilution with the highest relative proportion of starting template (determined by C_q values) was selected for subsequent metabarcoding using fusion-tagged primers. All negative control samples that showed positive amplification were included in the high-throughput sequencing library preparation.

2.2.5. Library preparation and high-throughput sequencing

A single step PCR with fusion-tagged primers was used to amplify the barcoding sequence and add technical sequences required for high-throughput sequencing. Forward fusion-tagged primers consisted of the P5 sequencing adaptor, a custom forward sequencing primer, a 7 bp Multiplex Identification (MID) tag, and the forward 12SV5 primer. Reverse fusion-tagged primer contained the P7 sequencing adaptor, a custom reverse sequencing primer, a 7 bp MID-tag, and the reverse 12SV5 primer. To minimize cross-contamination, no primer-MID combination had been previously used, nor were combinations re-used. Triplicate PCRs were run for each sample using the reaction conditions and thermal cycling profile described previously. Based on the average quantitation cycle value (Cq values) of each sample, amplicon libraries of 8–10 samples were pooled using equal volumes of each PCR replicate to produce a single DNA library. All negative controls were pulled together into a single unique library. Tagged amplicons were purified (to remove excess fusion-tagged primers and primer dimers) using Agencourt™ AMPure™ XP Beads (Beckman Coulter, Brea, CA, USA) in a 1.2 volume ratio relative to the amplicon pool.

The size and concentration of the amplicons of each pool were estimated by electrophoresis on 2% agarose gel stained with SYBR safe (Invitrogen™ Life Technologies, USA) and NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Based on pools equimolar concentration, amplicons were combined to produce a single super pool. The super pool was constructed by combining approximately equal amplicon copy numbers from each initial pool (i.e., considering the number of samples combined during the

first pooling step and the amplicon size). A total of 209 uniquely labelled libraries from this study (i.e., 193 and 18 libraries originating from scat DNA and negative control samples, respectively) were included in the final superpool. The resultant library was purified as described above. All sequencing for the 209 libraries was performed using Illumina MiSeq® with the Version 2 reagent 1x200 bp reagent kit at the Ramaciotti Centre for Genomics (University of New South Wales).

2.3. Bioinformatics data processing

The technical sequences (i.e. sequencing adaptors and primers) from the sequencing reads were trimmed using Trimmomatic v.0.36 (Bolger et al., 2014). Low-quality bases (Q-score < 30) at the end of the sequencing reads were filtered out and a sliding window of 4-bases was used to trim reads when the average quality per base was below 15. The OBITOOLS software (Boyer et al., 2016) was used for subsequent filtering of the sequences following the general workflow described by De Barba et al (2014).

The OBITOOLS *ngsfilter* and OBIGREP scripts were used to assign sequences records to the corresponding sample combination and remove any sequences shorter than 80 base pairs in length and with abundance below 10 (Shehzad et al., 2012), as they could potentially be sequencing errors and/or chimaeras. OBICLEAN and OBIGREP scripts were used to remove PCR and sequencing errors. The ECOTAG script was used to assign the sequences to their corresponding taxonomic information using a reference database built using the standard vertebrate sequences from the EMBL data repository (release 138; https://www.embl.de/) and a 12SV5 custom reference database built specifically for our target species: cheetah, horse, rabbit, deer, quail, chicken and turkey. ECOTAG output files were imported into in R version 3.5.1 (https://www.R-project.org/) for further filtering and statistical analyses using tidyverse (Wickham, 2016), lubridate (Vitalie et al., 2018), JAGS (Plummer, 2003) and jagsUI (Kellner, 2015).

During ECOTAG, some sequences were assigned to higher taxonomic ranks than the species level. Since all the species in our feeding experiment were known and all sequences assigned to higher taxonomic ranks had variant sequences assigned to species level with a higher occurrence, these incorrect assignments were reassigned to the species. Unclear taxonomic assignments were either modified or corrected based on the relative sequence abundance, the sequence information, and the prior knowledge of the expected species. For example, all sequences assigned to the Felidae family were combined into a single species level assignment *Acinonyx jubatus*, as it is likely they are from the cheetah. Additionally, all sequences assigned to the Leporidae Family were reassigned to *Oryctolagus cuniculus* species, all sequences assigned to Equidae family were reassigned to *Equus caballus* species and those assigned to Cervidae family combined into Cervus species. All other sequences from non-target species (not from cheetah or prey species in the cheetah feeding experiment) or without a taxonomic assignment were excluded from downstream analyses.

2.3.1. Data analysis

Due to differences in the sequencing depth among samples, the ECOTAG output data was transformed into binary data on the presence or absence of each prey species in each scat subsample. A prey species was considered to be present in a scat subsample if its sequence reads were detected but were missing or less than ten in the corresponding negative control.

Quail (spike diet) was detected in scats up to three days post feeding. Based on this knowledge, we excluded from analysis scats that were collected in the first three days of the feeding trial as we did not know what the cheetahs had been fed in the days prior to the start of the experiment. This resulted in, one prey species (Turkey *Meleagris gallopavo*) being excluded from the analysis because it was only fed to one cheetah within the first three days.

For each scat we had data on what the cheetah had consumed on the day of defaecation and for three consecutive days prior to scat collection, and for each subsample taken from each scat we had data on the presence or absence of prey species in that subsample. We modelled the presence of prey species in each scat subsample as a function of the proportion of each prey type that was fed to a cheetah in each of the previous three days, the number of days since a scat was defecated (degradation days) and the individual cheetah. The response variable was detection of prey species in a subsample from scat *i* on degradation day *j*, $Y_{s,ij,}$ coded as $Y_{s,ij}$ = 0 (if the prey species was not detected) or $Y_{s,ij}$ =1 (if the prey species was detected). We modelled the probability of detection, $p_{s,ij,}$ as a function of six fixed-effect covariates: an intercept term; the proportion of prey species fed to the cheetah on the day of defecation and on each of the three days prior to that, the number of days after defecation that the scat was subsampled (degradation days) and the individual cheetah. We also included a random effect term α with a different value for each scat that accounted for repeated measures in the data with multiple subsamples taken from each scat. Our model was:

$$Y_{s,ij} \sim \text{Bernoulli}(p_{s,ij})$$

$$\text{Logit}(p_{s,ij}) = \beta_{0,s} + \beta_{1,s} * \text{pr0}_{s,i} + \beta_{2,s} * \text{pr1}_{s,i} + \beta_{3,s} * \text{pr2}_{s,i} + \beta_{4,s} * \text{pr3}_{s,i} + \beta_5 * \text{degradation day}_j + \beta_6 * \text{cheetah} + \alpha_i$$

Where *i* indexes scats (1-26), *j* indexes degradation days (1-60) and *s* indexes prey species (1-5). $\beta_{0,s}$ is the baseline probability of detection for prey species s, $\beta_{1,s} - \beta_{4,s}$ are parameters that describe how the probability of detection depends on the proportion of each prey species eaten on the day of defaecation ($\beta_{1,s}$) or in the preceding three days ($\beta_{2,s} - \beta_{4,s}$), β_5 is a parameter that estimates how probability of detection changes as a function of scat degradation day, β_6

estimate the effect cheetah has on detection, and α is a random-effect term that allows a different overall detection probability for each scat.

We fit the models using Bayesian methods and estimated the posterior distribution for all parameters using Markov Chain Monte Carlo (MCMC) implemented in JAGS (Plummer, 2003) within the package jagsUI Version 1.5.0 (Kellner, 2015) in R environment (R Core Team, 2015). The β and α_i parameters were modelled hierarchically, assuming these were drawn from normal distribution with means and variance estimated from the data for the β parameters, and mean zero and variance estimated from the data for the α_i parameters. We used non-informative priors for the means (mean 0 and variance 100) and variances (uniform prior in the range 0-10 on the standard deviation). The models were run using three Markov chains of 20,000 iterations after a burn-in of 5000 iterations until all parameters were judged to have converged based on Gelman-Rubin statistic (Rhat statistic), for which all values were <1.1 (Rubin and Gelman, 1992). To assure full reproducibility of our data analyses we have provided all datasets and workflow as supporting information (S1, S2, S3, S4 and S5 files). The raw metabarcoding data and R code used for the analysis are available in the Dryad Digital Repository https://doi.org/10.5061/dryad.2z34tmpgs (Thuo et al., 2019).

2.4. Results

2.4.1. Environmental variables

During the study period, the study site received rain on 37 days for a total of 290mm. The temperature ranged from 2.5°C to 40.6°C with an average temperature of 26.9°C. The average minimum temperature over the entire study period was 12.8 °C and the average maximum was 28°C. Relative humidity ranged from 11.7% - 100%, with an average relative humidity of 60.7%.

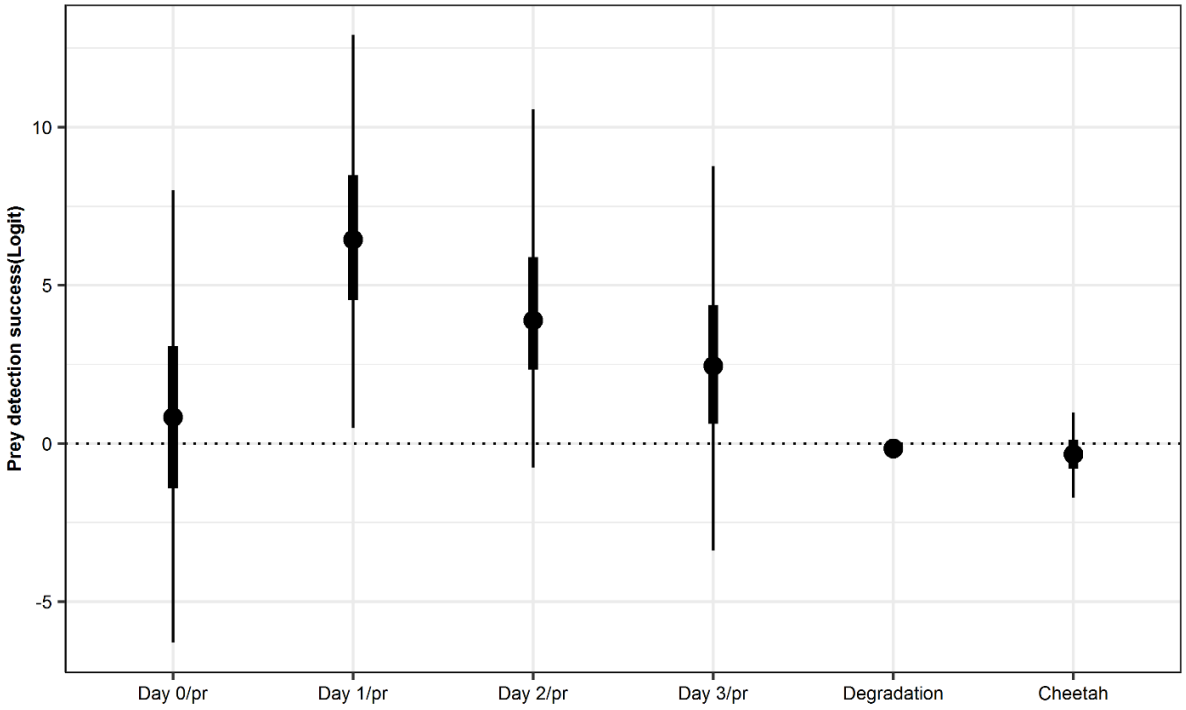
2.4.2. Bioinformatics

After quality filtering and removal of chimaeras, a total of 15,306,489 sequence reads were obtained of which 12,254,953 reads (80%) included perfectly matching MID. The remaining 20% either did not have MID or had MID tag with numerous mismatches to be reliably assigned. Overall, the quality of run was high (PhredQ30 score ≥ 90.53, error 1.04 ± 0.03). As expected, more than half of the sequence reads (54%) were assigned to the consumer (cheetah), while 33% were assigned to prey items and the remaining 13% of the total sequence reads being assigned to other. These findings are consistent with the literature (Forin-Wiart et al., 2018; Kaunisto et al., 2017; Piñol et al., 2014), this is due to the high number of epithelial cells/cells of the intestinal mucosa from the defecating animal and probable prey DNA decay due to digestion process (Deagle et al., 2006). Two of the extraction controls that had shown positive amplification did not result in assignment during ECOTAG process possibly because the initial positive amplification was due to the 12SV5 primers amplifying non-target (e.g. microbial) DNA or due to primer dimer formations.

2.4.3. Diet

The number of days since consumption and proportion of prey fed strongly influenced prey DNA detection in the cheetah scats. Averaged across all prey species, there was a positive relationship between the probability of detection per proportion of prey consumed, although this effect was weak on day 0 (the day of consumption), peaked on day 1 (the day after consumption) and then declined in the following two days (Figure 2.1 and Table 2.3).

Figure 2.1. The relative success of prey DNA detection on a given day after feeding (according to the proportion of prey consumed), degradation day and individual cheetah. The points are the posterior means and the bold and thin lines represent the 50% and 95% credible intervals around the means, respectively.



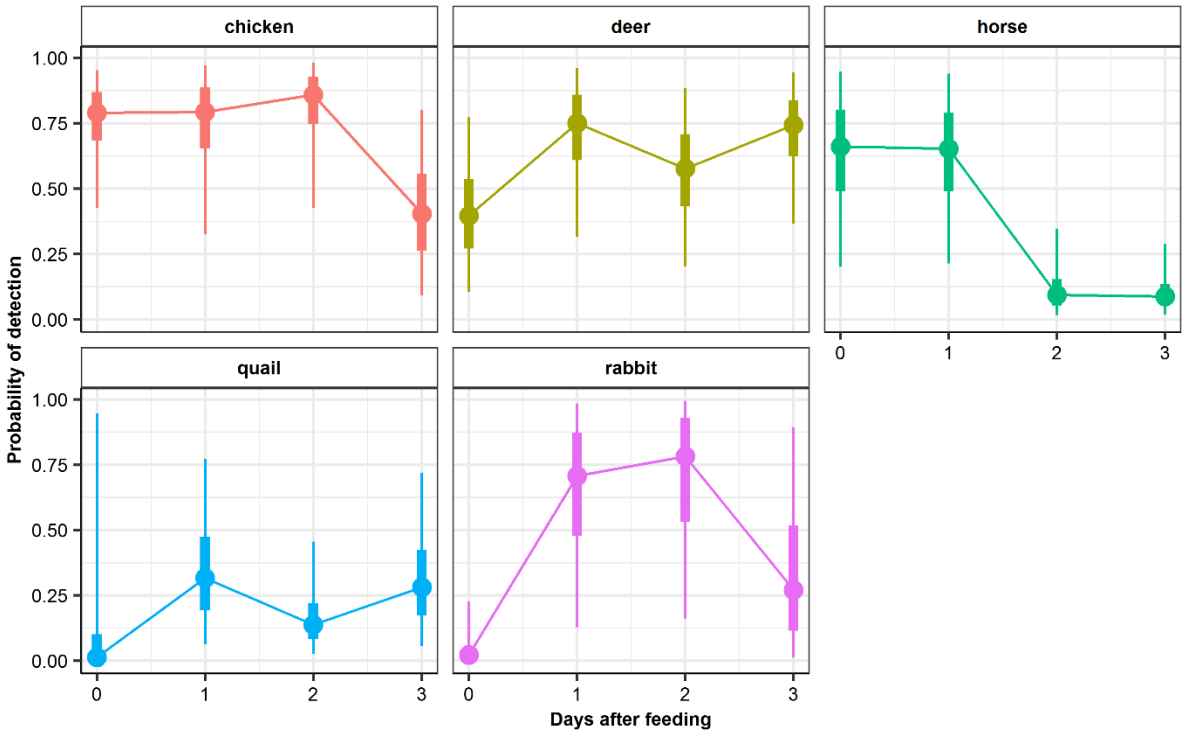
Nevertheless, these relationships also appeared to vary depending on the prey species consumed (Figure 2.2): chicken, deer and horse were more readily detected on the day of consumption compared to quail and rabbit, while horse was difficult to detect after day one.

