DNA Metabarcoding Diet Analysis of Leopards (*Panthera pardus*) in Bicuar National Park, Angola

Diogo Emanuel Pereira de Jesus

Master in Biodiversity, Genetics and Evolution

Biology Department

2021

Supervisor

Raquel Godinho, Principal Researcher, ECOGEN, CIBIO-InBIO

Co-Supervisor

Pedro Monterroso, Principal Researcher, CIBIO-InBIO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas. O Presidente do Júri,

Porto,_____/_____/_____

Acknowledgements

During the last five years, there have been several people who have been part of my academic journey. In a way or another, I would like to thank them all for making me grow by either influencing me positively or negatively. This journey has made me evolve both intellectually and personally, and I can tell I am a better person thanks to that.

Firstly, I would like to thank my parents and sister for all the unconditional support they gave me during the 5 years. To my parents for always allowing me to pursue and do what I love in pursuit of a dream that I see coming true. For realizing how hard I work to be someone they are proud of. I hope I have succeeded. To my sister for annoying me for so long. No, not just for the 5 years, but since we've been together. I grew up knowing that even though we have our disputes, we want to see ourselves succeed. My success is your success, and it is the success of our parents. Thank you for helping me make the charts, graphs, and PowerPoints. The papers and presentations wouldn't look as good if it wasn't due to your help. Thank you to the " 4 paws" family I have at home, you know how much I love you. Family, I dedicate this thesis to you.

Next, I would like to thank one of my main pillars, the person who has supported me since the first day we met. I remember it like it was yesterday. Alana, thank you for always being there for me and never giving up. Thank you for making me grow so much and for realizing that with two, everything is easier. Thank you for all the love you have given me during these 6 years. Thank you for understanding me, and for always knowing how to make my day better. You know that, without you, none of this would have been possible. Yes… I do not forget that you helped me to make all the charts, graphs and works I need. Love you.

I also thank the rest of my family for supporting me every day and being proud of the person I have become. Additionally, to Alana's family, for welcoming me so well. I love you all very much.

To Raquel Godinho, my supervisor, I would like to thank you for accepting me to work on this project, trusting me to conduct it successfully. Thank you for guiding me throughout the project and helping me to produce this thesis. Moreover, I would also like to thank Pedro Monterroso for accepting and co-supervising me during this project. Thank you for teaching me everything that was necessary for the preparation and development of the thesis.

Without forgetting, this study was not possible without the support of the CTM of CIBIO. I am particularly grateful to Maria Magalhães, Patrícia Ribeiro, Sofia Mourão and Susana Lopes who helped me during the whole laboratory process of the thesis from DNA extraction to sequencing. I learned a lot from you, thank you very much.

Abstract

Mammalian carnivores are a group of species in which global management and conservation problems have been identified. They are strong interactors and play an important role in ecosystem structure and functioning, controlling prey communities with cascading effects in the environment where they occur. Despite their importance, carnivore populations have significantly declined due to pressures caused by global climate change, habitat degradation, fragmentation, and direct persecution. Relevant efforts have been implemented to study carnivores' status, distribution, abundance, among many other population parameters, but their elusive and scarce nature limits the collection of data. A key factor influencing carnivores' ecology is prey availability. According to the optimal foraging theory, individuals should adjust their foraging behaviour to maximize the net rate of energy intake per unit effort, i.e., should choose prey that provide higher energetic benefits.

Several methodologies can be used to study the diet of carnivores, some of them requiring handling the animals, which may cause stress or potential injuries raising ethical and moral concerns. Because of that, non-invasive approaches are very much applied in diet analysis, among which the use of scats is the most used source of data by either analysing the undigested remains or by performing molecular identification of prey. Currently, the latter is outperforming the other methodologies. Specifically, the use of DNA metabarcoding approaches allows the parallel identification of prey and predator with unprecedented detail and accuracy.

Under the scope of current research programs being carried in Angola since 2017, 140 leopard (*Panthera* pardus) scat samples were collected in the Bicuar National Park and identified to species and individual levels using molecular approaches. The DNA available from these samples was used here to characterize the diet of leopards through the implementation of a DNA metabarcoding approach. This included the DNA amplification by a two-step PCR approach to construct DNA libraries with individual indexing of samples, followed by Illumina MiSeq sequencing. Output reads followed a bioinformatic pipeline of quality control and species identification. Prey species were identified against a reference database built for this work, which included all putative leopard prey present in Bicuar NP. Data was analysed for the frequency of occurrence of each prey species in the leopards' diet and was additionally explored for dietary differences between males and females, and between individuals to assess possible dietary specialization.

Results showed that the common duiker (*Sylvicapra grimmia*) was the most consumed prey by leopards in the Bicuar NP, which was expected given its abundance

and its suitable nature to fulfil the energetic requirements of leopards' preferred prey. The bushpig (*Potamochoerus larvatus*), the malbrouck (*Chlorocebus cynosuros*) and the greater kudu (*Tragelaphus strepsicerus*) were the other most consumed prey species identified in leopards scats. Consumption of ungulates represented a total of 86.67% of leopard's diet, which is in accordance with the literature. Leopards did also consume small-sized carnivore species such as genets, eventually due to intraguild predation, interspecific competition for smaller prey or opportunistic events. A few rodents, birds and reptiles were also identified in leopards' diet.

Differences in the diet of males and females were found to be significant, with females having a broader dietary niche than males. However, considerable overlap was observed between the diet of both sexes, as the most consumed prey for both was the common duiker. Finally, no evidence of individual dietary specialization was found, which will need further research using larger sample sizes to assess this objective.

Keywords: DNA Metabarcoding, Diet Analyses, *Panthera pardus*, Non-invasive, Optimal-Foraging Theory, Angola, Scat Analysis, Dietary Diversity, Frequency of Occurrence, Sexual Differences, Trophic Niche Overlap

Resumo

Os carnívoros são um grupo de mamíferos com problemas globais de gestão e conservação. São agentes muito interativos no ecossistema, desempenhando um papel importante na sua estrutura e funcionamento, nomeadamente controlando as comunidades de presas o que produz efeitos de cascata nos ambientes onde ocorrem. Apesar da sua importância, as populações de carnívoros têm diminuído significativamente devido a pressões humanas causadas pelas alterações climáticas globais, degradação e fragmentação de habitats bem como perseguição direta. Têm sido implementados esforços relevantes para estudar o estatuto, distribuição, abundância, entre muitos outros parâmetros populacionais em espécies de carnívoros, mas a sua natureza elusiva e escassa limita a recolha de dados. Um fator chave que influencia a ecologia dos carnívoros é a disponibilidade de presas. De acordo com a teoria do forrageamento ótimo, os indivíduos devem ajustar o seu comportamento de forrageamento para maximizar a taxa líquida de consumo de energia por unidade de esforço, ou seja, devem escolher presas que proporcionem maiores benefícios energéticos.

Várias metodologias podem ser utilizadas para estudar a dieta dos carnívoros. Algumas dessas requerem a manipulação dos animais, potencialmente causando stress ou eventuais lesões, o que levanta preocupações éticas e morais. Devido a isso, as abordagens não invasivas são muito mais comuns na análise da dieta, entre as quais a utilização de excrementos é a mais utilizada, quer através da análise dos restos não digeridos, quer efetuando a identificação molecular das presas. Atualmente, esta última está a superar em popularidade as restantes metodologias. Especificamente, a utilização de abordagens de “metabarcoding” de DNA permite a identificação paralela de presas e predadores com detalhe e exatidão nunca antes conseguidas.

No âmbito dos programas de investigação em curso em Angola desde 2017, foram recolhidas 140 amostras de excrementos de leopardo (*Panthera pardus*) no Parque Nacional de Bicuar, confirmadas a nível de espécie e identificadas a nível individual utilizando abordagens moleculares. O DNA disponível destas amostras foi aqui utilizado para caracterizar a dieta dos leopardos através da implementação de uma abordagem de “metabarcoding” de DNA. Isto incluiu a amplificação de um fragmento do gene 12S do DNA mitocondrial através de uma abordagem de PCR em duas etapas para construir bibliotecas de DNA com indexação individual de amostras, seguida de sequenciação de alto rendimento num sequenciador MiSeq da Illumina. As sequências obtidas seguiram um pipeline bioinformático de controlo de qualidade e identificação de espécies. As espécies de presas foram identificadas contra uma base de dados de

sequências de referência construída neste trabalho, que incluiu todas as presas putativas de leopardo presentes no Bicuar. Os dados foram analisados quanto à frequência de ocorrência de cada espécie de presa na dieta dos leopardos, e foram adicionalmente exploradas as diferenças alimentares entre machos e fêmeas, e entre indivíduos para avaliar a possível especialização alimentar.

Os resultados mostraram que o bâmbi-comum (*Sylvicapra grimmia*) é a presa mais consumida pelos leopardos no Bicuar, o que era esperado dada a sua abundância e a sua natureza adequada para satisfazer as necessidades energéticas dos leopardos. O porco-do-mato (*Potamochoerus larvatus*), o macaco-de-cara-preta (*Chlorocebus cynosuros*) e o kudu gigante (*Tragelaphus strepsicerus*) foram as outras espécies mais consumidas identificadas nas fezes dos leopardos. O consumo de ungulados representou um total de 86,7% da dieta dos leopardos, o que está de acordo com a literatura. Os leopardos consumiram também espécies carnívoras de pequeno porte, como as genetas, eventualmente devido a competição interespecífica por presas mais pequenas, predação intraguilda ou eventos oportunistas. Foram também identificados alguns roedores, aves e répteis na dieta dos leopardos.

As diferenças na dieta dos machos e das fêmeas foram consideradas significativas, com as fêmeas a terem um nicho de dieta mais amplo do que os machos. No entanto, observou-se uma considerável sobreposição entre a dieta de ambos os sexos, uma vez que a presa mais consumida por ambos foi o bâmbi-comum. Finalmente, não foram encontradas provas de especialização alimentar individual, que necessitarão de mais investigação utilizando amostras de maior tamanho para avaliar este objetivo.

Palavras-Chave: DNA Metabarcoding, Análise de Dieta, *Panthera pardus*, Não-invasivo, Teoria do Forrageamento Ótimo, Angola, Análises de excrementos, Diversidade Alimentar, Frequência de Ocorrência, Diferenças Sexuais na Dieta, Sobreposição de Nicho Trófico

Index

List of Tables

Table 2_SI. List of elutions used, dilutions perfmoed and amount of DNA used for PCR for the testing of 12S fragment and 12S-V5 primers – This list comprises the sample names and respective elution used, dilution performed and DNA amount of DNA used for PCR testing of primers and 12S fragment. Sample name is matched to the species in Table 2. * samples kindly offered by Professor Bettine van Vuuren (Centre for Ecological Genomics & Wildlife Conservation, University of Johannesburg); &samples available at CIBIO tissue collection.

Table 3_SI. PCR Mix used for 12S fragment and primer testing – The mix was prepared by adding 5 µL of Master Mix, 0.3 µL of both primers and 3.4 µL of H2O per sample (1x).

Table 4_SI. Thermocycling conditions for the PCR for testing primers and the 12S fragment – The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95ºC for 15 minutes; then 40 cycles of denaturing, annealing and elongation at 95 ºC, 47 ºC and 72 ºC respectively, during 30, 20 and 30 seconds, respectively; and a final elongation at 60 ºC for 10 minutes

Table 5_SI. Sanger Sequencing Mix used for 12S fragment and primer testing – The mix was prepared by adding 0.5 µL of TRR Mix, 1 µL of TRR Buffer, 0.5 µL of one of the primers and 6 µL of H2O per sample (1x).

Table 6_SI. Index PCR Mix - This mix was prepared by adding 7 µL of 2x Kappa HiFi Hot Start enzyme, 0.7 µL of each index and 2.8 µL of H2O and DNA per sample (1x).

Table 7_SI. NGS Library Prep Index PCR - The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95ºC for 3 minutes; then 8 cycles of denaturing, annealing and elongation at 95 ºC, 55 ºC and 72 ºC respectively, during seconds each; and a final elongation at 72 ºC for 5 minutes.

Table 8_SI . Results from Qubit Quantification – Table showing the results of the Qubit quantification for the pools of both replicates. Quantification was done two times (#1 and #2) and the mean was calculated.

Table 9_SI. Normalization for a final pool of 4nM

Table 10_SI. Results from qPCR validation of the NGS library

List of Figures

carried out after each step. Quantification, normalization, and pooling are carried out before sequencing on Illumina MiSeq. Figure retrieved from Bourlat et al. (2016).

Figure 10. – Results of filtering steps performed–Results of the filtering steps performed from MiSeq sequencing until the detection of prey. Each row indicates a filtering step.

Figure 11. Frequency of Occurrence (%) of each detected prey item – (a) Frequency of Occurrence (%) of the 5 taxonomic groups detected in leopard diet; (b) Frequency of Occurrence (%) of the 16 different species detected in leopard diet. Green bars represent Ungulates, Dark Blue represent Carnivores, Light Blue represent Primates, Orange represents Rodents and Black represents the remaining detected species.

Figure 12. Cumulative dietary diversity indexed by the Shannon index diversity for leopards against an increasing number of sample size - The curve reaches an asymptotic value around 37 samples meaning that this is the minimum number of samples that can be representative of the overall leopard diet .

Figure 13. Trophic niche diversity calculated with the Shannon's index for females, males and the total population diet – The overall population diet (H = 1.45 +/- 0.15) has higher value of trophic niche diversity when compared to both females (H = 1.22 ± 0.29) and males (H = 0.60 ± 0.09) diet .However, females have significantly higher values when compared to males.

Figure 1_SI – Results of the PCR test of the CIBIO samples, with electrophoresis on agarose gel at 2%

Figure 2_SI - Results of the PCR test of the samples from Centre for Ecological Genomics & Wildlife Conservation, University of Johannesburg, with electrophoresis on agarose gel at 2%

Figure 3_SI. Qubit standard/sample preparation workflow. Image retrieved from Quick Reference Qubit ASSAYS PDF file (Pub. No. MAN0017210), Thermo Fisher

Figure 4_SI -> 10000 TapeStation Results. Library showed a clear peak in 295 bp

List of Abbreviations

BNP - Bicuar National Park

bp – Base pairs

C_f - Final concentration

C_i - Initial concentration

CIBIO - Research Centre in Biodiversity and Genetic Resources

CITES - Convention on International Trade in Endangered Species of Wild Fauna and Flora

COI - Cytochorme c oxidasde subunit 1

Conc. - Concentration

DNA - Deoxyribonucleic acid

FO - Frequency of Occurrence

H - Shannon's diversity index

HTS - High-throughput sequencing

IUCN - International Union for Conservation of Nature and Natural Resources

kg - Kilograms

km - Kilometres

mm - Millimetres

MOTUs - Molecular operational taxonomic units

mtDNA - Mitochondrial DNA

NCBI - National Centre for Biotechnology Information

ng - Nanograms

nM - Nanomolar

OFT - Optimal Foraging Theory

PCR – Polymerase Chain Reaction

pM - Picomolar

PSi - Proportional Similarity index

qPCR - Quantitative Polymerase Chain Reaction

rRNA - Ribosomal RNA

V_f - Final Volume

V_i - Initial Volume

W - Mann-Whitney U Test

µl – Microliters

Introduction

1.1 General Introduction

Understanding how wildlife communities work is a focal part of ecology and conservation. Community ecology studies aim to understand systems, including assessing and integrating information from spatial distributions, abundances, number and kind of species and the interaction among these in the same geographical area (Karanth *et al*., 2004; Kotler & Brown, 2007). Currently, the ongoing 6th mass extinction caused by climate change, habitat degradation and fragmentations, has been causing considerable impacts on ecosystems, reinforcing the need for studies relating to habitat and species biology including, for example, the trophic interactions among them (Barea-Azcón *et al*., 2007; Østby, 2019). This necessity is even more important in large carnivore species (order *Carnivora*), for which hunting and direct persecution due to conflicts with humans, magnify the already problematic situation (Gitari, 2018).

Usually, large mammalian carnivores are apex predators known to be on the top of the food chain and thus, controlling prey communities and creating cascading effects in their habitat (Atkins *et al*., 2019; Ripple *et al*., 2014; Havmøller *et al*., 2020a). These apex predators generally include species occurring at low densities, having large home ranges and movements, and with large body size (Ordiz *et al*., 2013). They are generally strong interactors and have significant effects on the structure and functioning of the habitats they occupy (Gitari, 2018; Monterroso *et al*., 2019: Ripple *et al*., 2014). They can regulate ecosystem functions through the effects of consumer-resource and competitive interactions that shape the abundance, diversity and distribution of other species (De Barba *et al*., 2014). Subsequently, information about the trophic interaction between large carnivores and their prey is crucial to understanding not only how species adapt to a rapidly changing world by, for instance, switching their diet, but also to evaluate the conservation efforts needed to preserve species diversity (Flather & Sieg, 2007; Østby, 2019).

1.2 Prey Availability

One of the main factors influencing consumer's population dynamics is prey availability (Østby, 2019; Ordiz *et al*., 2013). Prey species are heterogeneously distributed due to physical (e.g. landscape structure) and biological (inter- and intraspecific interactions) features (Watanabe & Takahashi, 2014). As a result, understanding how animals explore and exploit their food patches has further been a major objective in ecology (Watanabe & Takahashi, 2014). When population sizes are

growing towards the ecosystem's carrying capacity, the inter- and intraspecific competition for food, space and other resources tends to increase (Holt & Kimbrell, 2008; Østby, 2019). This effect generally affects large carnivore species, with a much higher impact since they are strongly limited by food resources (Karanth *et al*., 2004). Several studies have shown that there is a positive relationship between predator density and the biomass of their preferred prey and therefore, predator species density is closely related to the availability of food, making this an important and limiting factor for population growth (Athreya *et al*., 2016; Østby, 2019; Hayward & Kerley, 2008). Thus, in the absence of other factors, predator densities may be determined by available prey biomass (Chakrabarti *et al*., 2016). Alongside, prey density and biomass are influenced by their social structure and abundance (Husseman *et al*., 2003; Gitari, 2018; Hatton *et al*., 2015). Therefore, changes in the population density of a single prey have the potential to trigger demographic changes in predators' populations and initiate critical effects cascading over entire ecological communities (Wegge *et al*., 2009).

Prey abundance and landscape features are the two main factors that drive the resource selection by carnivores (Stephens & Krebs, 1986; Balme *et al*., 2007). The choice of feeding habitat for predators may be influenced by either where prey is most abundant (prey abundance hypothesis; Hopcraft *et al*., 2005) or where they are easier to catch (landscape hypothesis; Balme *et al*., 2007). For instance, lions, *Panthera leo*, in the Serengeti, preferred to hunt in areas with good cover rather than in the ones with high prey density (Hopcraft *et al*., 2005). Likewise, wolves *(Canis lupus*) selected areas where prey detectability and encounter rates were higher to kill their primary prey easily (Hebblewhite *et al*., 2005). Balme *et al*. (2007) showed similar results in leopards (*Panthera pardus*). In contrast, several studies (Palomares *et al*., 2001; Spong, 2002) have shown that some predators select their feeding habitat according to the prey abundance present in that location (Balme *et al*., 2007). The theory behind both hypotheses is identical: predators need to select their hunting habitats where 'energy requirements can be met at minimum energy expenditure and, simultaneously, pose the least risk' (Balme *et al*., 2007).

1.3 Foraging theory

A proper comprehension of foraging behaviour is crucial to understand population dynamics and community ecology and, in the 1960s, the interest in foraging theories developed quickly among ecologists (Ydenberg *et al*., 2008; Østby, 2019). Foraging theories consist of models describing animal foraging behaviour and how foraging transforms the ecosystem of the animals that are looking for prey (Østby, 2019). Individuals may change their foraging strategies and decisions according to different

situations to meet energetic, nutritional and avoidance (of other animals) requirements (Newman, 2007; Østby, 2019). Through foraging, individuals are also influenced by the community they belong to (Østby, 2019). One of the main foraging theories, the Optimal Foraging Theory (OFT), postulates that predators adjust their foraging behaviour to maximize the net rate of energy intake per unit effort (Pyke, 2019). Hence, optimally foraging carnivores are expected to choose prey that provides higher benefits in terms of net biomass intake while reducing the handling costs of chasing and subduing prey, as well as minimizing injury risks (Mukherjee & Heithaus, 2013; Chakrabarti *et al*., 2016).

When moving across the landscape, a predator may find a variety of different prey with different intrinsic energic and nutritional values (Yahnke, 2016). Predators choose to prey upon a trade-off between the energy spent to hunt and the energy gained from consumption (Figure 1; Keim *et al*., 2011). Thereupon, they are expected to preferentially use habitats where they can maximize the probability of encountering and successfully hunting those prey (Keim *et al*., 2011; Hatton *et al*., 2015). Different individuals may have different perceptions of the intrinsic value of different prey, which can lead to different individual decisions regarding prey hunting. This decision-making process can result in dietary individual specialization (Balme *et al*., 2020).

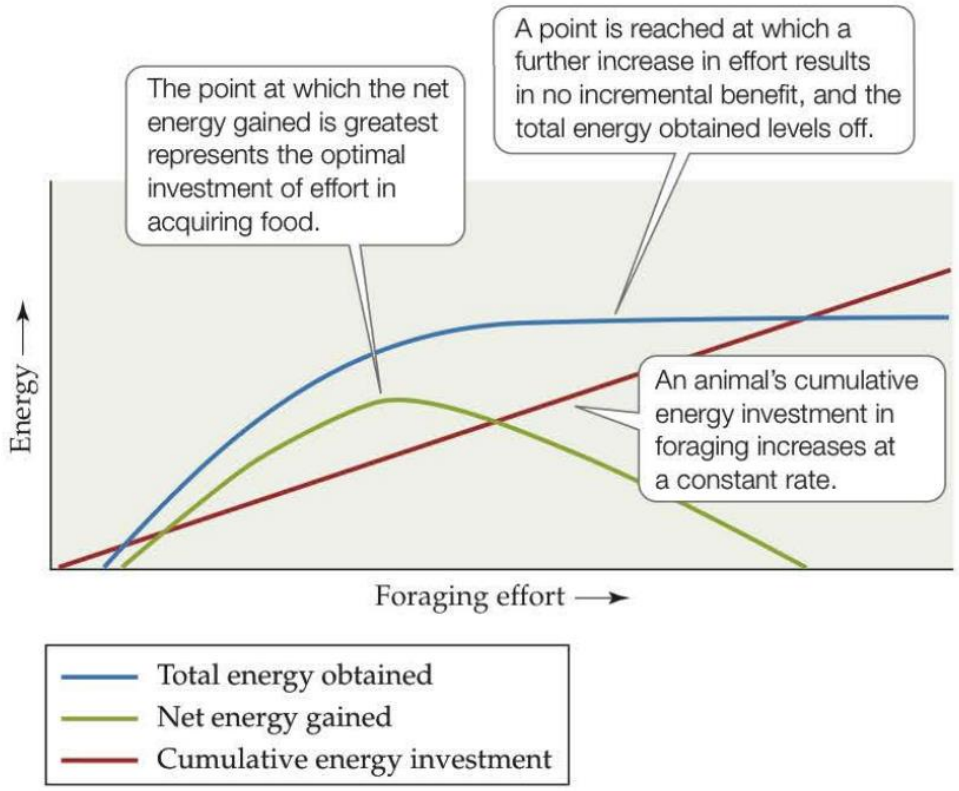


Figure 1 - Conceptual Model of Optimal Foraging – The net energy that predators gain from foraging (green line) equals the total energy obtained from food (blue line) minus the total energy invested in obtaining that food (red line). Figure retrieved from Bowman *et al*. (2017).