Table 2.3. Posterior summary of the model.

			95% credible interval	
Parameters	Posterior means	Standard deviation	Lower limit	Upper limit
Day 0/pr fed	0.01	3.24	-6.72	6.51
Day 1/pr fed	4.43	2.55	-0.56	9.85
Day 2/pr fed	1.82	1.69	-1.32	5.67
Day 3/pr fed	1.04	2.66	-4.30	6.58
Degradation	-0.16	0.09	-0.35	0.02
Cheetah	-1.19	0.64	-2.51	0.05

Degradation day (number of days the scat was exposed to the environment) was weakly negatively associated with detection probability for scats exposed to natural conditions for up to 60 days (Figure 2.1 and Table 2.3). There was no clear difference between individual cheetahs in the probability of prey detection (Figure 2.1 and Table 2.3).

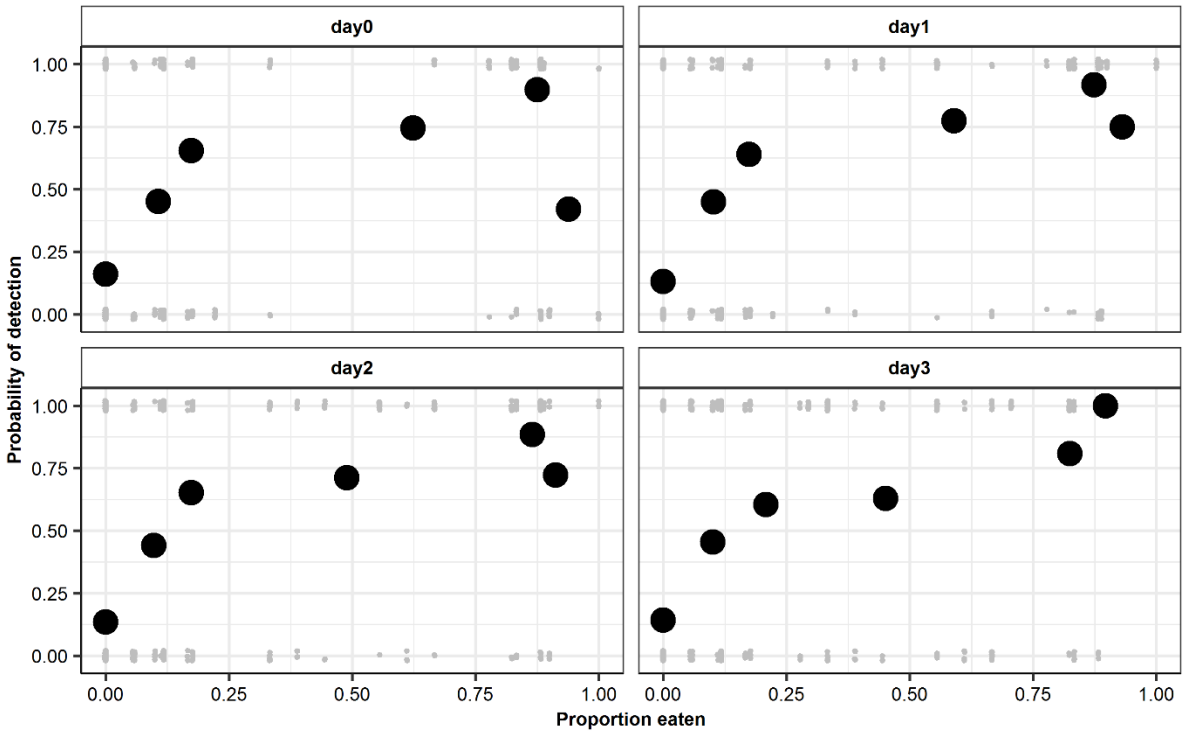
Figure 2.2 Estimates of mean detection probability of each prey species in scat samples relative to time since feeding. The bold and thin lines represent the 50% and 95% credible intervals around the means.



Detectability varied among prey species indicating the need to account for this bias when evaluating the cheetah diet (Figure 2.3). Chicken showed the highest probability of detection (75% SD: 0.18) while quail and rabbit (13% SD: 0.25 and 4% SD:0.06) showed the least probability of detection in day zero respectively i.e. the same day the cheetah was fed. The probability of detection declined after day one for horse and after day two for chicken and rabbit. Quail and deer showed no clear differences in detection probabilities among days.

Using the raw dataset to evaluate the relationship between meal sizes and the probability of prey detection, the results supported a positive correlation, where the probability of detection increased with increase in meal size (Figure 2.3).

Figure 2.3. Probability of prey detection as a function of meal size. The grey dots at 0.00 and 1.00 indicate absence or presence of detection of prey items respectively, and the black circles shows the proportion of prey detection relative to proportion fed.



The initial detection of the spike diet was possible within 24 hours post feeding (minimum gut transition time) and could still be detected until 72 hours (maximum gut transition time). We did not detect the spike diet in scats collected after 72 hours.

2.5. Discussion

Our results demonstrate that scat DNA metabarcoding provides a sensitive method of prey detection in cheetah scats. All the prey species fed to the cheetahs during the feeding experiment were detected and therefore show the potential utility of this approach in field

studies where prior information on diet of cheetah is not known. However, this study did show that prey DNA detection was influenced by different variables namely feeding day, degradation (scat age), consumed prey species, and the meal size consumed by the cheetah, which also need to be considered when making interpretations from field samples.

Our hierarchical model showed that prey detection was greatly influenced by the amount of time since being fed. Food items consumed by the cheetahs one day prior to scat collection had the strongest positive effect while a food item consumed the same day the scat was collected had the least influence on prey DNA detection. This trend follows expectations as more than 50% gastric emptying in most mammals happen within 40 hours (Clemens and Stevens, 1980). Moreover, it is also likely that cheetahs have high digestibility efficiency similar to that observed in domestic cats (Peachey et al., 2000; Sá et al., 2014). If this holds true, the errors or bias introduced by feeding day could affect prey inferences, especially when diagnosing rare prey species or economically valuable prey e.g. livestock which may not be a common prey species in the wild. Given that scat collection in the wild is not sequent and it is difficult to determine the time since the prey species was consumed, drawing a conclusion from scat DNA metabarcoding data by only estimating the frequency of occurrence could bias the diet estimates. Frequency of occurrence summarizes the proportion of samples containing a certain diet item, hence false negatives could arise if a scat was collected either too soon or too late after the consumption of prey (Klare et al., 2011; Weaver, 1993). These findings highlight the need for a more stringent scat collection protocol when planning for wild cheetah dietary studies perhaps by conducting an intensive scat collection within a short time period or by using a large number of scat samples collected over time.

We assessed whether degradation days (number of days a scat was exposed to the natural environment) had a significant impact on prey DNA detection on cheetah scats. Overall, this parameter showed a negative effect on prey detection. Similar results were reported earlier in

scat analysis studies showing that detection of prey DNA is higher in fresh than in old scats (Deagle et al., 2005; McInnes et al., 2017a; Oehm et al., 2011). However, contrary to the short maximum degradation time reported in the previous studies (e.g. 5-7 day old scats in Steller lion *Eumetopias jubatus* and 5 days old scat in carrion crows *Corvus corone*), our results indicate that prey detection is possible in cheetah scats that have been exposed to the open environment for up to 60 days under spring-summer conditions which have been shown to reduce prey detection success (Oehm et al., 2011). These results could indicate a potential species-specific food DNA detection success in old scats. This observation holds true as the diet of extinct ground sloth (*Nothroptheriops shastensis*) has been successfully inferred from fossilized scats (Hofreiter et al., 2000). During the degradation experiment, some samples were completely eaten or removed from the study site presumably by birds, foxes and/or small mammals, this is particularly relevant for field biologists planning a scat collection expedition as this would potentially affect the sample sizes.

The prey species consumed by the predators are recognized as an important consideration in scats dietary analysis and have been shown to influence the detectability of food DNA in scats (Thomas et al., 2014). Tissue composition and amount of DNA per gram of tissue vary across prey species hence some tissues are easy to digest and detect in scats (Alberdi et al., 2018). Similarly, our study showed variation in probabilities of detection among prey species. We found that detection success of chicken and horse was higher than that of deer, rabbit and quail. Of interest, our results showed that it is nearly impossible to detect some prey species on the same day they were consumed while it is highly feasible for others (Figure 2.3). The intuitive explanation is that the chicken and horse body parts fed to the cheetahs had high digestibility and contained high protein and lipid content and therefore could have reduced mitochondrial DNA decay during digestion. Thomas et al. (2014) in a feeding trial on harbour seals showed that fish with high protein levels tends to be overrepresented during diet recovery in scats.

Other alternative factors that could explain our finding includes the meal sizes and frequency of feeding of a particular food item within the study period or they had high amount of bones and hair which may have increased their detection rates (Hart et al., 2015).

Estimate of prey DNA detection window from the spike diet results showed that the maximum passage time is 3 days post-feeding after which the spike diet DNA could no longer be detected in the scats. However, we could not explicitly determine the minimum passage time as the initial scat after feeding the cheetah on the spike diet was defecated at night and the exact time of defecation was therefore unknown. Consequently, we estimated the minimum passage time to be 8-22 hours post feeding. Although this conclusion is based on one spike diet, these findings were supported by the species-specific prey detection in our model that showed the probability of detection depends partly on the prey species with some species being detectable sooner after feeding and some being possibly detectable after 3-4 days (Figure 2.3). Maximum and minimum passage time in vertebrates is known to vary depending on diet composition, sex, physiological and satiation status of the consumer (Markman, 2006; Oehm et al., 2011; Peachey et al., 2000). For cheetahs, gut transition time appears to be within the range of a few hours after feeding up to several days, meaning that a sample collected in the wild could potentially provide information on the cheetah's diet over the past 4 days. However, a lack of detection of a potential prey species may not necessarily mean its absence as food item, but possibly a failure to sample within the detection window.

The meal size can greatly influence the estimation of trophic ecology as large meals tend to have high detection rates as well as longer detection time span compared to small meals (Greenstone et al., 2014; King et al., 2010; Thalinger et al., 2017). In our study, there was a positive relationship between meal sizes and the probability of prey detection. However, the relationship was also dependent on the feeding day, with the proportion of food consumed one day prior to scat collection having the highest positive effect on the detection, implying that

the detection rate increases when a large meal size is consumed one day before a scat is collected (Figure 2.2).

We also showed that for 50% detection probability of prey in a scat, the prey item should have constituted approximately 20% of the cheetah's total daily consumed diet which in our study was approximately 300 grams. If these results hold true then this approach may be adequate in dietary studies of the wild cheetah as the maximum rate of consumption for wild cheetahs is estimated as 5.5 kg/day (Eaton, 1974) implying a higher probability of prey detection per scat. The plausible explanation for the uncertainty around the effects of the consumers (cheetahs) on prey detection is that the number of participating animals in our feeding experiment was small and biased towards males. To accurately account for this bias, further research is needed to explore the effects of sex and age by potentially using more cheetahs of different age groups. This is likely to be of particular importance as male cheetahs in the wild frequently occurs in coalitions and are larger than solitary females hence they kill larger prey (Broekhuis et al., 2018; Mills, 1992). Based on this, our hypothesis is that cheetah's sex and age may also affect prey DNA detection, with detection rates being higher for males as their meal size will likely be larger than that of females and, consequently, might result in a higher quantity of prey mitochondrial DNA in scats.

In summary, scat DNA metabarcoding provides an efficient and accurate non-invasive tool to robustly assess the diet of cheetahs, but there are several confounding factors that should be considered when designing an optimal cheetah diet study. Our finding showed that the majority of sequence reads will emanate from the consumer and this could potentially reduce the prey information, therefore we recommend the use of blocking primers (Vestheim et al., 2011) to prevent the amplification of cheetah DNA templates. In addition, factors such as the meal size, prey species and the feeding day may drastically affect prey detection rates and thus, the inferences drawn from scat metabarcoding data may over or underestimate the prey breadth

and diversity. To circumvent these limitations, we recommend the development of correction factors that would simulate field setup to maximise the usability of this approach.

2.6. Acknowledgements

The authors thank the National Zoo and Aquarium management and staff for permitting us to conduct this study in their facility and for assistance in scats collection. We particularly thank Richard Duncan for statistical advice and commentary on the manuscript, and two anonymous reviewers for comments/suggestions that greatly improved the previous version of the manuscript. We are indebted to Jonas Bylemans for his helpful guidance in wet and dry laboratories.

Chapter 3

An insight into the prey spectra and livestock depredation by cheetahs in Kenya using faecal DNA metabarcoding

*The following research is published in Zoology and, can be cited as:

Thuo D, Broekhuis F, Furlan E, Bertola LD, Kamau J, Gleeson DM (2020). An insight into the prey spectra and livestock predation by cheetahs in Kenya using faecal DNA metabarcoding. https://doi.org/10.1016/j.zool.2020.125853.

Photo: Construction of a predator proof boma (livestock corral) using recycled plastic poles. An initiative of Kenya Wildlife Trust and African Wildlife Foundation aimed to prevent Human-predator conflicts at night and encourage people to adopt sustainable boma construction materials that will reduce pressure on forest-based products.

Abstract

Dietary composition is a fundamental part of animal ecology and an important component of population dynamics. Therefore, obtaining accurate information on what an animal consumes is important for conservation planning, especially for wild large carnivores that exist in human-dominated landscapes where they are prone to direct conflicts with local people. We used faecal DNA metabarcoding to identify the vertebrate taxa commonly predated on by cheetahs (*Acinonyx jubatus*) with an emphasis on domestic taxa and determine the drivers of livestock predation by cheetahs residing in the Maasai Mara and Amboseli ecosystems which are important population strongholds in southern Kenya. From 84 cheetah faeces that we analysed, a total of 14 prey taxa were identified, including birds, wild and domestic mammals. The livestock taxa identified in cheetah faeces occurred at moderate frequency (12.8%) and the results showed that livestock predation was influenced neither by the sex of the cheetah nor by season. In general, our study shows that cheetahs prey on a diverse range of prey taxa including birds, wild ungulates of various sizes and occasionally on domestic animals, and that the faecal DNA metabarcoding approach represents a valuable complement to traditional dietary analysis methods.

Keywords: *Acinonyx jubatus*, diet, human-wildlife conflicts, carnivores, Amboseli, Maasai Mara

3.0. Introduction

Carnivore persecutions due to actual or perceived danger to human life and livelihoods are a central issue in carnivore management strategies worldwide (Treves and Karanth, 2003). Most carnivore species have large habitat requirements and are wide-ranging (Macdonald and Loveridge, 2010; Ripple et al., 2014), frequently roaming beyond protected area boundaries into human-dominated landscapes. In these non-protected areas, there is a high likelihood of carnivores encountering and killing livestock. As a result, retribution by local pastoralists has led to a rapid decline in carnivore populations, some to the extent of being locally extirpated (Gittleman et al., 2001; Treves and Karanth, 2003; Woodroffe and Frank, 2005). Consequently, mitigation of human-carnivore conflicts is a crucial conservation, social, and economic concern. Quantifying the role each carnivore species plays in human-carnivore conflict, especially in livestock predation, and determining which factors influence the likelihood of their involvement in conflict is key for their conservation. Nonetheless, reliable data on livestock predation are scarce in many areas where human-wildlife conflict occurs and are often inaccurate because of the methods used to assess predation events.

Feeding ecology of many carnivores is frequently studied using direct observations of feeding events or visual inspection of undigested matter in faecal samples (Marker et al., 2003; Davidson et al., 2013; Gómez-Ortiz and Monroy-Vilchis, 2013; Breuer and Breuer, 2015). However, these data are not always reliable as rare, small and soft-bodied species can be missed (Pompanon et al., 2012). In addition, livestock predation is most likely to occur outside protected areas. Here, it is difficult to observe carnivores as they could be adjusting their behaviour to avoid detection by people, e.g. using thicker vegetation or hunting at night (Gaynor et al., 2018). These limitations have been overcome recently by the development of molecular tools which utilise non-invasively collected faecal samples (e.g. Farrell et al., 2000). More specifically, application of high-throughput sequencing enables the identification of

multiple species contained within a single faecal sample (Shehzad et al., 2012; Xiong et al., 2017).

Because of their large home ranges, 77% of the cheetah's *Acinonyx jubatus* range falls outside protected areas (Durant et al., 2017). Here, cheetahs come into contact with people where they can be a threat to small livestock (sheep and goats), calves, and important game species (Woodroffe et al. 2007; Dickman et al. 2018). Some farmers pre-emptively kill cheetahs when they experience losses of livestock or game species (Voigt et al., 2014) regardless of the lack of direct evidence that cheetahs were responsible. Livestock predation events are rarely directly observed and even if the predator is sighted, the species involved can be misidentified. In addition, cheetahs are often blamed for predation events because they are diurnal and more visible, compared to other predators.

Here, we use faecal DNA and high-throughput sequencing for the first time in wild cheetahs to identify which vertebrate prey species are present in their diet and to quantify the levels of livestock predation in southern Kenya. In addition, we test whether livestock predation is influenced by season (dry or wet) and sex of the cheetah (male or female). Finally, we examine the power of faecal DNA metabarcoding by comparing the number and frequency of occurrences of prey species detected in this study to those identified using direct observation of feeding events in the Maasai Mara ecosystem (Broekhuis et al., 2018). Southern Kenya is an ideal place to conduct this study because it hosts high wildlife densities which are increasingly threatened by a rapidly increasing human population. This comes with associated environmental impacts such as land subdivision, conversion of natural land for agriculture and an increase in livestock farming (Lamprey and Reid, 2004; Waithaka, 2004; Okello, 2005). The region also consists of both wildlife areas and community land, with no physical barriers separating the two, so both wildlife and livestock move freely. In addition, the region is ranked as the most popular tourist destination in Kenya and hence is critical to Kenya's economy. We

predict that i) livestock predation will be high during the wet season when wild prey are dispersed, and ii) female cheetahs will kill more livestock than males because of their larger home ranges (Marker et al., 2008), which increases the likelihood of overlap with human

3.1. Materials and Methods

3.1.1. Study areas

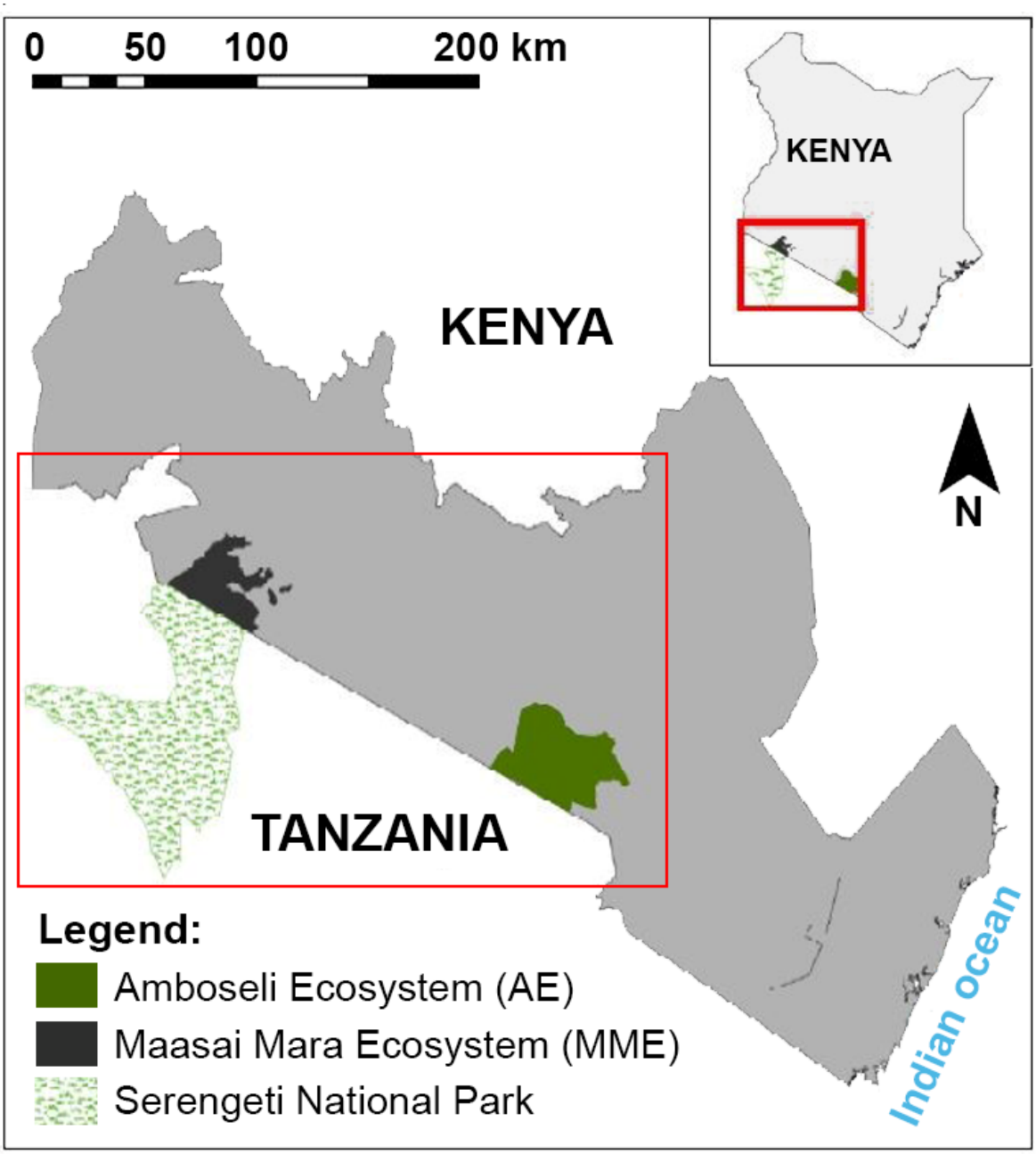
The study was carried out in southern Kenya (Maasai Mara and Amboseli ecosystems; Figure 3.1). The Maasai Mara ecosystem (MME) is centred at 1° S, 35°E, elevation ca. 1,700 m, in southwestern Kenya; and makes up the northern section of the larger Mara-Serengeti ecosystem. The MME covers approximately 2,600 km^2, which includes the Maasai Mara National Reserve (MMNR) and six adjacent community conservancies. The MMNR is managed by the Narok County Government while the community conservancies are managed by different management firms. Prey species preferred by cheetah (Thomson's gazelle *Eudorcas thomsonii*, impala *Aepyceros melampus*, Grant's gazelle *Nanger granti* and wildebeest *Connochaetes taurinus*) are generally present in the MME in high densities throughout the year (Ogutu et al., 2016) while migratory ungulates including the wildebeest, plains zebra *Equus quagga* and Thomson's gazelle from the Serengeti frequent the area annually during and after the long rains.

The Amboseli Ecosystem (AE, centred at 2° S, 37°E, elevation ca. 1,180 m) lies immediately to the north-west of the Mount Kilimanjaro, on the border of Kenya and Tanzania. The area covers approximately 5,700 km^2, including Amboseli National Park (ANP) and six communally owned group ranches. Administratively, the ANP is managed by the Kenya Wildlife Service, the Kenyan state corporation mandated to conserve and manage Kenya's wildlife, while the group ranches are communally managed. Within the AE lies the Amboseli Basin, a Pleistocene lakebed. The basin provides a permanent source of water from Mount

Kilimanjaro that attracts high numbers of animals during the dry season (Tuqa et al., 2014). Due to reliable rainfall and fertile volcanic soils, this ecosystem has experienced a rapid conversion of wilderness areas into crop farming. Similarly to MME, this area experiences recurrent droughts due to irregular rainfall (Campbell et al., 2005). Unlike in the MME, ungulates in AE are not migratory, and cheetah prey is available all year round. Both study sites host similar prey species for the cheetah.

The climatic conditions in both areas are similar with a dry season that spans from July to October and a wet season from November to June with two distinct periods of rain (November–December) and long rains (March-June; Campbell et al., 2005; Ogutu et al., 2008)

Figure 3.1. Map showing the Maasai Mara and Amboseli ecosystems in Kenya.



In both study areas, wild herbivore populations have declined by more than 78% within the last few decades which is largely attributed to a rapid increase in the human populations (Ogutu et al., 2016, 2011).

Southern Kenya is home to the Maasai people, the pastoralist community who keep cattle (*Bos taurus*), sheep (*Ovis aries*), and goats (*Capra hircus*). For Maasai people, livestock are an

important livelihood and a symbol of wealth and success (Homewood et al., 2009; Nkedianye et al., 2019). The Maasai generally use a traditional system of livestock husbandry where cattle and small stock are grazed in community areas during the day and kept in bomas (local enclosures made from branches and twigs) at night (Conroy, 1999). Over the years, the number of people and livestock have increased at a rapid rate. The populations living in and adjacent to the MME (Narok county) and the AE (Kajiado county) are estimated to be 1,149,379 and 1,107,296 people respectively, with an annual growth rate of 3–4% (https://www.knbs.or.ke, 2019).

As a consequence, the current livestock biomass is estimated to be more than eight times higher than that of wildlife compared to about 3.5 times higher in 1977–1980 (Ogutu et al., 2016). This has resulted in an increase in resource competition and human hostility towards wildlife in these areas (Okello and Kioko, 2010).

3.1.2. Faecal sample collection

Fresh cheetah faeces were collected between June 2013 and July 2017 in the MME and between May 2017 and July 2018 in the AE. Cheetahs were located using a search-encounter method (Broekhuis and Gopalaswamy, 2016). Whenever a cheetah was sighted, photographs were taken for the purpose of individual identification and the GPS location, time, and date were recorded. If the cheetah was moving, it was followed at a distance (approximately 30 m) until all the necessary data were collected. Whenever a cheetah defecated, a portion (approximately 5 g) of faeces was collected, labelled, placed in a brown paper bag, and stored in a cool dry place until further analysis. The photographs taken were used to identify the cheetahs based on their unique spot pattern (Caro and Durant, 1991). For some individuals, faeces were collected multiple times, but on different days.

3.1.3. DNA extraction and PCR inhibition evaluation

Total DNA was extracted from about 20 mg of each individually homogenized faecal sample using Invitrogen ChargeSwitch® Forensic DNA Purification Kit (ThermoFisher Scientific, USA) following the manufacturer's instructions and using overnight digestion at 55ºC rocking, at 850 rpm in a thermomixer (protocol optimised as in Thuo et al., 2019). Samples were extracted in batches of 23 plus a negative extraction control to monitor for possible contaminations.

Prior to preparation of high-throughput sequencing (HTS) library efficiency and inhibition of PCR amplification were assessed by conducting real-time PCR on dilution series (neat, 1:10 and 1:100) for each sample. The dilutions were amplified using universal vertebrate primers (12SV5; Riaz et al. 2011) in 25 µl reaction volumes consisting of; 0.20 µl of AmpliTaq Gold DNA Polymerase (Applied Biosystems, USA), 2.5 µl of GeneAmp 10× Gold Buffer (Applied Biosystems, USA), 2 µl of $MgCl_2$ (25 mmol/l; Applied Biosystems, USA), 0.2 µl UltraPure BSA (50 mg/ml; Invitrogen), 0.65 µl of GeneAmp dNTP Blend (10 mmol/L; Applied Biosystems, USA), 0.6 µl SYBR Green I Nucleic Acid Gel Stain (5X; Invitrogen), 1 µl each of forward and reverse primer (10 µmol/l), and 3 µl of template DNA made to volume with DEPC-treated water (Invitrogen™ Life Technologies, USA). All qPCRs were run using Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Hercules, USA) under the following thermal cycling conditions: initial activation at 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min and a final extension of 10 min at 72°C, and a melting curve with a stepwise increase of 0.1°C/5 s from 60 to 95°C completed the reaction. The PCR set-up was performed in a Trace DNA laboratory at the University of Canberra, Australia.

3.1.4. Library preparation and sequencing

The best performing DNA dilutions (determined by the cycle quantification values) were selected for subsequent metabarcoding using fusion-tagged primers (FTP). All negative controls that showed positive amplification were included in the preparation of the HTS library.

Gene-specific primers that amplify a ~100 bp fragment of the V5 loop of mitochondrial 12S rRNA gene incorporated with sequencing adaptors and Multiplex IDentifier (MID) tags, known as FTP, were used to construct the HTS library. The reaction was run using a one-step real-time PCR amplification following the thermal cycling conditions described above. The forward and reverse FTP consisted of P5 and P7 Illumina sequencing adaptors, a 7-bp MID tags and mitochondrial 12SV5 forward and reverse primers, respectively.

A unique combination of MID tags was assigned to each DNA extract to allow for the assignment of unique sequences to individual samples after pooling and HTS. PCR reactions were performed in triplicate for each sample and 8–10 samples were pooled to construct a single library based on the average qPCR Ct values. All pooled libraries were purified using Agencourt™ AMPure XP Beads (Beckman Coulter Genomics, USA) following the manufacturer's instructions. The concentration and amplicon sizes were estimated by electrophoresis on a 2% agarose gel stained with SYBR Safe (Invitrogen, USA) with a run time of 30 min at 120 volts. Amplicons were combined into a single DNA library based on equimolar concentration and band size estimates. The resultant library was purified as described above and quantified using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purified library consisted of a total of 88 uniquely labelled amplicons. Single-end MiSeq sequencing was conducted at the Ramaciotti Centre for Genomics (University of New South Wales, Australia) using version 2 reagent kit.