1.4 Individual Dietary Specialization

Within wildlife populations, individual animals can considerably vary in the use of their resources ("niche variation"; Araújo *et al*., 2011). These variations were historically attributed to ontogenetic niche changes or ecological sexual dimorphism (Schoener, 1986; Araújo *et al*., 2011). Nonetheless, resource-use variation is observed even among individuals of the same age and sex (Araújo *et al*., 2011). Individual specialization, i.e., individuals exploring and exploiting a subset of the population's niche, has been shown to be common in many vertebrate and invertebrate species (Araújo *et al*., 2011; Bolnick *et al*., 2003; Balme *et al*., 2020). It provides the basis for adaptation and speciation, and it can also have critical implications for community and population dynamics (Barabás & D'Andrea, 2016; Bolnick *et al*., 2011; Forsman & Wennersten, 2016; Balme *et al*., 2020). This subject has been increasingly studied as evolutionary biologists and population ecologists seek to understand the elements causing and maintaining individual specialization (Balme *et al*., 2020).

The optimal foraging theory considers that the degree of individual dietary specialization is influenced by both phenotypic constraints and environmental conditions (Robertson *et al*., 2015; Balme *et al*., 2020). The former comprises attributes that affect the individual's ability to access and exploit specific dietary items, such as age- or sex-related differences in size, experience, or dominance of consumers (Votier *et al*., 2017; Balme *et al*., 2020). The latter is related to ecological factors that influence the amount and types of food available for individuals (Balme *et al*., 2020). Several studies have concluded that resource limitation, which induces intraspecific competition, promotes individual dietary specialization (Robertson *et al*., 2015; Svanbäck & Bolnick, 2005; Tinker *et al*., 2008; Balme *et al*., 2020). Consequently, as the abundance of prey decreases, predators may choose to switch to alternative prey items not hunted by their conspecific or interspecific competitors (Balme *et al*., 2020). An alternative factor that can induce specialization is the ecological opportunity, or resource diversity, since it expands the spectrum of foraging options available to individuals, allowing dietary niches to diverge (Balme *et al*., 2020). However, empirical support remains limited.

Even though knowledge on the extent and possible causes for individual specialization has improved, it is still a matter of strong debate among ecologists (Robertson *et al*., 2015; Balme *et al*., 2020; Otterbeck *et al*., 2015; Voigt *et al*., 2018; Araújo *et al*., 2011). Specialists are usually assumed to be more efficient foragers than generalists, and subsequently, individual specialization is maintained in populations by disruptive selection, or, in other words, specialists have a fitness advantage over generalists (Bolnick *et al*., 2003). Notwithstanding, this is not frequently supported by

empirical evidence. Some studies have shown that the degree of individual specialization and body condition (Robertson *et al*., 2015), survival (Darimont *et al*., 2007) and/or reproductive success (Authier *et al*., 2012; Otterbeck *et al*., 2015) are correlated, while others fail to find a relationship between the degree of individual specialization and fitness components (Van de Pol *et al*., 2010; Woo *et al*, 2008). In a recent study, Balme *et al*. (2020) revealed that both generalists and specialists can coexist in the same population and that dietary specialization appeared to be disadvantageous, at least for male individuals.

Nonetheless, studies estimating the contribution of different prey in the diet and how predators switch between their resources, are important for evaluating predators' impact in wildlife communities and its conservation implications for maintaining species interactions, which are essential for ecosystem functioning (De Barba *et al*., 2014, Havmøller *et al*., 2020b). Fundamentally, what a predator hunts in a particular environment depend upon what is available (either in quantity and quality) and accessible (Piñol *et al*., 2014), and therefore, studies on this subject need accurate knowledge of the predator's diet to estimate the predator effects on ecosystems (Monterroso *et al*., 2019). Dietary data on large carnivore populations can be collected using various approaches, the most used being non-invasive methodologies.

1.5 Non-invasive Methods

Studying carnivore populations and their interaction with the environment often depend on the availability of information about parameters such as population size, diet, demography, gene flow and population structure (Mhuulu, 2015). Different methodologies can be implemented to access ecological parameters, namely traditional methods based on live captures, for example, telemetry or tissue sample collection (Kelly *et al*., 2012; Mhuulu, 2015). However, these methods are difficult to be implemented on rare, elusive, and nocturnal species like large carnivores as they require handling the animals which may cause stress, disturbance and potential injuries raising ethical and moral concerns (Kelly *et al*., 2012; Mhuulu, 2015). Additionally, large carnivores generally occur at low densities and the sampling effort to collect a reasonable number of samples is high, often making surveys unfeasible (Geyle *et al*., 2020). Contrastingly, the use of non-invasive approaches allows researchers to study ecological parameters like survival rates or population sizes, historic and current events of movement across habitats, genetic identification of individuals and many intrinsic aspects like carnivore stress, without catching, handling or even seeing the animals (Beja-Pereira *et al*., 2009; Mhuulu, 2015).

Non-invasive approaches include methods like remote camera trapping or collection of available biological material (e.g., scats, hairs, regurgitations, blood, urine, chewed food, spoor tracking; Karanth & Nichols, 1998; Perez *et al*., 2006; Boitani & Powell, 2012; Mhuulu, 2015) that provide the sources for different analysis. Genetic non-invasive methodologies have become commonly applied in carnivore studies (Kitano *et al*., 2007; Chaves *et al*., 2012; Mhuulu, 2015), providing accurate and reliable information about carnivore populations, including individual movements, population size, gene flow, demographic histories, diet and even assessing mating systems and behavioural ecology (Mhuulu, 2015; Monterroso *et al*., 2019). When compared to other genetic non-invasive samples, faeces arise as the easiest to collect and provide more information about biological parameters (Beja-Pereira *et al*., 2009).

1.6 Diet assessment

Diet examinations of carnivores provide key information to estimate biological parameters ranging from trophic niche breadth to trophic specialisation or prey selection. According to Mata *et al*. (2019), diet studies usually try to address three types of questions regarding animal populations: 1) what the number of different prey species consumed is (dietary diversity); 2) what the identity of the prey species consumed is (dietary composition); and 3) what the proportion either in number, biomass or energetic content of each prey species to the diet of a given predator is. Nonetheless, these studies have also been used to estimate required resources for carnivores (Carbone *et al*. 1999), evaluate the potential effects of habitat and community changes on carnivores (Novaro *et al*., 2000; Phillips *et al*., 2007; Larson *et al*., 2015), study interspecific competition (Jones & Barmuta, 1998; Fedriani *et al*., 2000), predict human-wildlife conflict and effects on prey populations (Risbey *et al*., 1999; Bagchi & Mishra, 2006; Morin *et al*., 2016).

Data on carnivores' diet obtained through direct observation of feeding events are generally limited (Lumetsberger *et al*., 2017) considering their scarce, elusive and nocturnal nature, especially on dense habitats where they often resign (Nilsen *et al*., 2012; Havmøller *et al*., 2020b). As such, alternative methods to study the dietary patterns of carnivores have emerged, including examination of the stomach or scat contents, or the analysis of stable isotopes from animal hair and fatty acid signatures (Putman, 1984; Iverson *et al*., 2004; Dalerum & Angerbjörn, 2005; Azevedo *et al*., 2006; Monterroso *et al*., 2019; Morin *et al*., 2019). For mammalian carnivores, in particular, the most traditional non-invasive approach to study diet is the identification of prey present in scats by its undigested remains (Chaves *et al*., 2012; Monterroso *et al*., 2019). It has been considered the simplest and least expensive method, and therefore the most commonly used (Morin *et al*., 2016). However, the use of these traditional technics often relies on

taxonomic expertise, and it is time-consuming (Kocher *et al*., 2017), as it demands the recovery of structures that can be identified using guides or catalogues (Bowen and Iverson, 2013). Thereby, when using morphological methods, the successful identification of prey relies upon a variety of factors that should be taken into consideration such as the prey size, the durability of identifiable parts, the degree of digestion that prey has been subjected to before the examination, the parts of prey that are ingested by the predator and the level of trituration and mastication by the predator (Kasper *et al*., 2004; Egeter *et al*., 2019).

1.6.1 DNA-Based Techniques

Over the last decade, many diet studies of mammal species, including herbivores, bats, carnivores, rodents, and other taxa, have been conducted successfully using molecular techniques. Approaches using region-specific primers for mitochondrial (mtDNA) or nuclear DNA and Sanger sequencing technology that were developed to identify species, known as DNA Barcoding, have been successfully applied to identify the DNA of prey and predator species in diet remains (Soininen *et al*., 2009; Zeale *et al*., 2011; Galan *et al*., 2012; Shehzad *et al*., 2012; Latinne *et al*., 2014: Gillet *et al*., 2015; Monterroso *et al*., 2019).

Molecular diet analysis yields a better taxonomic resolution and conceivably discriminate more taxa, contrasted with the conventional techniques (Soininen *et al*., 2009; Gillet *et al*., 2015). Indeed, several studies comparing the efficiency of both approaches have found that DNA-based methods typically improve prey detection either by detecting prey more frequently or by detecting a higher number of different prey species (Boyer *et al*., 2011; Carreon-Martinez *et al*., 2011; Casper *et al*., 2007a; Casper *et al*., 2007b; Purcell *et al*., 2004; Soininen *et al*., 2009; Tollit *et al*., 2009; Gillet *et al*., 2015).

The utilization of non-invasive molecular methodologies highlighted that identification of carnivore species based only on scat morphology is prone to errors, particularly in regions with sympatric predators of equal size (Morin *et al*., 2016). This can lead to biases in ecological inferences specifically under- or overestimating the distribution or density (Brassea-Pérez *et al*., 2019; Martínez-Gutiérrez *et al*., 2015, Morin *et al*., 2016, Weiskopf *et al*., 2016; Monterroso *et al*., 2019). For instance, Morin *et al*. (2016) found that reliably distinguishing scats of carnivore species of similar size like a coyote (*Canis latrans*) and bobcat (*Lynx rufus*) is challenging. The same happens with several other sympatric carnivores that have similar sizes (Farrell *et al*., 2000, Davison *et al*., 2002; Morin *et al*., 2016) which increases the likelihood of occurring false negatives or positives (Monterroso *et al*., 2019)

Molecular dietary studies can be performed using different sources of DNA specifically stomach contents, regurgitations or faeces that can be collected from the animal itself or the environment (King *et al*., 2008; Alberdi *et al*., 2019; Beja-Pereira *et al*., 2009). The choice of the type of samples is normally driven by biological attributes of the target species (e.g. elusiveness), practical (e.g. ethical limitations) (Alberdi *et al*., 2019) and logistical constraints (e.g. budget or available equipment).

The classical approach of DNA barcoding on diet analysis is based on prey-specific primers, meaning that the analysis must be repeated for every potential, often unknown and different prey (Piñol *et al*., 2014), which is often a limitation, especially in generalist predators. As such, the emergence of high-throughput sequencing (HTS) technologies, which allow the identification of many species at the same time, greatly improved the efficiency of molecular approaches on diet analysis (De Barba *et al*., 2017; Monterroso *et al*., 2019). The HTS approaches have three main advantages over traditional DNA barcoding methods in diet studies: first, it permits the simultaneous identification of the predator and approximately all the prey species present in a single, low concentrated and degraded DNA sample by using universal primers (Piñol *et al*., 2014; Monterroso *et al*., 2019); secondly, it allows parallel processing and sequencing of numerous DNA fragments from various samples for a minimal price (Alberdi *et al*., 2019; Shokralla *et al*., 2012; Taberlet *et al*., 2018; Hawlitschek *et al*., 2018; Østby, 2019); finally, enables the taxonomic identification of prey species more accurately (Østby, 2019). The use of HTS to the simultaneous identification of multiple sources of DNA is known as DNA metabarcoding and has been gaining great popularity among ecologists (Figure 2).