3.1.5. Data filtering and bioinformatics

Sequencing adaptors and primers were trimmed from sequencing reads using Trimmomatic v.0.36 (Bolger et al., 2014). Simultaneously, bases with Q-score below 30 at the end of each sequence read were removed and a sliding window of 4 bases was used to clip reads when the average quality per base fell below 15. Subsequent bioinformatics filtering of the trimmed sequence reads followed the protocol described in De Barba et al. (2014). The OBITOOLS functions were used to assign sequence reads to their respective samples by identifying the forward and reverse primers and tag combination using the *ngsfilter* script (Boyer et al., 2016). Using the OBIGREP function, sequences shorter than 80 bp in length and count below 10 were discarded. OBICLEAN function (threshold ratio -r 0.05) was used to remove all sequences resulting from PCR and sequencing errors. Finally, all sequence records were taxonomically assigned using the ECOTAG function (Boyer et al., 2016) and the reference database of standard vertebrate sequences from the EMBL database (release 139; https://www.embl.de/). The ECOTAG function was set to provide no taxonomic information if there was no reference sequence with a similarity equal or higher than 0.95 for a given sequence.

The ECOTAG output data were imported into R version 3.5.1 (R Development Core Team, 2015) for further filtering and analysis using packages tidyverse, gridExtra and vegan (Oksanen, 2011; Wickham, 2016; Auguie, 2017). Some samples returned reads assigned to *Lepus californicus* and *L. europaeus*. Since these reads were similar to *L. microtis* (≥ 85) *which* is present in our study areas, their reads were reassigned to Africa savanna hare (*L. microtis*). All the other ambiguous taxonomic assignments were either reassigned or discarded based on relative abundance and prior knowledge of the expected prey species within the study areas. For example, all sequences assigned to genus *Suidae* were reassigned to a single species level assignment *Phacochoerus africanus*.

3.1.6. Data analysis and statistics

Cheetah diet was expressed as the frequency of occurrence (FO), calculated as the percentage of the number of faecal samples containing a particular prey species (n) to the total number of faecal samples (N) analysed for each study area. Based on the detection of a prey species in each faecal sample, we assessed shared and unique prey species between the MME and the AE.

We tested whether the probability of livestock predation in our study areas was influenced by sex of cheetahs or season (wet and dry) by fitting five independent univariate generalized linear models implemented in lme4 (Bates et al., 2014) in R Environment (R Development Core Team, 2015). The presence (1) or absence (0) of livestock in a faecal sample was the dependent variable while, intercept only, sex only, season only, sex + season, and sex * season represented the fixed effects.

Dietary diversity within the MME and the AE was calculated using the Shannon's diversity index $H = -\sum p_i Ln\,(p_i)$ and the evenness of the diet was estimated using the Pielou's $J = \frac{H}{ln(n)}$, where n is the total number of prey taxa and p_i is the proportion of occurrence of prey species $_i$, calculated using $\%p_i = \left[\frac{Ni}{\sum Ni}\right] \times 100$. N_i is the number of faecal samples containing the *i-th* food item. The $\%p_i$ is different from %FO as p_i of all prey species sums to 100% while %FO of all prey species can be larger than 100% due to the presence of multiple prey species in a faecal sample.

Dietary richness and the number of faecal samples needed to detect all the prey species that cheetahs consumed in the two study areas were estimated using the species accumulation curves with 10,000 permutations. The power of faecal DNA metabarcoding was evaluated by comparing the total number of prey species in MME identified from 194 feeding events collected between 2013 and 2016 (Broekhuis et al., 2018) and in this study.

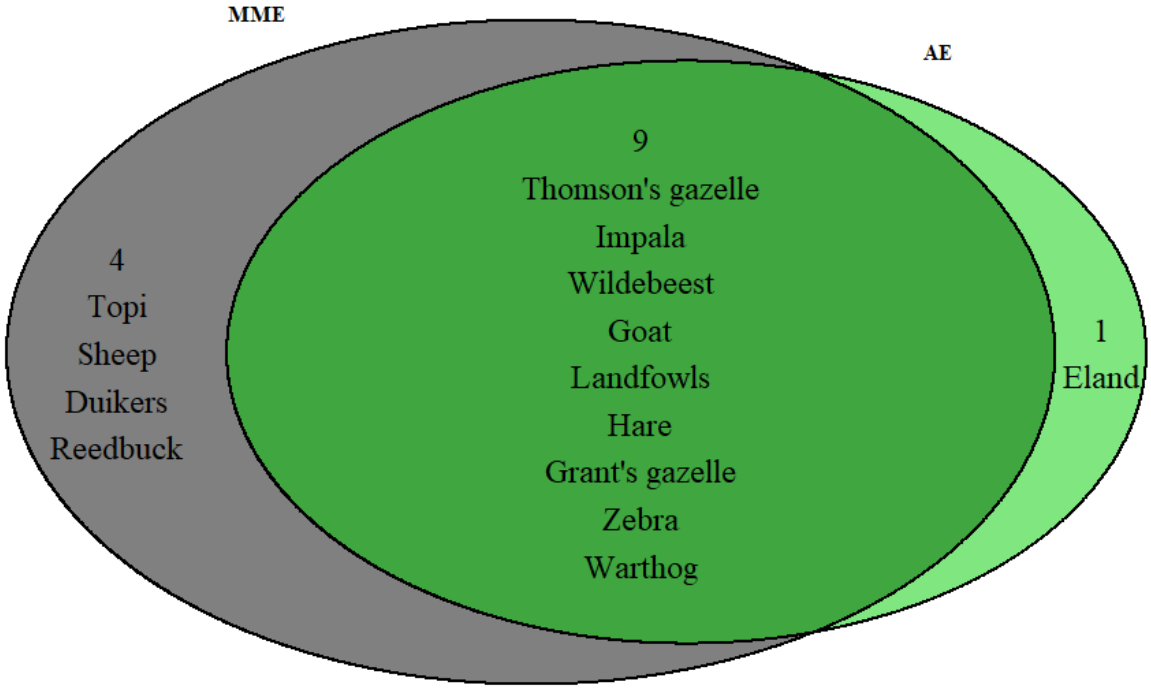
3.2. Results

A total of 84 cheetah faecal samples were collected in the two study areas: 67 in the MME and 17 in the AE. Of these, 78 samples were from known individuals – with 33 samples from males and 45 from females. Faecal samples collected from dependent cubs were classified as female as they were assumed to have fed on the mother's kill. Five individuals were sampled twice but on different days with resampling occurring two months to three years apart, therefore, these samples were treated independently.

After removing the low-quality sequences from the raw reads, we obtained 17,513,909 sequence reads of which 14,055,039 were perfectly assigned to their respective samples with an average of 43,235 reads per sample. The average quality of base calls was high (PhredQ30 score ≥ 89.16). The analysis revealed that 30% of samples produced only cheetah reads and no prey sequences. Excluding cheetah, a total of 14 different vertebrate taxa were identified: 57% to species, 29% to genus, 7% to subfamily, and 7% to family level.

Thirteen prey taxa were identified from MME and nine from AE. Nine of the prey taxa (Thomson's gazelle, impala, wildebeest, goat, African savanna hare, Grant's gazelle, common zebra, landfowls *Phasianinae spp.* and warthog *Phacochoerus africanus*) were detected in both study areas, while four (topi *Damaliscus lunatus*, sheep, duikers *Cephalophinae spp*. and reedbuck *Redunca redunca)* were unique to MME and one (common eland *Taurotragus oryx*) was unique to the AE (Figure 3.2). The number of prey items detected per faecal sample ranged from 1–6, with detection of only a single prey item being the most common occurrence (38.8%, n = 50).

Figure 3.2. Venn diagram showing prey taxa identified in faecal samples that were either shared or unique among Maasai Mara and Amboseli cheetahs.



Thomson's gazelle was the most frequently detected prey species, present in 27.9% (n = 24) of faecal samples, followed by impala (%FO = 15.1) and wildebeest (%FO = 12.8). Reedbuck and duikers were only detected in a single faecal sample (Figure 3.3).

The dietary diversity and evenness were similar for the two study areas (MME: H = 2.36, J = *0.8*; AE: H = 2.08, J = *1.1*).

Figure 3.3. Frequency of occurrence of prey species. The silhouettes images represent the domestic animals (goat and sheep)

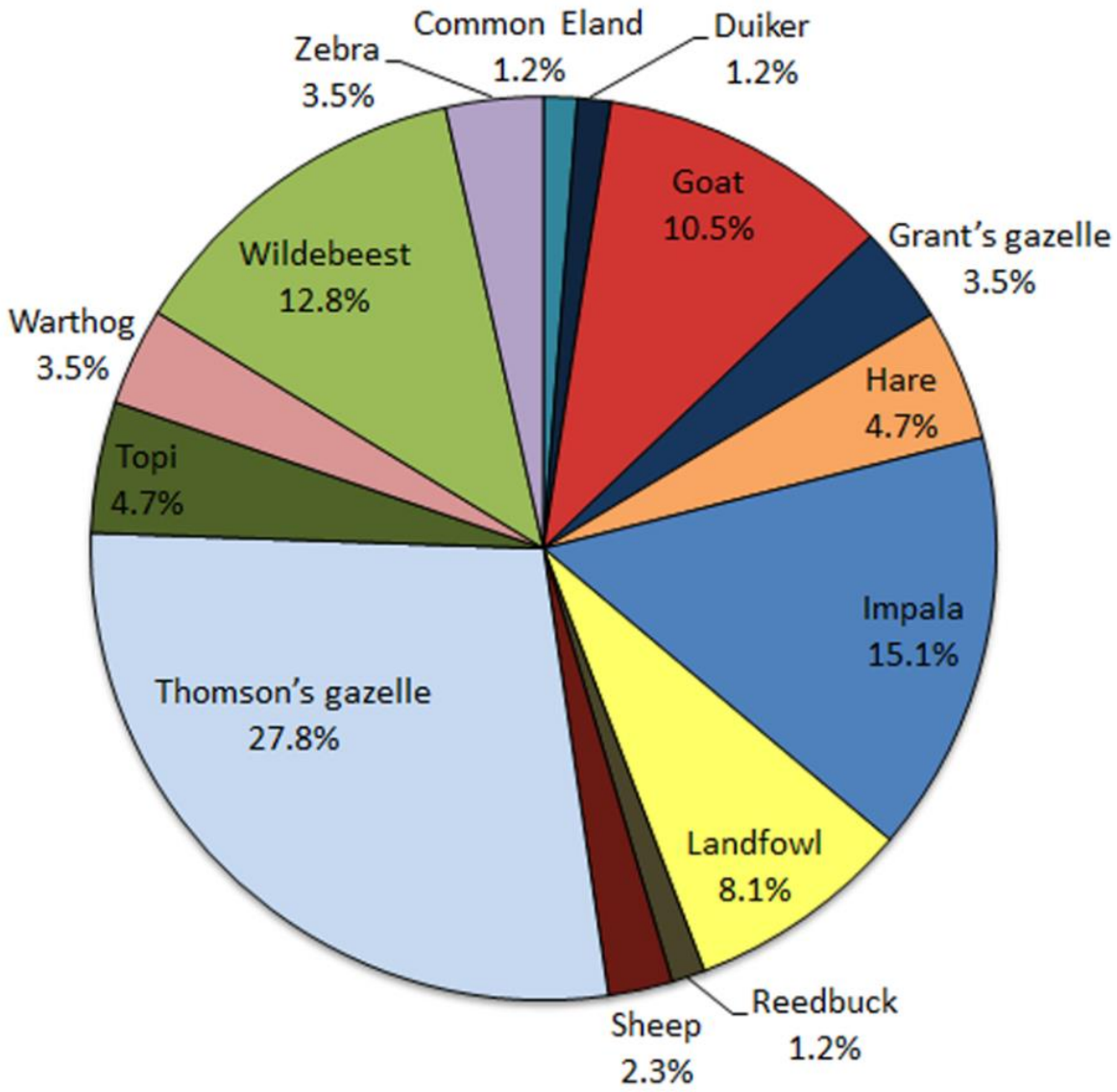
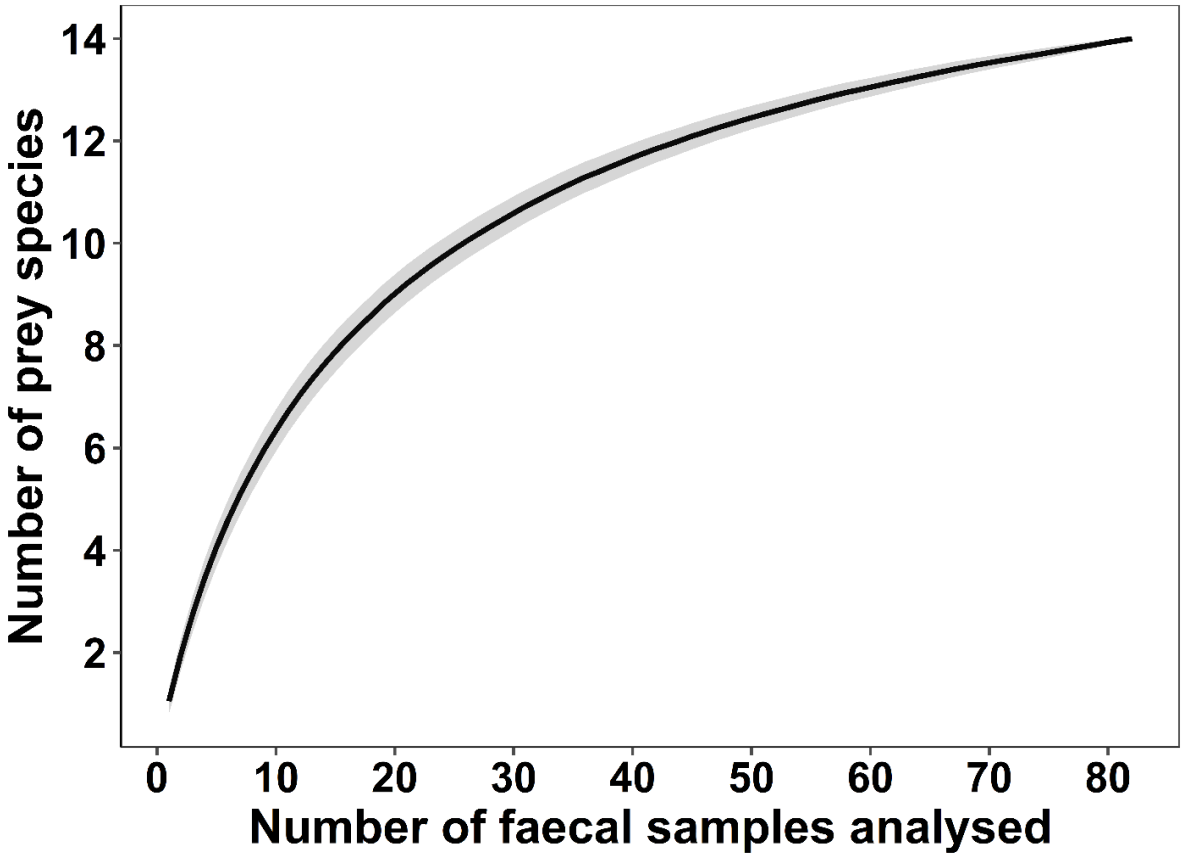


Table 3.1. Summary of the generalized linear models output showing the livestock predation depending on the sex of cheetah, season, season and sex, and season*sex interaction. Note: Males are in comparison to females and the wet season is in comparison to the dry season.