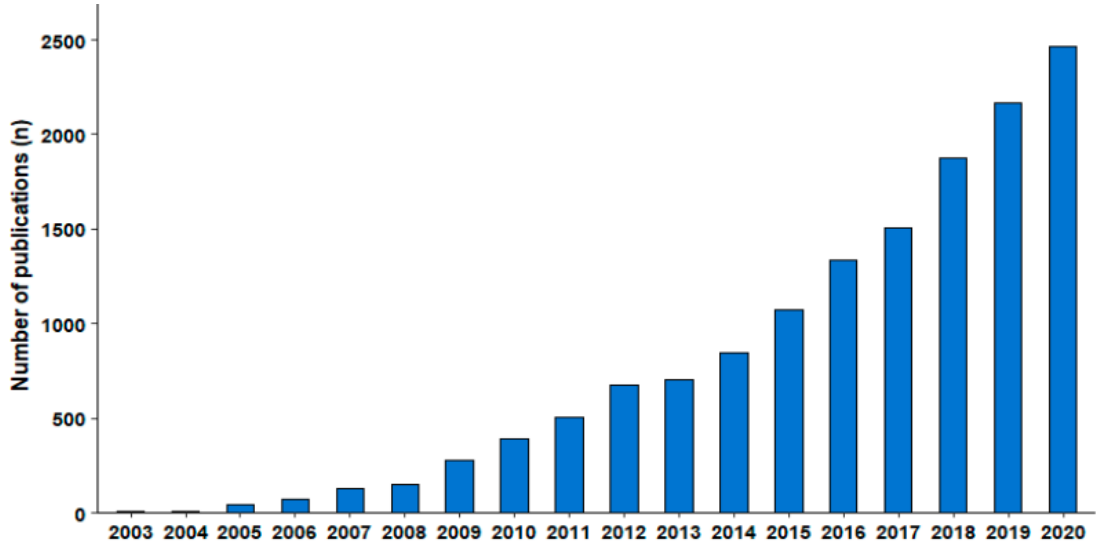


Figure 2. DNA barcoding and DNA metabarcoding publications through time – Publications per year registered in Scopus®, containing "DNA barcod*" or "DNA metabarcod*" in the title, abstract, or key words. Figure retrieved from Grant *et al*. (2021).

DNA metabarcoding revolutionized the diet studies and is currently highly applied, predominantly because: it hypothetically empowers solely the sequencing of the DNA of interest; numerous samples can be handled and sequenced in parallel; it does not need exorbitant profundity of sequencing; and it is not computationally heavy, implying that many, if not all, information preparing steps can be made on a personal computer (Alberdi *et al*., 2019). Additionally, metabarcoding allows the genetic analysis of pooled samples from different sources by indexing each samples' DNA with unique combinations of index primers before pooling the samples (Dobrovolny *et al*., 2019; Østby *et al*., 2019). After sequencing, when tagged with those unique primer combinations, the samples can be demultiplexed with the intention to allocate the DNA sequences detected in the sequencer to its original sample (Taberlet *et al*., 2018).

One of the critical steps in a metabarcoding study is to decide which marker region and primers to use (Alberdi *et al*., 2017). These choices are usually guided by a DNA reference database and taxonomic coverage, and animal studies have usually relied on markers within the barcode region of the COI gene (Alberdi *et al*., 2017). However, this marker is frequently not the best choice for metabarcoding (Deagle *et al*., 2014; Kocher *et al*., 2017) because this coding gene has high mutation rates at the third codon position, which difficult to find a good conserved primer binding site, potentially leading to significant amplification bias or detection failures when DNA from different sources is amplified simultaneously (Bitanyi *et al*., 2011; Deagle *et al*., 2014; Kocher *et al*., 2017). In seek of minimizing these amplification biases while maximizing the divergence between taxa, researchers developed specific software, like "ECOPrimers" to find suitable metabarcodes and associated primers (Riaz *et al*., 2011; Kocher *et al*., 2017; Dobrovolny *et al*., 2019), as well as "ecoPCR" to test their characteristics (Taberlet *et al*., 2012; Bellemain *et al*., 2010; Ficetola *et al*., 2010). Currently, most animal metabarcoding markers that were developed using this approach are located within the mitochondrial ribosomal RNA (rRNA) genes like the 12S rRNA and 16S rRNA genes (Riaz *et al*., 2011; Clarke *et al*., 2014; Deagle *et al*., 2014; Kocher *et al*., 2017). These genes have highly conserved regions due to the secondary structure of their sequences, in which primers can be designed, before and after the variable regions (loops) that allow interspecific distinction (Kocher *et al*., 2017). Despite this, there is no real consensus about which metabarcoding markers to use, leading to scarce reference libraries (Pompanon & Samadi, 2015; Kocher *et al*., 2017).

Overall, metabarcoding is a technique in which taxonomic informative DNA markers (barcodes) are targeted for PCR amplification and sequencing (Alberdi *et al*., 2019). The workflow of metabarcoding by HTS comprises the extraction of DNA from samples, the PCR amplification of a specific region and data generation by an HTS

sequencing device (Taberlet *et al*., 2018). Thenceforth, computer programs such as OBITools (Boyer *et al*., 2016) are used to organize the sequences into clusters of identical sequences and comparing them to a reference sequence taxonomic database where all the clusters of sequences generated are assigned to specific taxa (Taberlet *et al*., 2018; Østby, 2019). These taxonomic reference databases can be obtained either by retrieving sequences from online databases such as GenBank or BOLD (Barcode of Life Data System) or by developing their own reference databases by amplifying the target regions from the species of interest. Each sequence from a metabarcoding analysis is assigned to its closest match in the reference library (Østby, 2019).

1.6.1.1 Metabarcoding Challenges

DNA approaches used for diet analysis do not require the presence of undigested prey remains and therefore mitigate one of the greatest limitations of morphological diet studies. However, different challenges need to be accounted for when using these approaches. One of the most relevant is the occurrence of false positives, i.e., when a sequence is misidentified as a sequence of a prey item, and false negatives, i.e., when a sequence of a prey is misidentified as something else. This may happen for different reasons, including PCR and sequencing errors, primer misannealing or even the inexistence of high-quality taxonomic reference databases (Taberlet *et al*., 2012).

DNA metabarcoding is a PCR-based method, which requires a DNA amplification step that has several limitations (Taberlet *et al*., 2012). Firstly, the degradation of DNA templates can generate errors during PCR amplification (substitutions and insertions/deletions; Taberlet *et al*., 2012). DNA from faeces has generally low quality and suffers degradation through time. Thus, when faeces are not fresh or have been in contact with water, errors during DNA amplification may be more frequent, which limits these studies to relatively fresh scats (Tollit *et al*., 2009; Gosselin *et al*., 2017; Morin *et al*., 2019; Gillet *et al*., 2015). Secondly, PCR relies on primers for amplification, and metabarcoding studies require finding a suitable metabarcode that holds a short variable DNA region, appropriate to target a specific taxonomic group, and that is flanked by two highly conserved regions to attach universal primers (Taberlet *et al*., 2012).

A further limitation of DNA metabarcoding approaches is the possible detection of items that were not intentionally consumed, such as prey of the consumed prey, known as secondary predation (Hardy *et al*., 2017; Sheppard *et al*., 2005). However, the identification of a secondary prey may be mitigated by double ingestion, and only recent consumed prey may be detected in a significant frequency (Brassea-Pérez *et al*., 2019). Additionally, environmental contamination of faeces by different DNA sources (e.g.

pollen, eggs) before sample collection can possibly be detected on metabarcoding diet analysis (Pompanon *et al*., 2012; Monterroso *et al*., 2019).

The genetic identification of species is generally based on mtDNA due to its larger number of copies, lack of introns, limited exposure to recombination, haploid mode of inheritance and high rate of evolution when contrasted with nuclear DNA (Aksöyek *et al*., 2017; Hebert *et al*., 2003; Chaves *et al*., 2012; Monterroso *et al*., 2019). However, mitochondrial genes can have nuclear copies and be co-amplified during PCR, eventually resulting in the misidentification of species. The mtDNA introgression in closely related species is also a limitation because it can cause species misidentifications (Monterroso *et al*., 2019).

A common drawback of metabarcoding diet analysis is the high abundance of predator DNA in the DNA sample, which can mask prey DNA detections (Vestheim & Jarman, 2008; Egeter *et al*., 2019). To surpass this limitation, predator-specific blocking primers are being commonly used, hampering or limiting the amplification of the predator's DNA (Vestheim & Jarman, 2008; Piñol *et al*., 2014). Nevertheless, blocking primers can, to some extent, also block the amplification of prey species' DNA, especially if prey and predators are taxonomically related (e.g. felid feeding on another felid) (Piñol *et al*., 2014). Furthermore, species- or group-specific primers can be used, focusing on the prey of interest, as opposed to using universal primers that likely also co-amplify DNA from the predator species (Egeter *et al*., 2019). However, this is not the appropriate approach if prey are taxonomically diverse, as it would difficult the design of suitable primers (Shehzad *et al*., 2012).

An additional challenge on molecular diet analysis is the estimation of prey biomass or the number of prey individuals in a sample (Gillet *et al*., 2015; Monterroso *et al*., 2019). Although some studies have shown a relationship between biomass consumed by predators and prey weight (see Deagle *et al*., 2019 and Chakrabarti *et al*., 2016), it is still unclear how the relative size of prey may impact the dispersion of prey DNA inside a scat, or how scat size may impact detection probabilities for prey DNA under distinct sampling designs such as taking subsamples versus homogenization of the entire scat (Morin *et al*., 2019). Additionally, the different types of tissues that are digested by predators can influence the amount of DNA present in a scat (Alberdi *et al*., 2019). Because of this, metabarcoding studies usually quantify diet in terms of frequency of occurrence of a given prey, although it does not reflect the true intake of different prey species regarding numbers, biomass, or energy (Mata *et al*., 2019). Nonetheless, promising advances are being made in this study area (Carreon-Martinez *et al*., 2014; Bowles *et al*., 2011; Deagle *et al*., 2019; Deagle *et al*., 2013; Deagle & Tollit, 2007; Thomas *et al*., 2016; Thomas *et al*., 2014; Gillet *et al*., 2015).

Metabarcoding approaches have not been regularly applied to mammalian carnivore diet analysis, despite the falling in costs and the increasing power of sequencing and analysis strategies (Monterroso *et al*., 2019). Regardless, recent studies delineate their relevance in carnivore research (Shehzad *et al*., 2012; Dawson *et al*., 2016; Xiong *et al*., 2017). In this manner, it is currently conceivable to depend completely on non-invasive DNA and HTS technology to assess carnivore species and individual, in addition to all prey items, from DNA extracted from its scats (Monterroso *et al*., 2019).

1.7 Model species – *Panthera pardus*

Leopards (*Panthera pardus*) are the most widely distributed wild cat in the world, with a distribution range extending from South Africa, throughout the Middle East and Southeast Asia to the Russian Far East (Figure 4; Balme *et al*., 2007; Hayward *et al*., 2006). They are primarily distributed throughout sub-Saharan Africa where large and continuous populations still exist at densities ranging from 2.49 to 11.11 individuals/100km^2 (Stein *et al*., 2011; Balme *et al*., 2010; Hayssen & Stein, 2013). Smaller isolated populations occur in Morocco, south-eastern Algeria, the eastern desert of Egypt and Niger (Ray *et al*., 2005; Hayssen & Stein, 2013).

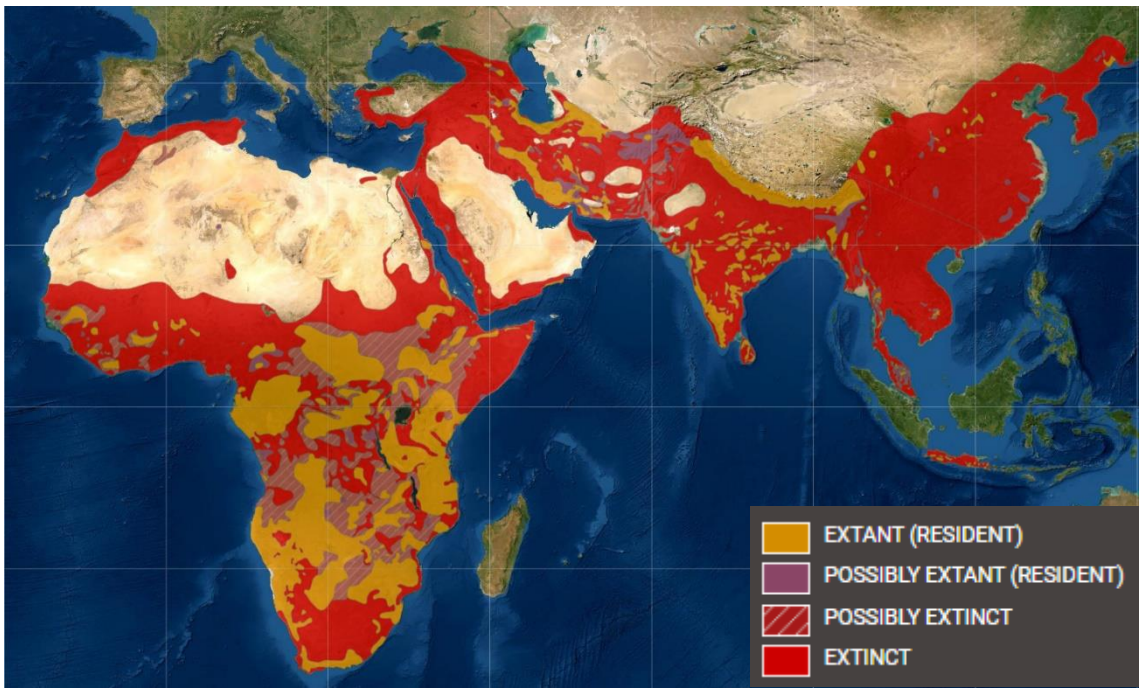


Figure 4. Distribution of the leopard, *Panthera pardus* – Map showing the distribution of leopards around the world. Figure retrieved from IUCN (2021).

This large spotted cat (Figure 5) can be distinguished from the other species of the *Panthera* genus (and other species from the Felidae Family) by their characteristic

dark, rosette spots which make them suitable for species and individual identification (Figure 4). This species is sexually dimorphic with males varying from 30 to almost 63 kg and females from 21 to 54 kg (Hayssen & Stein, 2013). They require between 1.6 and 4.9 kg of meat per day to maintain their body mass and consequently, they kill around 40 to 60 prey per year, depending on their location (Hayward *et al*., 2006).

Figure 5 – Leopard Figure. Leopard photograph retrieved from ©GP232

Leopards exhibit a highly adaptable hunting and feeding behaviour. The species has been recorded to prey on approximately 200 species of vertebrates (Hunter *et al*., 2013) of which 110 are mammals (Hayward *et al*., 2006; Shehzad *et al.,* 2015) and is considered a dietary generalist (Balme *et al*., 2020). Leopard's diet varies considerably between different studies depending on habitat, prey availability, vegetation cover available and competitors (Hayssen & Stein, 2013; Havmøller *et al*., 2020a). These components of the landscape influence the way leopards use and move in their home ranges, which are generally smaller where prey availability is high and concealing cover is available (Hayssen & Stein, 2013).

In general, leopards are driven by their opportunity to catch and maintain possession of their prey. They prefer to prey on small to medium body size prey (10-40

kg) that can minimize the rate of kleptoparasitism and competition with other large carnivores such as lions, spotted hyenas (*Crocuta Crocuta*), and tigers (*Panthera tigris*) (Hayward *et al*., 2006; Hayward & Kerley, 2008; Karanth *et al*., 2004; Karanth & Sunquist, 1995; Havmøller *et al*., 2020a). Although the preference for prey ranges from 10 to 40 kg, in the absence of these and other large competitors, they may feed on larger prey (Hayward *et al*., 2006; Stein, 2008; Hayssen & Stein, 2013). However, the costs and risk they take when trying to kill a larger prey (> 150 kg) may restrict their diet (Scheepers & Gilchrist 1991; Hayssen & Stein, 2013). In the absence of preferred or larger prey, they can feed on a variety of smaller species including rodents, birds, reptiles, and even other smaller carnivores (Figure 6; Hayward *et al*., 2006; Hayssen & Stein, 2013). Females with cubs usually increase their foraging effort by killing smaller prey (Bothma and Coertze, 2004; Hayssen & Stein, 2013). The most frequently prey taken by African leopards are the impala, the common duiker, the steenbok, the common warthog and the bushpig (Hayward *et al*., 2006; Searle *et al*., 2020).

Figure 6. Photographic evidence of hare predation – Leopard moving with a prey probably killed by him.. Photograph taken from 1 of the cameras installed in the Bicuar National Park.

Their hunting strategy highly relies on cover to hide their approach, which will thus increase the success of killing their prey (Balme *et al*., 2007). In open habitat, they generally hunt alone at night where their camouflage permits them to stalk closer to their target before initiation a short sprint of up to 120 meters (Hayward *et al*., 2006). In

rainforests, leopards hunt diurnally with crepuscular peaks by ambushing their prey at fruiting trees and along trails instead of stalking (Hayward *et al*., 2006).

Leopards are strongly territorial. However, males that have large home ranges tend to share the external areas of their ranges with neighbour individuals, maintaining the core areas of their territories of exclusive use (Bothma & Le Riche, 1984; Steyn & Funston, 2009; Hayssen & Stein, 2013; Balme *et al*., 2020). Females have smaller home ranges than males and, therefore, their territories are usually overlapped by larger territories of solitary males (Hayward *et al*., 2006). Additionally, females share their territories with their young until males reach 12-18 months of age and disperse (Bailey, 1993; Hayssen & Stein, 2013). Young females can often take part in their mother's range (Bailey, 1993; Hayssen & Stein, 2013**)**.

After a kill, leopards will either eat their prey quickly if it is small or hide it to safely consume it (Bothma & Le Riche, 1984; Bailey, 1993; Hayssen & Stein, 2013). Leopards may drag their kills a few hundred meters to specific kinds of trees or bushes with a necessary stature, trunk thickness, and foliage thickness (Bothma & Le Riche, 1984; Bailey, 1993; Hayssen & Stein, 2013). Leopards can drag a carcass with more than their double body mass using only the mouth and hoist the carcass into tress where other large competitors cannot reach (Scheepers & Gilchrist, 1991; Hayssen & Stein, 2013).

Typically, leopards can exhibit nocturnal activity with their peaks during dawn and dusk, or diurnal with peak activity during late morning and late afternoon – early evening (Bailey, 1993; Hayssen & Stein, 2013). Individuals feeding on large prey may stay in a specific location for several days (Bothma & Le Riche, 1984; Bailey, 1993), but they usually move around their territory over 7 – 10 days depending on their home range size (Hayssen & Stein, 2013). Besides, human disturbances can influence the way leopards use and explore their territories as well as their activity patterns (Marker & Dickman, 2005; Hayssen & Stein, 2013)

Although they are viewed as one of the most adaptable of the world's large felid species due to their foraging strategies, *Panthera pardus* is listed as “Vulnerable” in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (Searle *et al*., 2020). This species’ main threats are habitat loss and fragmentation, prey depletion and direct persecution by humans which can lead to population isolation and species decline, challenging their continued existence (Figure 7; Di Minin *et al*., 2016; Wolf & Ripple, 2016; Inskip & Zimmermann, 2009; Jacobson *et al.,* 2016; Havmøller *et al*., 2020; Searle *et al*., 2020). In Africa, due to pressures carried out by humans, leopards have disappeared from more than 35% of their historical-geographical range (Jacobson *et al.,* 2016; Havmøller *et al*., 2020b).

QUICK FACTS:

Population Size:	Unknown, decreasing
Range:	48-67% of historic range lost
IUCN Red List:	Vulnerable (2020)
CITES:	Appendix I (since 1975)
International Trade:	7,155 leopard trophies traded internationally from 2009-2018
Threats:	Habitat fragmentation, reduced prey base, conflict with livestock and game farming, excessive killing for ceremonial use of skins and poorly managed trophy hunting.

Figure 7. Quick facts about leopards – Facts about population size and trend, lost range, IUCN Red List designation, CITES listing, International Trade and Main Threats of leopards. Figure retrieved from Humane Society International (2021).

1.8 Objectives and Working Hypothesis

Angola holds an extraordinary level of biodiversity that has been under-studied when contrasted with other southern African countries (Butler *et al*., 2019). This thesis intends to enhance the understanding of Angolan biodiversity and the ecological knowledge of key-species for conservation by leveraging the availability of leopard scats collected in the Bicuar National Park in the framework of CIBIO ongoing projects. For that, we implement a metabarcoding approach with the main objective of examining the diet of leopards in the Bicuar National Park. With this in mind, and with the purpose of producing useful and additional information about the diet of an African top predator, the following aims were defined:

1) Characterize leopards' diet in terms of prey species diversity and frequency of occurrence;

2) Understand whether there are significant dietary differences between sexes in this leopard population;

3) Examine whether individual dietary specialization is present in this under-studied leopard population.

In accordance with the aims of this thesis we hypothesise that:

1) Leopards in Bicuar have a less diverse diet when compared to other studies possibly due to lower prey diversity present in the study area. Additionally, we expect leopards to focus their diet mainly on medium-sized ungulates since they are usually the most preferred prey. Also, we expect to detect other less common prey related to opportunistic events;

2) Given that male leopards are larger than females, we hypothesise that males have a smaller dietary niche breadth as they probably can subdue larger prey and therefore, males can fulfil their energetic demands with less predatory events. Conversely, females' smaller size can be advantageous when hunting smaller prey which can increase their dietary diversity when compared to males;

3) Within the third aim of this thesis, we hypothesise that both generalist and specialist individuals will be found in this leopard population.

Methodology

2.1 Study Area

Angola holds an extraordinary level of biodiversity that has been under-studied when contrasted with other southern African countries (Butler *et al*., 2019). It represents a significant biogeographic transitional zone that connects the tropical rainforests of Congo to the deserts of Namibia (Leaché *et al*., 2014), and the Angolan Great Escarpment fills in as a buffer zone between the drier coastal lowlands to the more humid interior plateau (Crawford-Cabral, 1991). These characteristics raise high levels of endemism across taxa, making Angola a significant territory for additional exploration and conservation efforts (Butler *et al*., 2019).

Occupying 7900 km^2, the Bicuar National Park (BNP) is the only protected area within the borders of Huíla province. It is located in the south-central region of the province, bordered by the Cunene River to the east from Capelongo to Mulondo (Figure 3; Huntley, 1971; Overton *et al*., 2017). In 1938, the BNP was created as a hunting reserve due to the abundance of game species in the area, and in 1964 was elevated to a national park (Overton *et al*., 2020). However, most of BNP's mammalian diversity was hunted during the civil war that started in 1975 and ended in 2002 (Huntley & Ferrand, 2019; Overton *et al*., 2020). Since then, some of the remaining fauna has been able to recover within the boundaries of the park, with some game species such as elephants, elands, and roans persisting, although illegal poaching still remains a problem (Butler *et al*., 2019).