Model	**Parameters**	**Estimate**	***SE***	***p* values**
No variable/intercept only	Intercept	-0.792	0.343	0.000
Sex only	Intercept	-1.674	0.445	0.000
	Sex	-0.118	0.699	0.866
Season only	Intercept	-1.609	0.414	0.000
	season	-1.337	0.743	0.651
Sex + Season	Intercept	-1.595	0.476	0.001
	sex	-0.043	0.720	0.952
	season	-0.326	0.765	0.670
Sex + Season + Sex*Season interaction	Intercept	-1.792	0.540	0.001
	sex	0.406	0.957	0.561
	season	0.493	0.846	0.672
	season:sex	-1.672	1.555	0.283

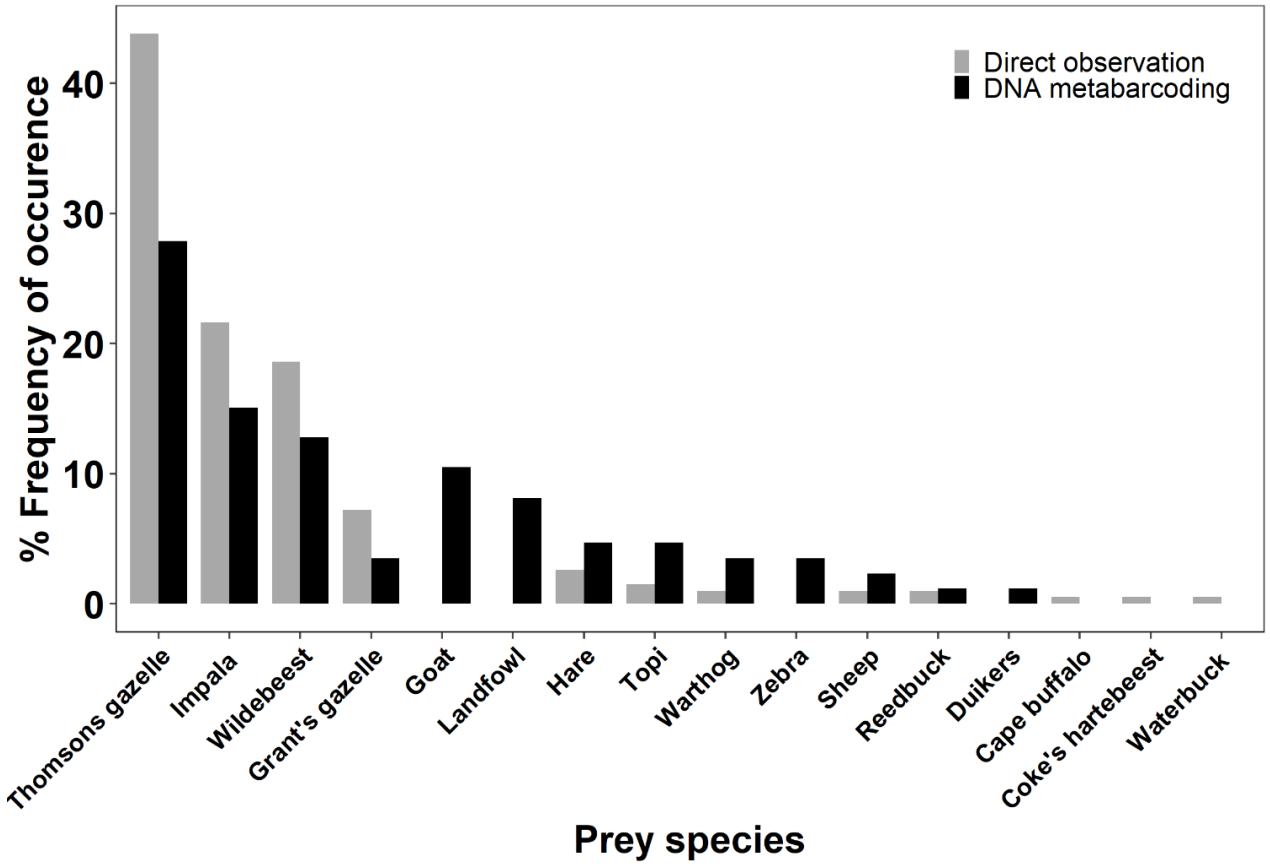
Only two domestic animal species (goat and sheep) were detected in the cheetah faecal samples, which accounted for 12.8% of the total diet of cheetahs. Neither sex nor season had an influence on the probability of livestock predation (Table 3.1). Species accumulation curves based on prey species identified in the faecal samples showed that the samples that were analyzed did not reach an asymptote (Figure 3.4). However, the results showed that over 80% of the prey species we detected were recoverable after analyzing 45 faecal samples.

Figure 3.4. Species accumulation curve based on all prey species identified in the faeces of cheetahs in Maasai Mara and Amboseli ecosystems. Grey areas represent the 95% confidence intervals.



Using faecal DNA metabarcoding, we detected four prey species (goat, landfowl, zebra and duikers) that were not identified in a previous study that used data of direct observations of feeding events in the MME (Broekhuis et al., 2018). Simultaneously, we did not identify three prey species that were detected in the direct observation of feeding events study (Cape buffalo, Coke's hartebeest *Alcelaphus buselaphus cokii* and waterbuck). The frequency of occurrences of the four main prey species (Thomson's gazelle, impala, wildebeest, and Grant's gazelle) was higher for the 194 feeding events compared to DNA metabarcoding of 37 faecal samples from the MME (Figure 3.5).

Figure 3.5. A chart showing the frequencies of occurrence of cheetah prey species calculated from the results of faecal DNA metabarcoding (this study) and direct observations of feeding events in the Maasai Mara ecosystem (Broekhuis et al. 2018) .



3.3. Discussion

This study is the first to assess the applicability of faecal DNA metabarcoding to determine the diet of wild cheetahs and to quantify their involvement in livestock predation. Our findings provide support for the premise that cheetahs prey on a diverse range of taxa including varying sizes of wild ungulates, birds, and domestic animals. In the 84 samples that we analyzed, we identified a total of 14 different prey, including domestic animals, at moderate frequencies.

Thomson's gazelle made up the largest part of the diet of cheetahs in southern Kenya. To the authors' knowledge, this is the first study to document cheetah diet in the Amboseli ecosystem

but similar findings were reported in another study in Maasai Mara based on kill observation data (Broekhuis et al., 2018). Although we did not have prey availability data, it is possible that Thomson's gazelle were frequently killed because they were the most abundant prey species in both areas as cheetahs are known to prey upon the most abundant small to medium-sized ungulates to minimize the cost of hunting (Hayward et al., 2006). Female cheetahs have also been shown to prefer Thomson's gazelle (Broekhuis et al., 2018) and the female biased sampling seen in this study may have contributed to the high proportion of Thomson's gazelle found in the diet.

The common eland was present in one sample, and this is the first time this species has been recorded in the diet of cheetahs in Kenya. It is a rare occurrence for cheetahs to kill large prey such as this, due to the high risk and energy required to subdue them (Clements et al., 2014). However, cheetahs have occasionally been reported to scavenge and kill large-sized mammals (Pienaar, 1969; Mills et al., 2004; Broekhuis and Irungu, 2016).

In terms of livestock predation, our results show that two domestic species, goat and sheep, occurred in 12.8% of the samples with goat occurring more frequently than sheep (10.5% and 2.3% respectively). The frequency of domestic animals detected in the cheetah faecal samples of this study was higher than elsewhere, e.g. 5.9% in Botswana (Boast et al., 2016). This high frequency of domestic species in the cheetah faecal samples was surprising as several studies have shown that cheetah prefer free-ranging wild species over domestic prey (Marker. et al., 2003; Boast et al., 2016; Broekhuis et al., 2018). Our findings could be attributed to several factors. First, rapid conversion of wild land to agriculture may have displaced natural prey or alternatively increased the abundance of domestic stock (Ogutu et al., 2016; Okello and D'Amour, 2008), thereby increasing cheetah attacks on domestic animals. Secondly, unattended sheep and goats during the day and poor husbandry could also be a plausible reason why domestic animals are more vulnerable to cheetah attacks. Whilst livestock was detected

in only a few independent faecal samples (11 out of 84), this suggest that at least 11 sheep and goats were killed by cheetahs. Although the number of livestock killed is relatively small, it has significant financial implications for the rural communities and may jeopardize local people's support and participation in cheetah conservation.

Factors such as sex and season are considered key determinants of cheetah diet (Winterbach et al., 2015; Broekhuis et al., 2018). Contrary, our study showed that neither sex nor season had an influence on the probability of livestock predation. However, these findings were based on a small sample size (n = 11) and a low detection rate, therefore further research using more samples across different seasons is needed in order to substantiate this finding. Notably, one cheetah in the MME, who was a member of a two-male coalition had both sheep and goat in a single faecal sample. This could indicate a conflict individual who has modified its feeding habit and preference to survive.

We observed up to six different prey species from a single faecal sample: wildebeest, sheep, Thomson's gazelle, Grant's gazelle, topi and duiker. These six prey species may have been consumed across a three day period (Thuo et al., 2019) or, alternatively, may be the result of multiple kills within a day in order to meet high energetic needs (especially mothers with cubs) or to account for kills kleptoparasited by other predators. It may also be as a result of 'surplus' killing, where cheetahs kill more prey individuals than they can eat (Hilborn et al., 2012). Thirty one samples contained only cheetah DNA with no prey DNA; similar findings have been reported in snow leopard *Panthera uncia* (Shehzad et al., 2012). A plausible explanation for this is the irregular hunting behavior of carnivores or unsuccessful hunts resulting in individuals going for several days between meals, hence some faeces only contained the predator's own metabolic waste products and hair from grooming (Shehzad. et al., 2012). Alternatively, the 12SV5 vertebrate primers used in this study may not have amplified all the taxa in faecal samples due to the lack of specificity or inefficient amplification.

This study has highlighted the strength of DNA-based techniques in assessing the feeding ecology of wild carnivore species. DNA-based techniques proved to be more successful than direct observations of feeding events to identify livestock predation: 12.8% of faecal samples contained DNA of domestic animals whereas livestock predation was only observed on 0.5% occasions using direct observations of feeding events. Species of the subfamily *Phasianinae* were detected in samples from both the Maasai Mara and Amboseli. Birds, specifically guinea fowls, have only been reported as cheetah prey in a single study in East Africa (Hamilton, 1986). It is possible that other studies did not identify birds as prey items because most were based on kill observation data which tend to underestimate small prey species (Broekhuis et al., 2018; Marker et al., 2018), although the exact origins of *Phasianinae* DNA must be careful deduced (Furlan et al., 2020).

Our study identified four prey species (zebra, goat, member(s) of subfamilies *Phasianinae*, and *Cephalophinae*) that were not previously reported using direct observation of feeding events (Broekhuis et al., 2018) but also missed three prey species (Coke's hartebeest buffalo and waterbuck) that were identified through direct observation of feeding events. Broekhuis et al. (2018) detected higher frequency of occurrence of the four main cheetah prey species (Thomson's gazelle, impala, wildebeest, and Grant's gazelle) possibly because of the higher sample size (194 feeding events). To obtain a complete prey assemblage, we recommend combining all available information on cheetah diet from data of both the direct observations of feeding events and faecal DNA metabarcoding. In addition, biases introduced by sampling or molecular analysis must be considered to enable accurate interpretation of cheetah diet (Thuo et al., 2019).

It is worth noting that DNA-based methods using faecal samples collected without direct observations of the predator will limit the identification of the size of the prey consumed as well as the social grouping of the predators. This may be a caveat in carnivore feeding ecology

studies, as these parameters are important when assessing the prey selection and competition within the carnivore guild (Rostro-García et al., 2015; Broekhuis et al., 2018) . The species accumulation curve did not reach a plateau, suggesting that the 14 prey species that we detected do not represent the complete diet of cheetahs. Due to the overall small sample sizes in this study, it is difficult to determine the minimum number of faecal samples needed to reveal all prey species, but based on our analysis, 45 faecal samples appear to be sufficient to provide substantial results on diet compositions. Future studies intending to investigate the feeding ecology of cheetahs should therefore aim at collecting more faecal samples.

3.4. Conservation implications

Our study demonstrates that the systematic molecular identification of prey in the faeces of predators can effectively detect food items that may be difficult if not impossible to obtain using other methods. We showed from our samples that cheetahs mainly prey on wild ungulates, but small domestic stock provided a significant alternative food source. This is especially important as it shows that cheetahs are either modifying their foraging behavior to include livestock or are opportunistically taking these species and this might have an implication for cheetah-human coexistence management. Based on this, adequate conservation strategies, especially those oriented toward local pastoralists, are required to reduce livestock predation and promote tolerance to the presence of cheetah in their areas. Since cheetahs are more likely to attack unattended grazing livestock, we suggest that cheetah-livestock conflicts mitigation programmes should improve livestock husbandry (e.g. not using children to herd small stock). Cheetah diet appears to be predominantly comprised of wild prey and therefore measures to restore habitats for wild ungulates will likely help increase natural predatory behavior and consequently improve cheetah conservation.

3.5. Acknowledgements

Permissions for this study were granted to David Thuo and Femke Broekhuis (Permit No: KWS/BRM/5001 and NACOSTI/P/16/69633/10821). We are grateful to Narok County Government, the Maasai Mara Wildlife Conservancies Association, and the Mara Conservancy for granting permission to conduct research in the Maasai Mara. We would like to thank the current and former research staff of Mara Predator Conservation Programme and Sakimba ole Kimiti, Senteu ole Karuma, Sankuyan ole Mingati and Ole Parmari for assisting with cheetah sighting data and sample collection. We would also like thank the local communities within our study areas for supporting our research.

3.6. Funding

David Thuo is a recipient of the Research Training Program Scholarship provided by the Australian Commonwealth Government. This study was funded by the African Wildlife Foundation, BAND foundation, Vidda Foundation, and numerous private donors.