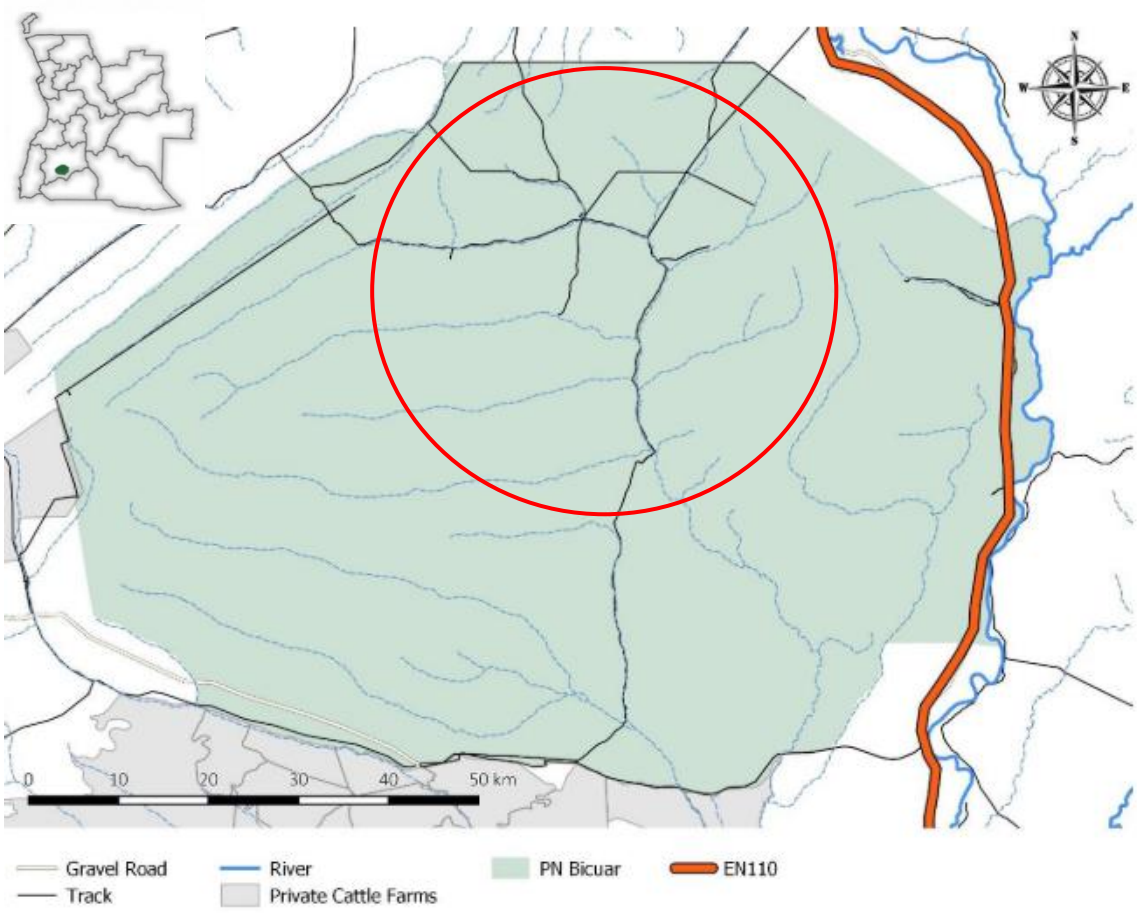


Figure 3. Map of Bicuar National Park – Location of the Bicuar National Park in Angola (small inset) and map of the park itself (large inset). The main conservation area of the park is situated where the density of tracks is higher (Zone within the red circumference). Figure retrieved from Parque Nacional do Bicuar – Plano de Gestão 2020 – 2029 (2020).

The landscape of the park is roughly flat with a sandy substrate, and with numerous low depressions draining eastwards and nearly no exposed rock in many spots (Overton *et al*., 2017; Butler *et al*., 2019). Generally, the actual climate bears a nearer similarity to wetter parts of the Kalahari basin in eastern Angola and Namibia than it does io western Huíla Province (Butler *et al*., 2019). Altitude ranges from 1150 to 1500 meters above sea level, annual precipitation ranges from 1200 mm in the northeast part of the park to 800 mm in the south and mean average temperature ranging from 20 to 22 ºC (Huntley, 1971; Overton *et al*., 2017). BNP is inserted in the Zambezian phytogeography region with a regular pattern of open grassland plains and a variety of savannah, woodland and ticket communities (Huntley, 1971: Overton *et al*., 2017). The habitats present in Bicuar are a transition between Angola miombo woodland and the drier Baikiaea woodlands of south-central Africa with open grasslands (Ron, 2015; Monterroso *et al*., 2020).

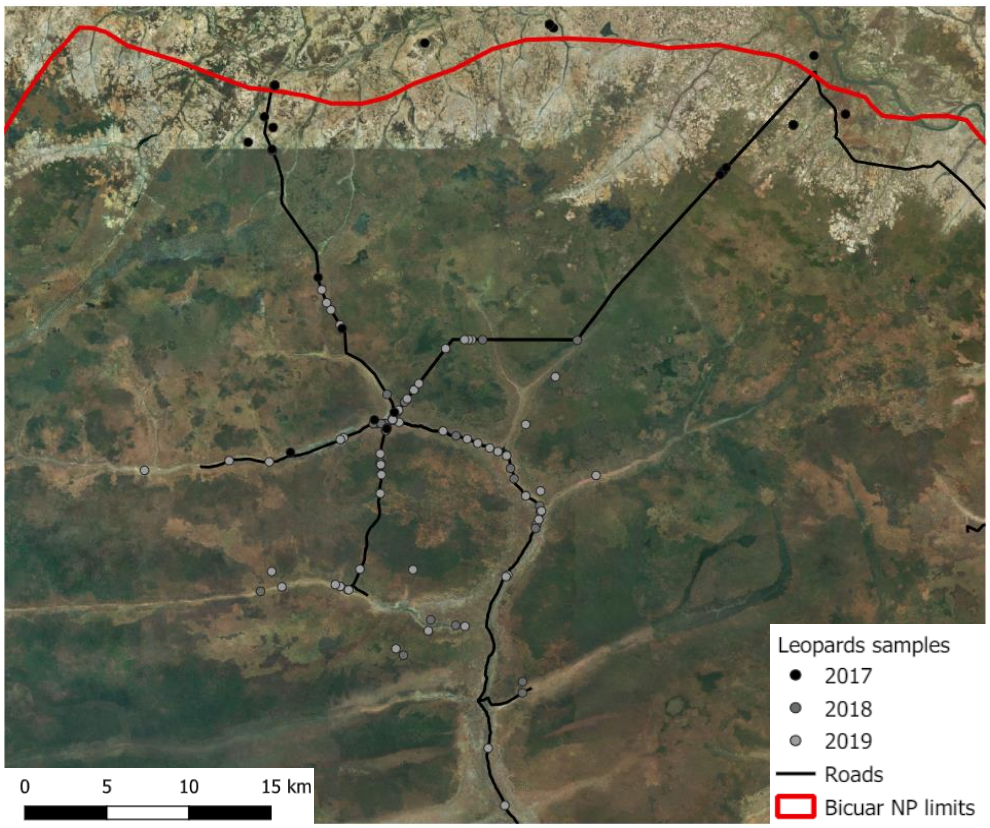
In the past, several large and medium-sized mammals were reported including: “side-striped jackal (*Canis adustus*), African wild dog (*Lycaon pictus*), spotted hyaena (*Crocuta Crocuta*), lion (*Panthera leo*), leopard (*Panthera pardus*), cheetah (*Acinonyx jubatus*), elephant (*Loxodonta africana*), Burchell’s zebra (*Equus quagga burchellii*), common warthog (*Phacochoerus africanus*), bushpig (*Potamochoerus larvatus*), eland (*Taurotragus oryx*), vervet monkeys (*Chlorocebus sp.*), greater kudu (*Tragelaphus strepsiceros*), bushbuck (*Tragelaphus scriptus*), roan antelope (*Hippotragus equinus*), reedbuck (*Redunca arudinum*), blue wildebeest (*Connochaetes taurinus*), impala (*Aepyceros melampus*), common duiker (*Sylvicapra grimmia*), oribi (*Ourebia ourebi*), steenbok (*Raphicerus campestris*), Cape buffalo (*Syncerus caffer*), giraffe (*Giraffa camelopardalis*), black-faced Impala (*Aepyceros melampus petersi*), aardwolf (*Proteles cristata*), bat-eared fox (*Otocyon megalotis*), Defassa waterbuck (*Kobus ellipsiprymnus*), Krik’s dik-dik (*Madoqua kirkii*), thick-tailed greater galago (*Otolemur crassicaudatus*) and Cape pangolin (*Smutsia temminckii*)” where the most abundant species were elephant, Burchell’s zebra, common warthog, eland, roan antelope and wildebeest. (Huntley, 1973; Huntley, 1974). Overton *et al*. (2017), Balme *et al*. (2020) and Rocha *et al.* (2019), identified leopards and spotted hyenas as the most common large carnivores at Bicuar with both having good densities in the core area of the park and being widespread through the external areas of the BNP. Similarly, further ten species of small carnivores were identified including wild dogs, serval (*Leptailurus serval)*, caracal (*Caracal caracal*), black-backed jackal (*Canis mesomelas*), wildcat (*Felis silvestris*), miombo genet (*Genetta angolensis)*, honey badger (*Mellivora capensis*), bat-eared fox, Cape fox (*Vulpes chama*), aardwolf, Selous mongoose (*Paracynictis selousi*) and swamp mongoose (*Atilax paludinosus*), where serval, caracal, black-backed jackal and wild cat were the most common (Monterroso *et al*., 2020; Overton *et al*., 2020; Overton *et al*., 2017; Rocha et al., 2019). Despite not detecting side-striped jackal and only detecting 1 civet once (*Civettictis civetta;* Rocha *et al.,* 2019*)*, both species may also be present at Bicuar (Overton *et al*., 2017). Additional animals namely elephants and seven species of ungulates were recently observed. These include the common duiker, steenbok, roan antelope, bushpig, common warthog, greater kudu and eland, where the most common species were common duiker and roan antelope (Overton *et al*., 2017). It is important to notice that sightings were considerably lower in the outer area of Bicuar (Overton *et al*., 2017).

The currently threats that endangered species living in Bicuar are related with logging and fire which are associated with agriculture practices in the park perimeter (Overton *et al*., 2017). Alongside, poaching for bushmeat, either for subsistence or commercial use, has-been identified as a major threat (Ron, 2015; Overton *et al*., 2017).

Despite this, while there are no resident human communities inside the park area, they show a significant impact on its margins through invasion and unsustainable agricultural practices that limit the assess to water in the dry season (Overton *et al*., 2017). The main human-animal conflicts occur essentially in the south and east of the park with human-elephant conflicts, and in the north and east of the park which is related with the access of cattle to grazing inside the park area (Ron, 2015; Overton *et al*., 2017).

2.2 Scat Collection // DNA Extractions // Individual Identification

Under the scope of CIBIO ongoing research projects in the Bicuar National Park since 2017, scat surveys are being conducted twice a year in the park, mostly centred in an area of 364 km^2 where an intense camera-trapping survey is also ongoing (Figure 8). Scats have been sent to CIBIO laboratories where DNA was extracted and identified for species by CIBIO staff following routine procedures for non-invasive samples (Table 1_SI; Monterroso *et al*., 2013). Additionally, samples identified as leopard were screened for a set of 23 microsatellite markers originally developed in the domestic cat (Menotti-Raymond *et al*., 1999), and shown to be polymorphic in leopards (e.g., Mondol *et al*. 2009; Uphyrkina *et al*., 2001). Leopard DNA and individual identifications were already available to be used in the framework of this thesis.