Chapter 4

Patterns of genome wide SNPs reveal spatial genetic variation of free-ranging cheetahs

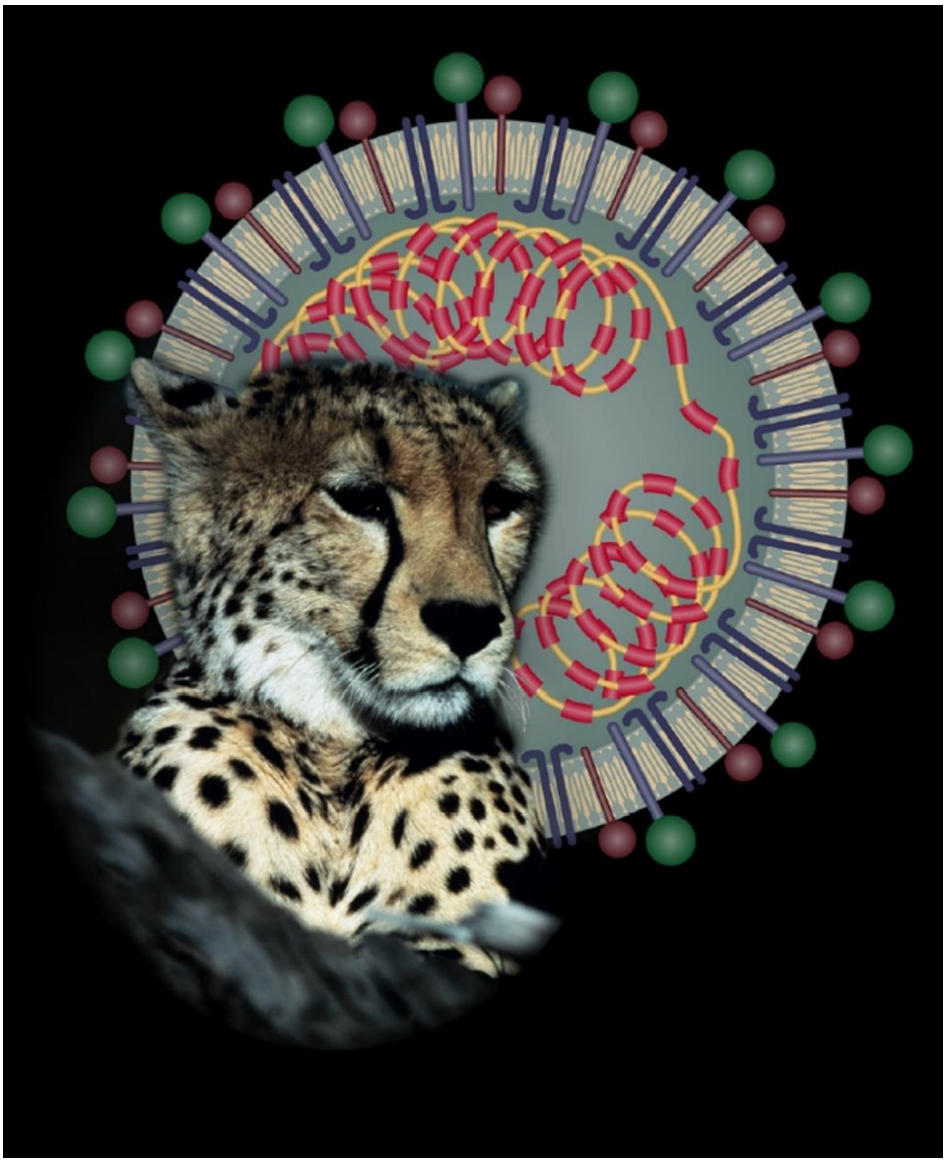


Image: Stephen O’Brien

4.0. Introduction

Cheetahs (*Acinonyx jubatus*) are among the most endangered large cats and umbrella species in ecosystems throughout the African savanna and central deserts of Iran. They were widely distributed across Africa and Asia, but modern anthropogenic impacts such as habitat fragmentation (Durant. et al., 2017; Jeo et al., 2018), persecutions due livestock predation (Dickman et al., 2018; Inskip and Zimmermann, 2009), and poaching for illegal pet trade (RWCP and IUCN/SSC, 2015; Tricorache et al., 2018) have reduced the total cheetah population to an extent that the remainder are confined in small, fragmented populations (Durant. et al., 2017). Such fragmentation of populations pose high risk of reduced genetic variability and subsequent inbreeding depression which are recipes for species extirpation (Frankham, 2005; Spielman et al., 2004). Thus, there is a growing need for wildlife managers to include the information of demographic parameters such genetic diversity in management actions and policies (Convention on Biological Diversity, 2007; Ralls et al., 2017).

Cheetahs living in Kenya provide an important example of a population that has experienced a rapid decline, are spatially fragmented and the genetic status is unexplored. Their distribution within Kenya is imperfectly known with the exceptions of Laikipia/Samburu cheetahs that falls entirely in Kenya and the southern transboundary subpopulation that spans the Maasai Mara, Masailand, and Tsavo and are connected to one another through contiguous areas of Northern Tanzania (IUCN/SSC, 2007a). These two Kenya subpopulations are considered to be a critical part of the global cheetah distribution as they are two of the only four remaining cheetah subpopulations in eastern Africa that have more than 200 adults and adolescents (Durant. et al., 2017; IUCN/SSC, 2007a). Nonetheless, human settlements and livestock in these areas are increasing at a rapid rate, and in turn has resulted in decline of wildlife numbers in the last few decades (Lamprey and Reid, 2004b; Ogutu et al., 2016). There are concerns that, cheetah subpopulations in Laikipia/Samburu and Maasai Mara are facing similar declines and their

genetic diversity and connectivity may have been subsequently impacted. However, no accurate estimates to support this or inform genetic management are currently available.

Over the last couple of decades, microsatellite and mitochondrial DNA markers have been predominantly used in genetic studies of cheetahs across Africa to estimate the genetic diversity (Charruau et al., 2011; L. L. Marker et al., 2008), evaluate kinships (Dalton et al., 2013; Gottelli et al., 2007; L. L. Marker et al., 2008), phylogeography (Charruau et al., 2011) and subspecies delimitation (Charruau et al., 2011; Freeman et al., 2001). In recent times, these markers have been replaced by single nucleotide polymorphisms (SNPs, Davey et al., 2011), in genetic studies of various large felids including lions (*Panthera leo*; Smitz et al., 2018), tigers (*Panthera tigris;* Natesh et al., 2017), and puma *(Puma concolor*; Fitak et al., 2016). Single nucleotide polymorphisms have shown unprecedented increase in the power and accuracy in most genetic analyses (Kleinman-ruiz et al., 2017; Morin et al., 2009, 2004) but they have not yet been applied in genetic evaluations of any free-ranging cheetah population.

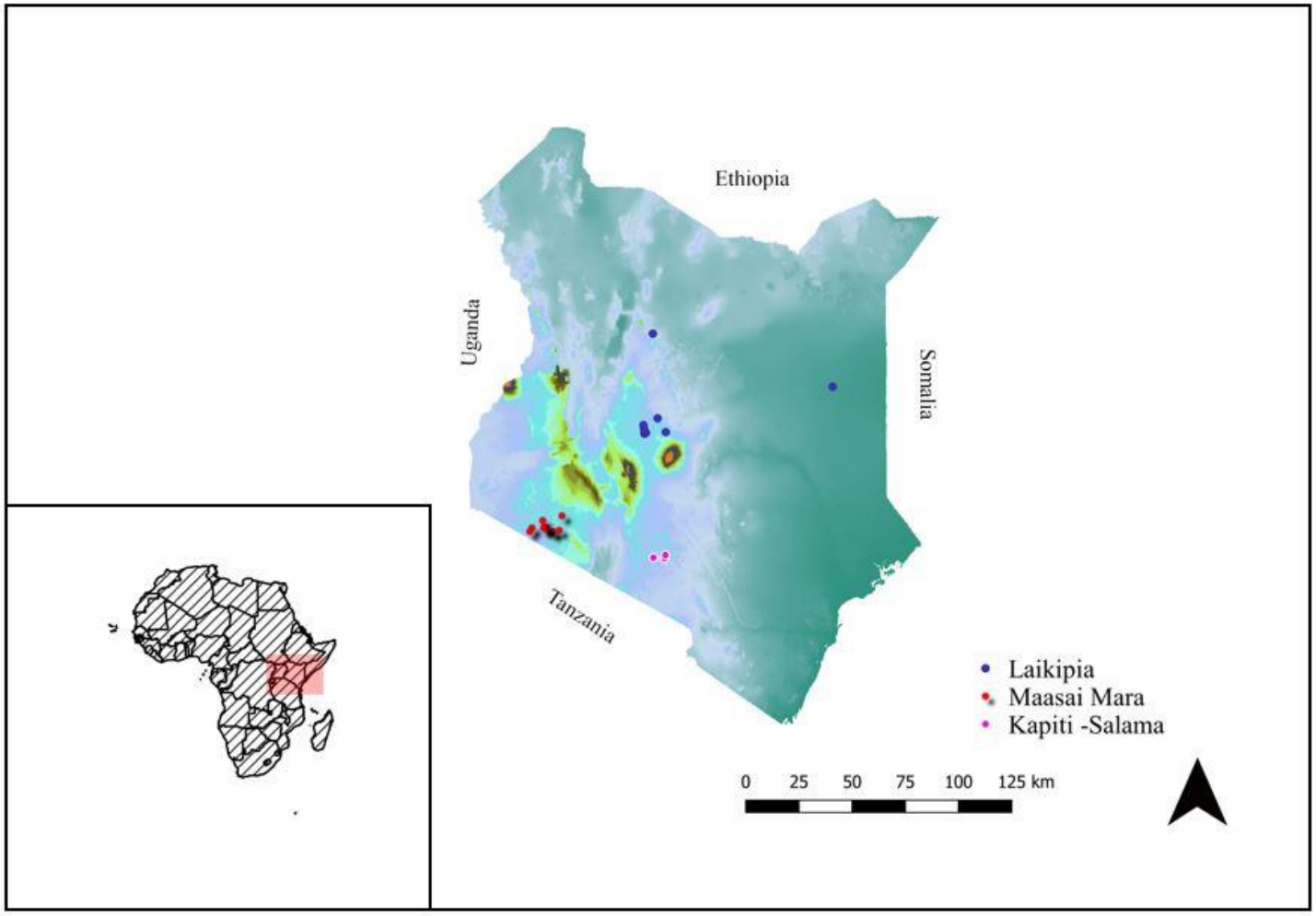
In the present study, we use genome-wide SNPs data to conduct the first population genomics assessment of cheetahs in Kenya. Our two main objectives are: (1) to assess whether the Laikipia/Samburu/Wajir and Maasai Mara cheetah subpopulations are genetically distinct and, (2) to estimate the genetic diversity and test whether the diversity will significantly vary between Maasai Mara and Laikipia/Samburu/Wajir. We predict that these subpopulations will show genetic differentiation as connectivity between them is unlikely considering the geographic distance (Broekhuis and Elliot, 2019). We also hypothesise that diversity will vary between Maasai Mara and Laikipia/Samburu/Wajir, with Maasai Mara showing higher diversity because of the geneflow with the Tanzania cheetah population. Critically, we consider the conservation implications of our study and hope this work will serve as a case study to inform policy on sustainable management especially in the light of the rapid human population grown in Kenya.

4.1. Materials and methods

4.1.1. Study sites and sample collections

We collected 20 blood and tissue cheetah samples consisting of 12 samples from Maasai Mara (1° S, 35°E; elevation ca. 1700m in the Southwest of Kenya) and 8 samples from Laikipia/Samburu/Wajir which lies on and to the north of the equator between 0.4°S to 2°N and 36.2°E to 38.3°E (Figure 4.1). Samples were collected between 2013 and 2018 using either remote biopsy darts or opportunistically when cheetahs were immobilized by the Kenya Wildlife Service veterinary team for disease surveillance, treatment, and deployment of telemetry collars. Eight additional samples were included in our analysis (4 samples from cheetahs of unknown origin housed at the Nairobi National Park-Animal orphanage and Nairobi Safariwalk and 4 samples from free-ranging cheetahs in Athi Kapiti-Salama area). Athi Kapiti-Salama lies at 1°S, 37°E, elevation 1600m. Athi Kapiti-Salama is approximately 220 kilometres from Laikipia/Samburu and 180 kilometres from Maasai Mara and are separated by numerous natural and manmade barriers (towns, rivers, human settlements, roads, agricultural lands, rugged hills etc) between these areas. Each of the 28 samples were identified as belonging to different individual cheetah based on their unique spot patterns (Caro and Durant, 1991) and this was confirmed by subsequent genetic analysis using patterns of SNP variation.

Figure 4.1. Map depicting the sampling locations in Kenya.



4.1.2. DNA extraction, library preparation and DArT sequencing

Genomic DNA was extracted from blood and muscle using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following manufacturer's protocol. The DNA quality and quantity of each sample were determined by electrophoresis in 1% agarose gel and Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The total DNA recovered for each sample was more than 2 μg and the optimal concentration was then adjusted within the range of 50-100 ng/μL for DArTseq technology.

A high-throughput genotyping method that employs DArTseq™ technology (Kilian et al., 2012; Sansaloni et al., 2011) was performed at Diversity Arrays Technology Pty Ltd (Canberra, Australia) to genotype the cheetah samples. Essentially, DArTseq™ technology relies on a complexity reduction method to enrich genomic representations with single copy sequences and subsequently perform next-generation sequencing using HiSeq. 2000 (Illumina, USA).

For this analysis, four combinations of restriction enzymes were tested initially to identify the best complexity reduction method with *PstI-Sph4* selected as the most appropriate restriction enzymes based on locus coverage, polymorphism, and reproducibility. DNA samples were processed in digestion/ligation reactions as described in Kilian et al. (2012), but the single *Pstl* compatible adaptor was replaced with *PstI-Sph4* adaptors which correspond to two different restriction enzymes overhangs. The forward-compatible adaptor (*PstI*) was designed to include Illumina flow cell attachment sequence, sequencing primer and a "staggered", varying length barcode region, similar to the sequence reported by Elshire et al. (2011). The reverse compatible adaptor (*Sph4*) contained the Illumina flow cell attachment region and Sph4-compatible overhang sequence. The ligated fragments were amplified by adaptor-mediated PCR using the following reaction conditions: an initial denaturation step of 94°C for 1min,

followed by 30 cycles with the following temperature profile: denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 72°C for 45 s, with final extension phase at 72°C for 7min. Based on equimolar concentrations, the amplicons from each sample were pooled and single-end sequenced for 77 cycles on an Illumina Hiseq2500.

The resulting sequence data output from the Illumina Hiseq2500 (Illumina) was processed using proprietary DArT analytical pipelines (Kilian et al., 2012; Sansaloni et al., 2011). In brief, the primary pipeline filtered out poor quality sequences while applying more stringent selection criteria to the barcoded region compared to the rest of the sequence (minimum barcode Phred score 30). This ensured the reliability of sequence assignment to specific samples. All identical sequences were then collapsed into "fastqcall" files for use in secondary pipeline analysis, using DArT PL software, applying proprietary calling algorithms (DArTsoft14) for SNP and SilicoDArT markers discovery (Kilian et al., 2012; Sansaloni et al., 2011). For SNP calling, all tags from all libraries included in the DArTsoft14 analysis are clustered using DArT PL's C++ algorithm at the threshold distance of 3, followed by parsing of the clusters into separate SNP loci using a range of technical parameters, especially the balance of read counts for the allelic pairs. In addition, multiple samples were processed from DNA to allelic calls as technical replicates and scoring consistency (repeatability) was used as the main selection criteria for high quality/low error rate markers. Calling quality was assured by the high average read depth per locus. The process described here is similar to that used for generating and filtering DArTseq data of yellowtail kingfish *Seriola lalandi* (Premachandra et al., 2019), Koala *Phascolarctos cinereus* (Schultz et al., 2018) and short-necked turtles *Chelidae: Emydura* (Georges et al., 2018).