2.3 Selection of DNA fragment and primers

Considering the available literature of metabarcoding diet studies targeting vertebrate species (Nowak *et al*., 2014; Riaz *et al*., 2011; De Barba *et al*., 2014; Gitari, 2018; Egeter *et al*., 2019; Shehzad *et al*., 2012; Xiong *et al*., 2017; Havmøller *et al*., 2020b; Kartzinel *et al*., 2015; Brassea-Pérez *et al*., 2019), we selected the 12S rRNA gene to be used within this study given its high rates of successful species identifications when compared to other markers like the COI (Nowak *et al*., 2014; Kocher *et al*., 2017). The chosen primers were described by Riaz *et al*. (2011), and their sequences are available in Table 1.

Table 1. Sequences of the 12S primers used – The sequences for the 12S-V5 primers were retrieved from Riaz *et al*. (2011).

Primer Name	Primer Sequences	
	Forward	Reverse
12S-V5	TAGAACAGGCTCCTCTAG	TTAGATACCCCACTATGC

2.4 12S rRNA gene and primers testing

A total of 24 potential leopard prey species (which DNA was available for extraction, amplification and sequences) were tested for the amplification of the 12S fragment using the selected primers (Table 2).

Table 2. ***List of Prey Tested for 12S rRNA gene and 12S-V5 Primers –*** List of prey species of whose DNA was extracted, amplified, and sequenced for the testing of the 12S gene and 12S-V5 primers. This list contains the species scientific name, common name, and respective sample name. * Samples kindly offered by Professor Bettine van Vuuren (Centre for Ecological Genomics & Wildlife Conservation, University of Johannesburg); [&]samples available at CIBIO tissue collection.

List of Prey Tested for 12S rRNA gene and 12S-V5 Primers

Scientific Name	Common Name	Sample Name
Ungulates		
*Sylvicapra grimmia**	Common Duiker	UJ2630
*Potamochoerus larvatus**	Bushpig	UJ1866
*Equus sp.**	Equine	UJ1868
*Phacochoerus africanus**	Common Warthog	UJ1869
*Taurotragus oryx**	Common Eland	UJ1888
*Oryx gazella**	Gemsbok	UJ1891

*Aepycerus melampus**	Impala	UJ1964
Hippotragus equinus[&]	Roan Antelope	26 HN150
Sylvicapra grimmia[&]	Common Duiker	A5842
Bubalus sp.[&]	Bufalo	C7097
Hippotragus niger[&]	Sable Antelope	HN565
Lagomorpha		
Lepus saxatilis[&]	Scrub Hare	Sax1a
Lepus saxatilis[&]	Scrub Hare	Sax4a
Carnivores		
*Otocyon megalotis**	Bat-eared Fox	UJ2418
*Canis mesomelas**	Black-backed Jackal	UJ2744
*Canis adustus**	Side-striped Jackal	UJ1850
*Caracal caracal**	Caracal	UJ2833
*Ictonyx striatus**	Striped Polecat	UJ2627
*Mellivora capensis**	Honey Badger	UJ1862
*Galerella pulverulenta**	Cape Grey Mongoose	UJ2742
Canis mesomelas[&]	Black-backed Jackal	6595
Felis silvestris lybica[&]	African Wildcat	Fli307
Genetta genetta[&]	Common Genet	8
Other		
*Manis temminckii**	Ground Pangolin	UJ1865

DNA extraction was performed for the 24 samples using the EasySpin® Column Protocol. DNA extractions were checked by electrophoresis on 0.8% agarose gels. A series of 1:2 and 1:3 dilutions were performed (Table 2_SI) in samples that had higher quantities of DNA visible by the brightness of the bands after electrophoresis.

Subsequently, PCR tests were conducted for each sample. All pre-PCR procedures were performed in a dedicated laboratory under sterile conditions and positive air pressure. Negative controls were included throughout the entire laboratory procedures to monitor for DNA contamination. PCR mixes were prepared according to the number of samples (Q) and including a negative control. This mix was prepared using [5 x (Q+2)] µL of QIAGEN PCR Mix (containing the polymerase and all the reagents necessary for its successful work), [0.3 x (Q+2)] µL of each primer (12S-V5 Forward and Reverse diluted 1:10) and adding H_2O until reaching [9 x (Q+2)] µL of final volume (Table 3_SI). After this step, 9uL of the global mix was distributed by PCR tubes and different amounts of DNA, depending on the evaluation of DNA concentration in each sample, were added to the respective tube (Table 2_SI).

The thermocycling conditions were set to an initial step of pre-denaturation of 1 cycle at 95ºC for 15 minutes; then 40 cycles of denaturing, annealing and elongation at 95 ºC, 47 ºC and 72 ºC respectively, during 30, 20 and 30 seconds, respectively; and final elongation at 60 ºC for 10 minutes (Table 4_SI). PCR products were checked by electrophoresis using 2% agarose gels (Figure 1_SI and Figure 2_SI).

Successful PCR amplicons were cleaned by ExoSap enzymatic reaction in the thermocycler for 30 minutes at 37 ºC and 85 ºC (15 minutes each) and prepared for Sanger sequencing using the BigDye chemistry (Applied Biosystems). Sequencing mix included [0.5 x (Q+3)] µL of one primer (either reverse or forward), [0.5 x (Q+3)] µL of TRR mix (containing DNA polymerase, dNTPs, ddNTPs associated with fluorochromes), [1 x (Q+3)] µL of TRR Buffer and H_2O until reaching [9 x (Q+3)] µL. To each 9 µL of sequencing mix was added 1 µL of cleaned PCR amplicon (Table 5_SI). The sequencing reaction was performed using the same thermocycling conditions of PCR amplification. Sequencing products were then cleaned with Sephadex, dried at 95 ºC for 20 minutes and re-hydrated in 15 µL of formamide. Finally, sequencing products were separated by capillary electrophoresis in a ABI3130xl genetic analyser (Applied Biosystems). Sequences were visualised and cleaned using BioEdit v7.2.5 (Hall, 1999).

2.5 NGS Library Preparation of DNA Extracted from Leopard Scats

All pre-PCR procedures were performed in a dedicated laboratory used for the manipulation of low-quality DNA under sterile conditions and positive air pressure. Negative controls were included throughout the entire laboratory procedures to test for DNA contamination. To construct a dual-indexed DNA library using leopard DNA previously extracted, a two-step PCR approach was conducted following Illumina procedures as shown in Figure 9 (Bourlat *et al*., 2016). According to this procedure, two types of primers are needed: the first composed of 12S-V5 universal primers with Illumina overhangs adapters attached, and the second composed of an overlapping region to the Illumina overhang adapters, indexes and P5/P7 adapter sequences to enable the binding to the MiSeq flowcell.

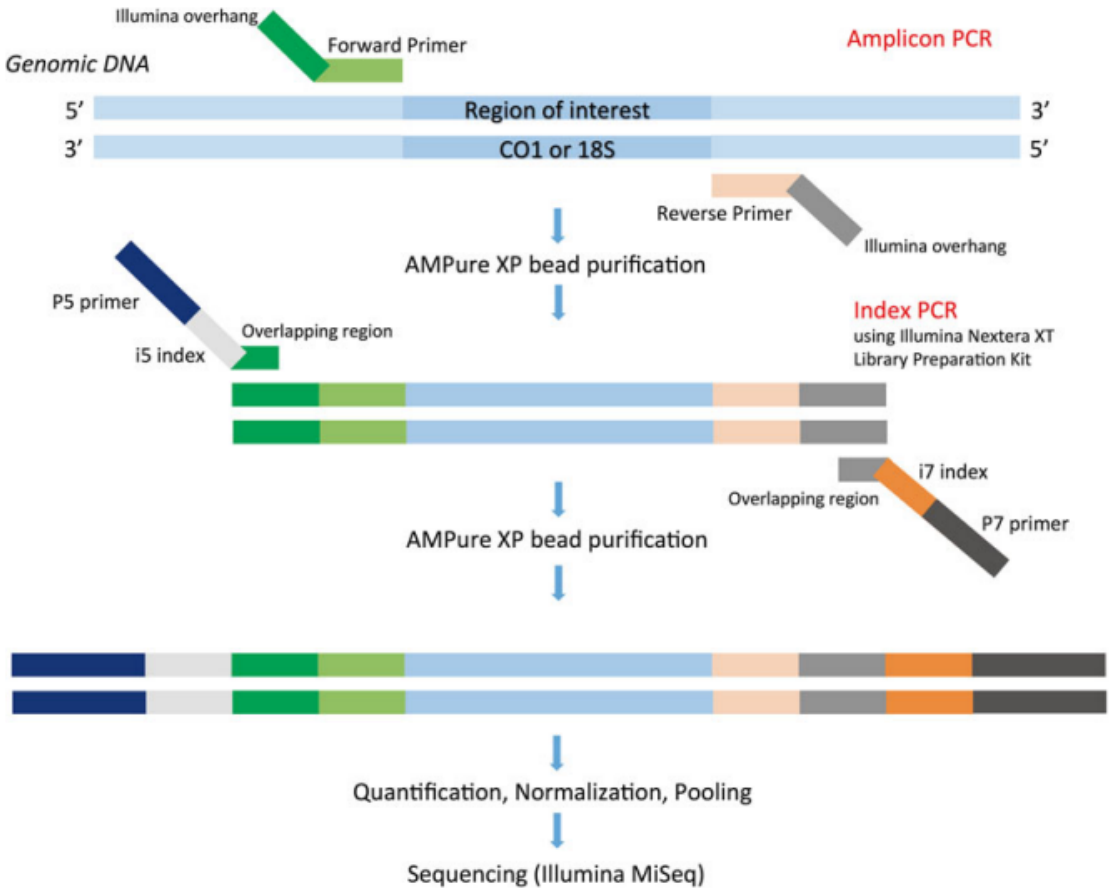


Figure 9. Schema for Illumina MiSeq library preparation using a two-step PCR approach – The first PCR uses amplicon-specific primers (in this case 12S specific primers) including Illumina adapter overhangs (Amplicon PCR). The second PCR allows the incorporation of Illumina index adapters i5 and i7 (Index PCR). Bead purifications are carried out after each step. Quantification, normalization, and pooling are carried out before sequencing on Illumina MiSeq. Figure retrieved from Bourlat *et al*. (2016).

During the first step of the PCR, the 12S fragment target was amplified using the first type of primers. To account for PCR stochasticity and monitor future sequencing errors (Alberdi *et al*., 2017), 2 replicates of this amplification were conducted. Each amplification plate had a negative and positive control (a *Felis s. lybica* sample used in sanger sequencing tests). The PCR followed the conditions optimized for the amplification of this fragment during Sanger sequencing tests. PCR amplicons were tested by electrophoresis using a 2% agarose gel prior to indexing. PCR products were diluted in 15 µL of water given the very successful amplification for all samples.

The index PCR, where P5/P7 Illumina adapters and indexes are incorporated into the PCR products of the first PCR step, was performed using 7µL of 2x Kapa HiFi Hot Start enzyme, 0.7 µL of both P7xx and P5xx indexes, 2.8 µL of H_2O and 2.8 µL of the PCR product from the first PCR (Table 6_SI). The thermal cycling conditions included an initial step of 95 ºC for 3 minutes, then 8 cycles of denaturing, annealing and elongation

at 95 ºC, 55 ºC and 72 ºC, each taking 30 seconds, a final step of 72 ºC for 5 minutes and hold at 10 ºC (Table 7_SI). To check the success of the Index PCR, amplicons from the Index PCR and the first PCR were run together in agarose gels at 2%. Since it was incorporated a small fragment (index) to the original first step PCR products, it is expected to observe a size increase for the amplicons from the Index PCR. This electrophoresis is also important to inform the size of the target fragment, which is important for the PCR Clean-up step that follows.