4.1.3. Filtering SNP loci and visualization

The SNP data and metafile containing cheetah identification and sampling locations were read into genlight object (adegenet, Jombart 2008) using function gl.read.dart for downstream

analysis with package DARTR (Gruber et al., 2018). Only loci with over 95% scoring consistency (repAvg) were retained for further analyses. Additional filtering was carried out to remove: any loci which had missing marker information for more than 5% of the population (call rate 95%, method = "loc"), any individual which had more than 2% missing information (call rate 98%, method = "ind"), any secondary SNP that shared a sequence tag, any monomorphic loci arising as a result of the removal of individuals and finally any alleles with minor allele frequency (MAF) < 5%. The loci were not filtered for linkage disequilibrium (LD) or deviation from Hardy-Weinberg equilibrium due to the low within-population sample sizes.

4.1.4. Genetic analysis and population differentiation

The dartR and Adegenet packages (Gruber et al., 2018; Jombart, 2008) were used to estimate observed heterozygosity (H_{OBS}), expected heterozygosity (H_{EXP}), inbreeding coefficients (F_{IS}) and F_{ST} values. Mean allelic richness with rarefaction was calculated using the PopGenReport R package (Adamack and Gruber, 2014). Due to the small sample sizes the genetic diversity parameters were only estimated for Maasai Mara, Laikipia/Samburu/Wajir and Maasai Mara and Laikipia/Samburu/Wajir combined. We also tested whether the differences in H_{EXP} between Maasai Mara and Laikipia/Samburu/Wajir subpopulations were significant by computing the differences of H_{EXP} between the two subpopulations in Adegenet package.

Principal coordinate analysis (PCoA, Gower 1966; Gruber et al., 2018) ordination was used to visualize the genetic differences among individuals and subpopulations. The number of informative axes to examine was determined by a scree plot of eigenvalues, derived from the average percentage variation explained by the original variables (Cattell, 1966; Georges et al., 2018).

The distribution of population structure was calculated and visualized using a Bayesian clustering approach performed for five independent Markov Chain Monte Carlo (MCMC)

simulations with K from 1 to 5 and each run with a burn-in length of 10,000, followed by 100,000 iterations, assuming an admixture ancestry model with correlated allele frequencies and 10 iteration of each K using STRUCTURE Version 2.3.4 (Pritchard, 2007). The optimal *K* (the number of putative populations) was determined using log-likelihood and the *DeltaK* approach (Evanno et al., 2005) using STRUCTURE Harvester online program (Earl and vonHoldt, 2012).

4.2. Results

In total, 4, 844 SNPs were retained after filtering on 95% call rate. Further filtering on repeatability, shared sequence tags, MAF and monomorphisms resulted in 3,425 high quality SNPs for the 28 samples.

The ordination of the full dataset using PCoA revealed four distinct clusters, which corresponds with the Maasai Mara, Laikipia/Samburu/Wajir, Nairobi Safariwalk (2 individuals), and Athi Kapiti-Salama subpopulations (Figure 2a). Notably, it was evident that individuals from Athi Kapiti-Salama and two particular cheetahs (Luk47, CH047) were significantly different. The first two principal coordinates of PCoA accounted for 23.3% of the total genotypic variation in the dataset.

Table 4.1. Comparative genetic diversity for Maasai Mara, Laikipia/Samburu/Wajir and total free-ranging subpopulation (Maasai Mara and Laikipia/Samburu/Wajir) expressed in (1) Hexp = expected heterozygosity, (2) Hobs = observed heterozygosity, (3) AR = mean allelic richness, and (4) Fis = inbreeding measure. *n* = the number of cheetah samples analyzed in each subpopulation and Fst is the measure of distinctiveness between Maasai Mara and Laikipia/Samburu/Wajir (0 indicate no distinction and 1 indicate complete isolation).

Population	***n***	**Hexp**	**Hobs**	**AR**	**Fis**	**Fst**
Maasai Mara	12	0.301	0.315	1.308	-0.027	
Laikipia/Samburu/Wajir	8	0.282	0.289	1.291	-0.031	0.115
Total free-ranging population	20	0.294	0.294	1.293	-0.053	

From the STRUCTURE analyses we observed that the genetic composition of the Kenya cheetah was best explained by three genetically distinct populations (K = 3; Figure 4.2a and 4.2b). The Nairobi Safariwalk individuals that had clustered into a separate population in PCoA (Figure 4.3) merged with Maasai Mara subpopulation in structure analyses (Figure 4.2a). Structure analysis also showed that Luk47 and CH047were significantly different from other cheetahs. The captive individuals in Nairobi animal orphanage grouped with the Maasai Mara subpopulation (Figure 4.2a and Figure 4.3)

Figure 4.2a

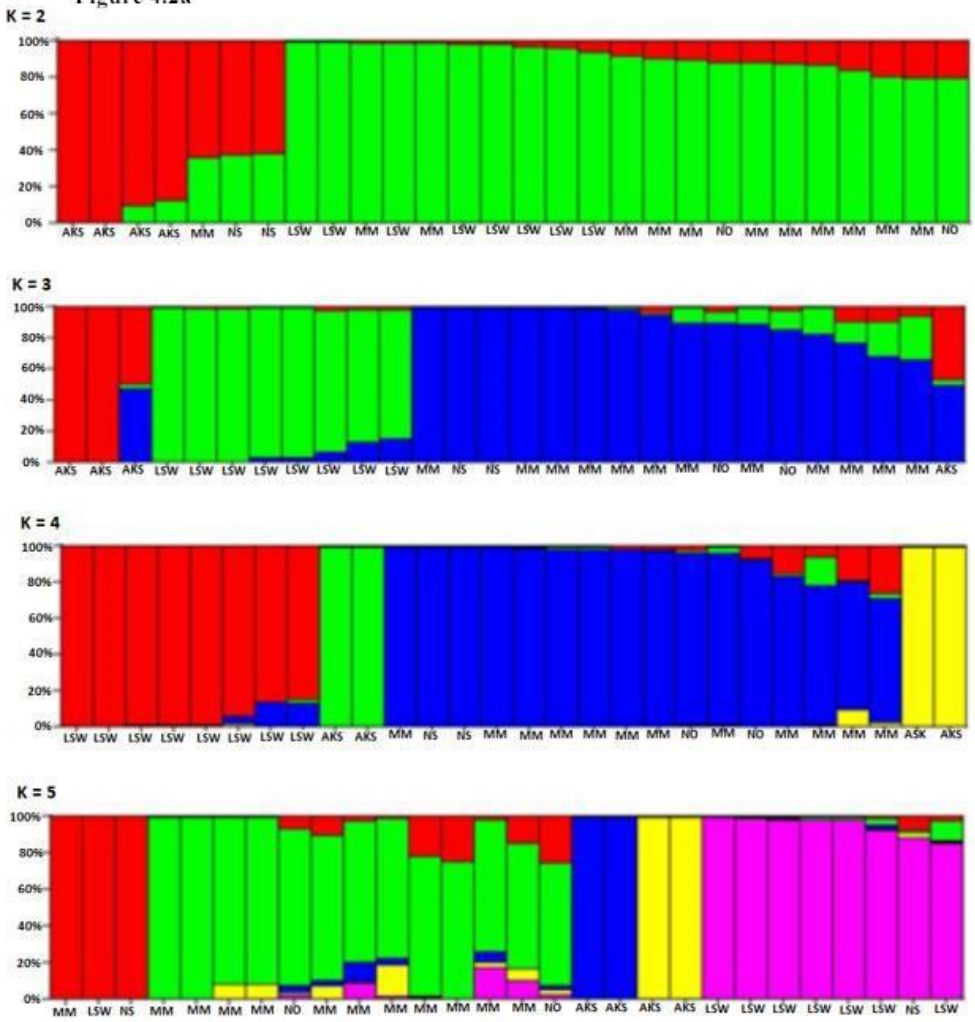


Figure 4.2b

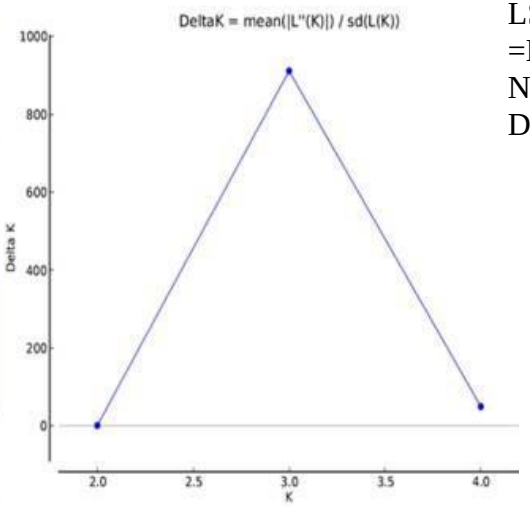
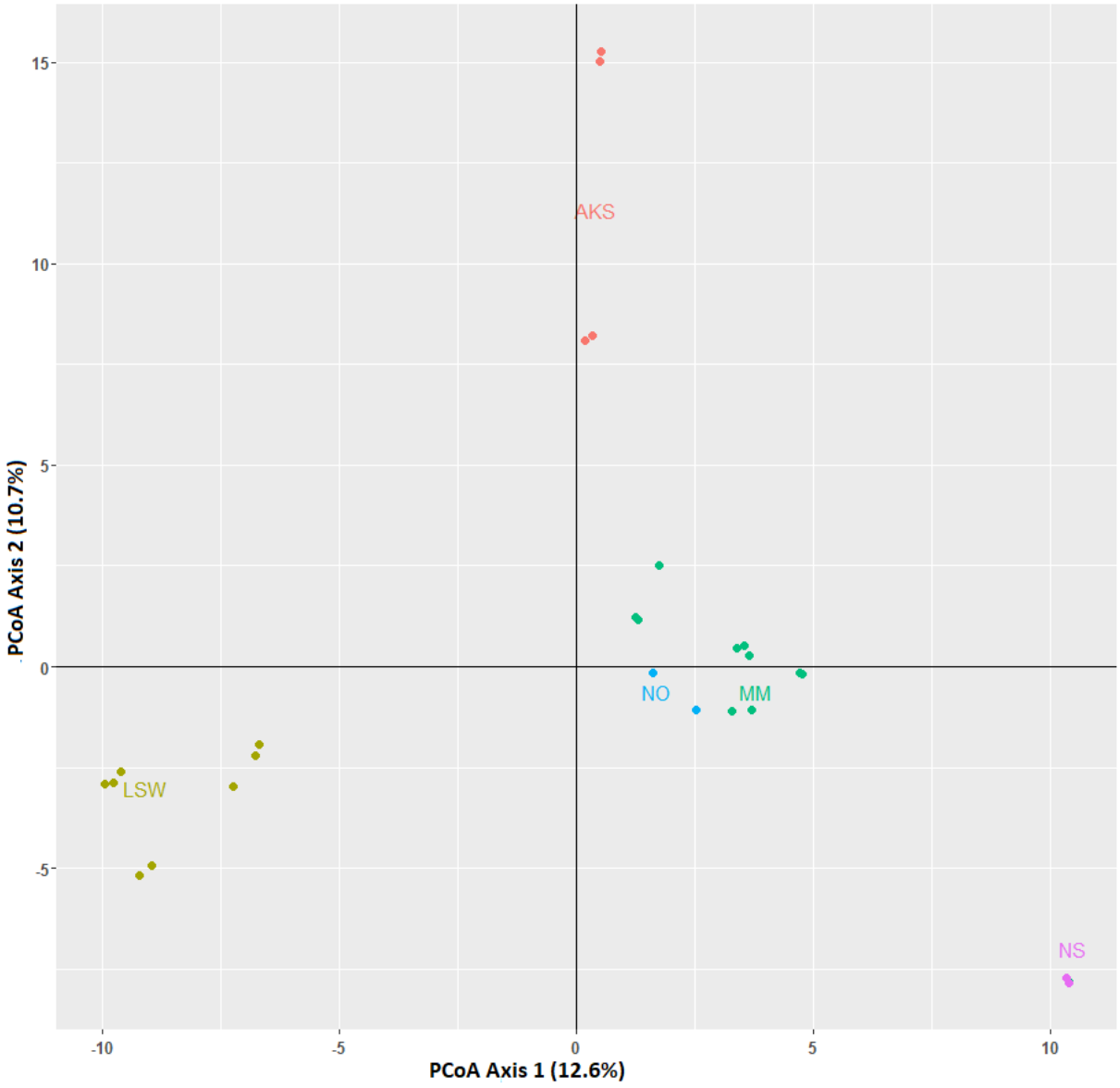


Figure 4.2a. Population clustering of cheetahs in Kenya simulated with 2–5 putative populations (K = 2–5). Each vertical bar represents, and individual cheetah and each colour represent percentage of ancestry of each individual originating from each of the 2–5 populations. MM = Maasai Mara, AKS = Athi Kapiti-Salama, LSW = Laikipia/Samburu/Wajir, NO =Nairobi animal orphanage and NS = Nairobi Safariwalk. Figure 4.2b. Delta K results

We observed a mean expected heterozygosity of 0.301 (Maasai Mara), 0.282 (Laikipia/Samburu/Wajir) and 0.294 (total free-ranging population). Mean allelic richness followed a similar trend – highest for Maasai Mara, intermediate for the two subpopulations combined and lowest for Laikipia/Samburu/Wajir (Table 4.1).

Figure 4.3. PCoA plot showing clustering of genetic clustering of cheetahs from different geographic regions in Kenya. A total of 23.3% of variation was explained by the first two principal coordinate axes. AKS = Athi Kapiti Salama, NO = Nairobi animal orphanage, MM = Maasai Mara, NS = Nairobi safariwalk and LSW = Laikipia/Samburu/Wajir.



The difference between the expected heterozygosities of Maasai Mara and Laikipia/Samburu/Wajir was not significant (*P* value = 0.06). Fst value (0.115) showed that there is modest genetic differentiation between the Maasai Mara and Laikipia/Samburu/Wajir

cheetahs (Table 4.1). Measured inbreeding coefficients (Fis) for Maasai Mara, Laikipia/Samburu/Wajir and total population were negative (Table 4.1).

4.3. Discussion

This study reports results of the first genome-wide population genetic analysis of free ranging cheetahs. Using SNPs generated by DArTseq platforms (Kilian et al., 2012; Sansaloni et al., 2011), the cheetah population in Kenya clustered into three distinct genetic groups corresponding to Maasai Mara, Laikipia/Samburu/Wajir and Athi Kapiti-Salama via Bayesian clustering analyses and four genetic groups via PCoA. The two individuals from Safariwalk that formed the fourth genetic group in PCoA clustered with Maasai Mara in Bayesian clustering analyses. Both PCoA and Bayesian clustering analyses provided consistent evidence that there is genomic differentiation between Maasai Mara and Laikipia/Samburu/Wajir subpopulations. This is not surprising as a recent study of cheetah movements have shown limited geographic connectivity between this two subpopulations (Broekhuis and Elliot, 2019). Although deduced from small sample sizes, we showed that two of the Athi Kapiti-Salama cheetahs were significantly different from Maasai Mara and Laikipia/Samburu/Wajir cheetahs. These two individuals may suggest the existence of another subpopulation within this area or that the samples were collected from recent immigrants that migrated from a more distant subpopulation. The remaining two individuals from Athi Kapiti-Salama were admixed with Maasai Mara cheetahs. This was expected as Athi Kapiti-Salama is geographically proximate to Maasai Mara and ecological theory predicts that large-bodied carnivores can cover large dispersal distances (Sutherland et al., 2000).

Examples from other species have shown little changes to estimates of genetic diversity as sample sizes increase beyond eight individuals provided 1000 SNPs or more are used (Bemmels et al., 2017). Based on this, we only estimated the genetic diversity of Maasai Mara

(n = 12) and Laikipia/Samburu/Wajir (n = 8). Overall, the genetic diversity of these two subpopulations were low relative to othe large cats, this pattern is consistent with previous studies of African cheetahs that show a substantial reduction in genetic diversity due to two or more historic bottlenecks (Charruau et al., 2011; Dobrynin et al., 2015a; L. L. Marker et al., 2008; S. J. O'Brien et al., 1983; Schmidt-Küntzel et al., 2018). The significance test of the differences in expected heterozygosity between Maasai Mara and Laikipia/Samburu/Wajir was not significant (P = 0.06). However, the expected heterozygosity and mean allelic richness were slightly higher for Maasai Mara subpopulation, perhaps due to the contiguity with the Serengeti cheetahs and potentially other Tanzanian cheetah populations (IUCN/SSC, 2007b). Our results showed negative values of inbreeding coefficients, most likely due to heterozygote excess. Heterozygote excess may be caused by small effective populations sizes resulting from historic bottleneck or due to a small number of individuals contributing to each generation (Luikart and Cornuet, 1998) which is a key feature of cheetahs (Dobrynin et al., 2015a; Menotti-Raymond and O'Brien, 1993).

The level of differentiation between Maasai Mara and Laikipia/Samburu/Wajir was also evident using *Fst* measures (*Fst = 0.115*). These levels of genetic differentiation are similar to those observed in other African cheetah populations by different genetic markers (Dalton et al., 2013; Kotze et al., 2008; L. L. Marker et al., 2008). Although our level of *Fst* was low, it could suggest a potential isolation by distance operating at a scale of 400km (distance between Maasai Mara and Laikipia/Samburu/Wajir through Athi Kapiti-Salama).

4.4. Conclusion

Genetic structuring observed within the Kenya cheetah populations has conservation implications. Increase in population structure is known to correlate with extinction (Orlando et al., 2011), suggesting that our results represent a potential conservation 'red flag' for cheetahs

in Kenya. Our results confirm that there are recent or historic barriers to geneflow between Maasai Mara and Laikipia/Samburu/Wajir and potentially even between geographically neighboring populations (Maasai Mara and Athi Kapiti-Salama). To avoid further isolation, connectivity between these populations should be promoted.

Genetic diversity is crucial to species' adaptation to environmental changes (Orlando et al., 2011), and judiciously it has been included to the wildlife conservation toolkit by the Convention on Biological Diversity (CBD, 2020). Although cheetahs have survived with low genetic diversity for thousands of years (Dobrynin et al., 2015a), the current rate of habitat loss and associated effects of climate change may further reduce their genetic diversity to irrecoverable extents and limit their potential to adapt to changes in their environment. Based on our results it is critical to improve within population variation as well as increase population connectivity. Even though natural connectivity for some cheetah populations in Kenya is not feasible due to the man-made barriers such as land conversion, erection of fences and large-scale development projects (e.g. SGR and the LAPSSET), translocations initiatives (Aitken and Whitlock, 2013; Ralls et al., 2018) could determine the future survival of cheetahs.

4.7. Acknowledgements

We would like to thank the Kenya Wildlife Service for granting us permission to conduct this study in Kenya and for providing biological samples. We would also like to thank the current and former research staff of Mara Predator Conservation Programme for assisting with cheetah sighting data and sample collection. We are grateful to Dedan Ngatia and Helen O'Neill for providing the Laikipia/Samburu samples used in this study. We are also grateful to Diversity Arrays Technology for advice on aspects related to SNPs discover.

Chapter 5

General discussion

Photo taken in Maasai Mara during a cheetah monitoring session (Naboisho Conservancy, Maasai Mara)

The roles of molecular genetic technologies in the conservation of endangered species are now commonly recognized, as they not only provide reliable information on patterns of genetic variation and diseases of the species in question but also in investigating the diets of elusive species and tracking down illegal wildlife trade (Blanchong et al., 2016; Ogden, 2011; Ouborg et al., 2010; Supple, 2018). While a large number of studies have applied the powerful new genetic technologies to assess the genetic composition and diets of numerous big cats around the globe (Fitak et al., 2016; Natesh et al., 2017; Saremi et al., 2019), comparatively little research has utilised emerging molecular approaches on free-ranging African large carnivores, which currently exist in relatively low numbers and are threatened by loss of habitat and prey, and persecution due to real or perceived livestock predation (Ripple et al., 2014; Wolf and Ripple, 2016). This scarcity of information especially for cheetahs, a species that lack genetic diversity and largely exists in human-dominated landscapes where they are perceived as pests can further endanger their long-term survival.

In this study, I generated robust datasets and information to enable scientifically informed conservation and management of cheetahs in Kenya. Threats to the long-term viability of the cheetah population in Kenya include habitat loss and mortality associated with cheetah-livestock-human interactions. The existing methods used to study cheetah diet and infer the prevalence of livestock in their diet typically returns uncertain results as they are prone to under and overestimation of prey items. I applied an advanced molecular approach to generate reliable cheetah diet information by initially validating the potential of DNA metabarcoding in cheetah diet analysis and subsequently applying this technique to study the diet of free-ranging cheetahs in Kenya. The cheetah is among the species that have exhibited relative paucity of overall genome variability for both zoos and wild populations (reviewed in Schmidt-Küntzel et al., 2018). Species with low variation are known to possess reduced mean fitness, resilience and adaptability (Lacy, 1997). For such species, accurate characterization of the remaining

genetic diversity is required to prevent extinctions. Past analyses of the genetic patterns in microsatellites and mitochondrial DNA have failed to fully categorise the genetic variability of cheetah populations in the wild. This has reduced the capacity to manage remaining levels of genetic diversity and population structure. To resolve these shortcomings, I used cheetah-specific SNPs generated by DArTseq platforms to assess the regional patterns of genetic variation in free-ranging populations in Kenya. This was the first-time SNP data has been used to assess the population structure of free-ranging cheetah.

5.1. Summary of research findings

Chapter 2 provided an evaluation of DNA metabarcoding as an efficient tool to study the diet of cheetahs and enabled the identification of limitations that should be considered when designing a dietary metabarcoding study. This chapter assessed factors that could potentially influence prey detection success in cheetah scats, including scat degradation, meal size, prey species consumed, and time of consumption. The general conclusions from this chapter were that i) scat DNA metabarcoding provides a sensitive method of prey detection in cheetah scats; ii) the days since consumption and proportion of prey fed had the greatest influence on prey DNA detection in cheetah scats and; iii) the efficiency of prey detection decreased with the age of scat. While it was possible for some information to be extracted from 60-day old scats, the optimal DNA detection window for prey in cheetah scats was between 8 hours and 3 days post-feeding. Some prey species were more readily detectable in scat on the same day they were consumed than others. These two observations, the optimal detection window and variability in detection probability of different prey species are especially important for large carnivores because they are usually implicated in domestic animal and rare species predation (Treves and Karanth, 2003b; Woodroffe et al., 2007). A lack of detection of any particular species including domestic animals in a scat does not necessarily mean they do not kill that species. The failure

to detect can be explained by the detection probability of the prey species consumed and the age of the scat. Based on these observations, the monitoring of cheetah diet for the purpose of managing human-wildlife conflicts using DNA metabarcoding needs to account for detection probabilities in the survey design. Sample sizes will need to be sufficiently large to ensure the identification of prey species with low detection probabilities and in particular scats older than 3 days will require larger sample sizes to account for DNA degradation.

In Chapter 3 DNA metabarcoding was used to determine the vertebrate taxa consumed by free-ranging cheetahs in two key wildlife areas in Kenya. While the diet of cheetahs in Maasai Mara ecosystem had previously been studied using observational data (Broekhuis et al., 2018), this was the first study to evaluate the diet of cheetahs in the Amboseli ecosystem. This study identified prey species that were previously not detected using other methods (Broekhuis et al., 2018). The analyses supported an earlier hypothesis (Clements et al., 2014; Hayward et al., 2006) that domestic animals form a small proportion of their overall diet. Sheep and goat were the domestic livestock identified in this study. The analyses did not detect an effect of sex or season on livestock depredation however the statistical power to detect such effects was likely low given the sample size. This study showed that more than 78 scat samples are required in order to completely characterize the diet composition of cheetahs.

Finally, in Chapter 4, a detailed genetic study was undertaken to assess the population and regional patterns of genetic variation of the cheetah population within Kenya. To my knowledge, this is the first study to use cheetah-specific genome-wide SNPs to investigate the population status in any area in Africa or elsewhere. Relative to the commonly used markers such as microsatellites and mitochondrial DNA, genome-wide SNPs are much more informative and reproducible (Morin et al., 2004; Ouborg et al., 2010). My study showed that there are low levels of heterozygosity in Kenyan cheetahs which is consistent with other studies of African cheetahs (reviewed in Schmidt-Küntzel et al., 2018). The sub-populations I studied

exhibited genetic partitioning, suggesting a possible recent or historic gene flow barrier between the southern (Maasai Mara) and the northern (Laikipia/Samburu/Wajir) cheetah sub-populations in Kenya. While the rarity of my study species (typical of cheetahs) prevented the collection of more samples within the timeframe of this study, I propose that SNPs derived from a larger sample size are likely to provide more information on geneflow that would greatly assist the conservation management of Kenyan cheetahs.

5.2. Implication for cheetah conservation

The findings from this thesis have implications for the conservation and management of free-ranging cheetahs, both in Kenya and throughout their range. The diet composition of wild cheetahs evaluated in this thesis using DNA metabarcoding identified a potential source of conflict between cheetahs and farmers. The presence of domestic animals in cheetah scats, albeit moderate, provides clear evidence that cheetahs do consume domestic animals, but that this tends to be restricted to smaller livestock such as sheep and goats. The communities that share their land with cheetahs in Kenya are mostly subsistence farmers and predation of even a single domestic animal can have significant consequences for their livelihood. Similarly, given the extremely low population size of cheetahs in Kenya, any inadvertent mortality can have a significant impact on the sustainability of the species in the wild. As such, management strategies that are oriented toward local pastoralists should be put in place to reduce livestock predation and promote tolerance to the presence of cheetah in community lands. Our results support previous studies that suggest that cheetah diet is mostly composed of wild prey species, therefore, conservation measures that restore habitats for wild ungulates and promote natural predatory behavior of cheetahs are likely to be effective in minimizing cheetah-livestock interactions. Increase in natural prey would also allow cheetah ecologists to measure if cheetahs

are modifying their foraging behavior to include livestock or are simply taking domestic animals opportunistically.

Monitoring and maintaining adequate patterns of genetic variability is important for the conservation of threatened species because genetically diverse populations have the potential to provide a buffer against catastrophic events like disease epidemics and enhance long-term evolutionary adaptions (Frankham et al, 2002). The levels of genetic diversity estimated using expected heterozygosity and mean allelic richness was relatively low in the Kenyan cheetah population while there was some evidence for genetic differentiation (Chapter 4). This observation could have significant implications for the ongoing viability of Kenyan cheetahs as it suggests there is a break in gene flow between the sub-populations. It is important to note that the patterns of genetic variability obtained in this study were based on relatively low sample sizes and therefore should be treated as approximations, as sample sizes have consequences on the reliability of inferences (Sánchez-Montes et al., 2017). If these results hold true, then management options that promote functional connectivity between populations should be put in place to improve the genetic status of cheetahs.

5.3. Concluding remarks and future research needs

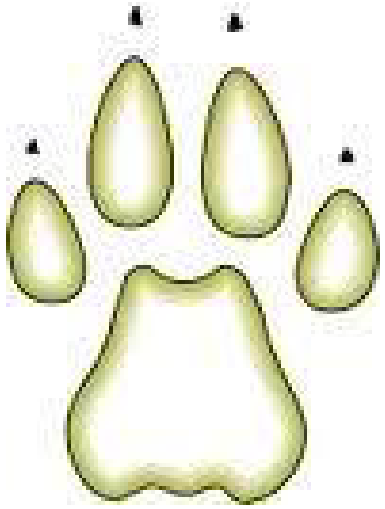
The research presented in this thesis contributes to the conservation of cheetahs and advances the field of conservation genomics for this species. The study demonstrated that domestic animals constitute a substantial proportion of the cheetah diet and the fragmentation of the cheetah populations have started to be reflected in their genetic composition. Therefore, urgent measures aimed at fostering human-cheetah coexistence are needed and the genetic status of Kenyan cheetahs should be closely monitored using more samples. The molecular techniques used in this research provide additional resources to the current cheetah conservation studies toolkit.

The future research needs for wild cheetahs are almost inexhaustible due to cheetah's remarkable position in evolutionary history, genetic variation and conservation (O'Brien et al., 1987). The conservation and management of this species in Kenya will require sound knowledge on their ecology and genetics while closely working with the local communities who bear the cost of living with them, this way, the decision-making process for conservation will be holistic. As technology evolves, similar questions will be easier to answer on a much finer scale. By using genome-wide SNPs data this research provides a solid platform for more research questions in future. There are many aspects that can build on this research to give a better insight into where wildlife corridors should be developed and which individuals should be translocated. It would be worthwhile investigating whether the *Acinonyx jubatus jubatus* is interbreeding with the *Acinonyx jubatus soemmeringii* subspecies, as a preliminary report has indicated that East African subspecies (that include Kenya's cheetah) are more closely related to *A. j. soemmeringii* than their current classification (*A . j. jubatus*; Prost et al., 2020). The results from this thesis show that northern Kenya subpopulation is genetically dissimilar to the southern Kenya cheetahs, but whether this differentiation is due to geographic distance and infrastructural developments between these two subpopulations or due to the northern subpopulation breeding with the *A. j. soemmeringii* which are geographically closer has not yet been investigated.

5.4. Epilogue

While my datasets answered most of the questions that I had laid out for this thesis, the question I am inescapably asked by the local people who share their land with the wildlife is: "Why should we listen to you while your animals (wildlife) always come to graze in our land yet when it is dry and we bring our cows to your park (protected areas) to graze or when your animals kill our livestock and we remove the culprits (predators), we get arrested?" As a local,

I completely understand the depth of this issue and I have put myself in their shoes and wondered the same thing, which is-why would farmers care about what is presumably the cause of their despair? Despite the magnitude of this issue, I have always answered this question with objectivity, empathy, and some reference to carnivore ecology. Carnivores generally kill domestic animals because they are easy to subdue as compared to wild prey whose antipredator behaviors are well developed. In addition, land-use changes and human population growth which is evident to every member of the community has rapidly increased the human-predator overlap and to some extent encroached to wildlife habitats. This has increased the likelihood of predators encountering and killing domestic animals. However, this is all ecological conjecture and the truth is that the reason large predator kill domestic animals is a complex conundrum which requires proper interrogation of the cause, extent and factors that influence predation so as to provide a framework for developing solutions to these conflicts. Possibly the reason the local people should listen to these research findings is because my study provided answers to some important questions that have implications on long-term coexistence.



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