The PCR Clean-up step removes DNA fragments that do not correspond in size to the Index PCR products (free primers, primer-dimers, spurious DNA products from different sizes). Clean-up was conducted using AMPure XP Beads following the TruSeq DNA Nano protocol from Illumina. The beads were used in a ratio of 0.7x for ~10 µL of Index PCR products to remove DNA fragments with a size below 200 bp. After the clean-up step, the PCR products were once more checked by electrophoresis in 2% agarose gels to assess the success of cleaning procedures.

2.6 MiSeq Sequencing on Illumina

The DNA libraries were quantified using an Epoch spectrophotometer (BioTek Instruments) and were normalized to a final concentration of 20 nM in a pool with a final volume of 20 µL using the concentration value and the size of the Index PCR product (280 bp), and following

$$\left[Conc._{(ng/\mu L)}/660 \cdot 280\ bp\right] \cdot 10^6 = C_i\ nM$$

and

$$\left(C_f \cdot V_f / C_i\right)/N = V_i$$

with N being the total number of samples per replicate, including negative and positive controls.

Each pool containing all DNA libraries (1 pool per replicate) was quantified on a Qubit fluorometer (Invitrogen) (Figure 3_SI; Table 8_SI) and were pooled together in a single pool with a final concentration of 4 nM and a final volume of 20 µL (Table 9_SI). The final pool was run on a 2200 TapeStation (Agilent Technologies), following the D1000 protocol, to quality control of DNA concentration and size of the DNA libraries (Figure 4_SI; Table 10_SI).

The pool was then prepared to run on a MiSeq platform. It was denatured with NaOH and adjusted to a final concentration of 12 pM. The PhiX control library was diluted to the same concentration and mix to the pool to a 7% ratio for a final volume of 600 µL. This mixture was then denatured by heating and loaded in the flowcell of a MiSeq Reagent Kit v3 (150-cycle) for sequencing.

2.7 Data analysis

2.7.1 Creation of a reference database

The 12S sequences of potential prey species (Table 3) were downloaded from GenBank and aligned together with the prey sequences generated during amplification tests using MEGA-X (Kumar *et al*., 2018). All sequences were carefully examined for potential errors. Sequences with errors were eliminated from the alignment. Only one haplotype per species was kept in the final alignment. At last, a reference database with the aligned sequences from all potential prey species of leopards was generated for further use during analysis.

Table 3. ***List of Potential Leopards' Prey in Reference Database*** - List of potential prey species of leopards used to construct the reference database. This list contains the species scientific name, common name, and respective sample name or GenBank code. * GenBank Accession number. [A] samples kindly offered by Professor Bettine van Vuuren (Centre for Ecological Genomics & Wildlife Conservation, University of Johannesburg); [&]samples available at CIBIO tissue collection.

Potential Leopards' Prey in Reference Database

Potential Prey	Common Name	Source of sequence
Ungulates		
Aepyceros melampus	Impala	This study (A5842 [&]) This study (UJ1964 [A])
Equus sp.	Equines	This study (UJ1868 [A])
Hippotragus equinus	Roan Antelope	This study (26 HN150 [&]) NC_020712 *
Hippotragus niger	Sable Antelope	AY670653 * This study (HN565 [&])
Raphicerus campestris	Steenbok	JN632693 *
Orycteropus afer	Aardvark	NC_002078 *
Oryx gazella	Gemsbok	This study (UJ1891 [A])
Phacochoerus africanus	Common Warthog	AJ010817 * DQ409327 * This study (UJ1869 [A]) GQ338939 *
Potamochoerus larvatus	Bushpig	GQ338939 * This study (UJ1866 [A])
Sylvicapra grimmia	Common Duiker	AF154269 * JN632701 * This study (UJ2630 [A])
Syncerus caffer	African Buffalo	KJ192644 * AF091688 *

		JQ235547 * KJ192643 * This study (C7097 &)
Tragelaphus oryx	Common Eland	JN632704 * This study (UJ1888 A)
Tragelaphus strepsiceros	Greater Kudu	AY667208 * AF091696 *
Carnivores		
Canis adustus	Side-striped Jackal	KJ192459 * This study (UJ1850 A) This study (6595 &)
Canis lupus familiaris	Domestic Dog	MN181405 *
Canis mesomelas	Black-backed Jackal	This study (UJ2744 A)
Caracal caracal	Caracal	This study (UJ2833 A) NC_028306 *
Civettictis civetta	African Civet	NC_033378 *
Felis catus	Domestic Cat	KM224283 * KM224282 *
Felis silvestris	Wild Cat	KP202275 * NC_028310 * KJ192507 * This study (Fli307 &)
Galerella pulverulenta	Cape Gray Mongoose	This study (UJ2742 A)
Galerella sanguinea	Common Slender Mongoose	NC_053972 *
Genetta genetta	Common Genet	NC_053968 * This study (8 &)
Ichneumia albicauda	White-tailed Mongoose	MW257213 *
Ictonyx striatus	Striped Polecat	EF472284 * U78334 * This study (UJ2628 A)
Leptailurus serval	Serval	KJ192509 * NC_028316 *
Mellivora capensis	Honey Badger	This study (UJ1862 A)
Otocyon megalotis	Bat-eared Fox	NC_036369 * This study (UJ2418 A)
Primates		
Chlorocebus cynosuros	Malbrouck Monkey	KU682693 * NC_024933 *
Chlorocebus pygerythrus	Vervet Monkey	MT481926 * KU682698 *
Others		
Hystrix africaeaustralis	Cape Porcupine	U12448 *

Lepus saxatilis	Scrub Hare	This study (Sax1a [&]) AY292704 *
Manis temminckii	Ground Pangolin	MF536686 * MF536685 * This study (UJ1865 [A])
Pedetes capensis	South African Springhare	AJ010817 * HE983623 *

2.7.2 OBITools Filtering

The dietary data analysis was performed using a bioinformatic pipeline denominated OBITools (Boyer *et al*., 2016). Before analysis, sequences were downloaded as fastq format files from the Illumina platform and uploaded to an internal server where OBITools is installed.

Firstly, forward and reverse reads were assembled and aligned running the "*python 1.run_illuminapairedend.py.fastq*" script. Thereafter, the aligned reads with <50 alignment score were filtered and discarded running "*python 2.run_aligned.py*" script. This step generated two types of files: the *paired.bad.fastq* which are sequences with <50 alignment score; and the *paired.good.fastq* which are sequences that passed this filtering step.

Thereupon, a demultiplexing per sample step was conducted. For this step, a *ngsfilter* file was created in a samplesheet using samples' information - names and replicates, both primers and the well where the pool was loaded. The samplesheet was converted into a *.txt* file to be uploaded and work properly on the server. Using the script "*python 3.split_ngsfilter.py ngs_filter.txt*", the samplesheet was distributed per well/index. Then each read was assigned to each sample running "*python 4.run_ngsfilter.py*". This script generates two types of files: *ngs.bad.fastq* which contain reads that were not assigned to any sample; and *ngs.good.fastq* containing all reads assigned to each sample. Finally, all *ngs.good.fastq* files were converted in a single file running "*cat *.ngs.good.fastq > all.ngs.good.fastq*".

Several filtering processes were further completed. First, unaligned sequences were removed using the "*obigrep"* script. Then, identical sequences were clustered into unique sequences using the "*obiuniq*" script. Sequences with less than 10 reads were removed from the data using the "*obigrep"* script once more. Sequences with a size smaller or longer than 95 and 110 bp, respectively, were removed from the dataset running again the "*obigrep*" script. Finally, the sequences were cleaned for PCR/sequencing errors running the "*obiclean*" code and the results were merged in a single tab-delimited file running "*obiannotate*".

Sequences were then Blasted against the reference database for 12S sequences of leopards and potential prey species running "*blastn*" code. Results from the blast were processed in R. The taxonomic category given by the blast depended on the probability of identity (pident) of the sequences regarding the reference database. Sequence with at least 98% of pident, would be assigned to a Species. Sequences from 98 to 95% were assigned to Genus and 95 to 92% to a Family. Assignments to Order/Class/Phylum/Kingdom were made only if >= 80% pident.

Following this, a single samplesheet was generated with all filtered sequences attributed to a specific taxonomic level. Furthermore, this file has the number of reads of a specific sequence (MOTUs – molecular operational taxonomic units) per sample which allowed to identify the main species present in that sample. As the negative controls had few reads due to some human- and possibly cross- contaminations, it was decided that MOTUs with less than 50 reads would be discarded. Then, all sequences were individually checked for taxonomic identification using the NCBI BLAST.

After this individual check, human and other contaminations from Iberian species (mainly Iberian small rodents that appeared due to contamination from CIBIO-InBio facilities) were removed from the sequence results. MOTUs that were not correctly attributed to a specific species were fixed. Finally, the results were organised in presence of species per sample. However, since *Sylvicapra grimmia* presented continuously high numbers of reads, sequences present in samples, where this species was detected with less than 150 reads, were also discarded to avoid miss readings from cross-contaminations from one sample to another. Species present in a sample with human contamination were similarly removed. This restrictive PCR replicate filtering was executed due to high certainty of correct taxonomic identifications being needed, despite decreasing the detected diversity (Alberdi *et al*., 2017). Finally, the frequency of occurrence (FO) was calculated as the number of times a diet category was present in a sample, over the number of scat samples with any prey identification for the predator.

2.8. Leopard Diet

The leopards' diet was analysed regarding the different species detected in each sample by calculating their frequency of occurrence (FO). This was calculated as the number of scats positive for a given prey item over the number of scat samples with any prey identification for the predator. Therefore, samples not containing any prey identification were discarded which correspond to 50 samples.

Then, to characterize the dietary diversity the Shannon's diversity index (*H*) was calculated (Joseph *et al*., 2007). This diversity index has been popular and combines two factors: the number of species (richness) and their relative abundance. Nonetheless,

when the value of this index approaches 0, it means that the predator feeds on only 1 prey species, and as the value grows, the higher the diversity of prey in the predator's diet. Therefore, *H* is low under strong dominance of one single species and higher as the number of species increases, especially if they are equally distributed (Thukral, 2017). However, before characterizing the dietary diversity, and to account for different sample sizes and their effect on trophic niche diversity, a bootstrapping resampling analysis was performed for 500 bootstrap samples (calculated with the Shannon's diversity index), in order to determine whether the sample size was adequate to describe the diet of the predator. When the difference between the dietary diversity of a given number of samples and the total number of samples of the overall dietary diversity is consistently lower than 5%, we reached the asymptotic value corresponding to the minimum number of samples representative of the overall dietary diversity.

2.9. Dietary Diversity Between Males and Females

Only scats for which we achieved the identification and sex of individual leopards were used. To characterize the dietary diversity of male and female leopards, the Shannon's diversity index (*H*) was calculated (Joseph *et al*., 2007). Because dietary diversity of both sexes did not follow a normal distribution, a Mann-Whitney U Test (W) was conducted to check if differences were significant (MacFarland & Yates, 2016). To estimate the degree of niche overlap between males and females' diets, the Pianka's index was calculated (Pianka, 1974). Niche overlap is referred as the partial or complete sharing of resources or other ecological factors (in this case diet) between two or more species (Eklöf & Allesina, 2012). This index varies from 0 (no diet overlap) to 1 (complete overlap in diet). All these analyses were conducted in R and the indices were calculated using the vegan R package (Oksanen *et al*., 2020).

2.10 Quantifying individual specialization

For this quantification, only samples where it was possible to identify the different individuals were used. Several different indices can be used to quantify the individual specialization in a given species (see Bolnick *et al*. (2002) and Araújo *et al*. (2008)). Hence, to assess whether leopard individuals within the BNP population are specialists or not, the R-package RInSp was used (Zaccarelli *et al*., 2013). This package calculates ecological parameters of within-population niche variation based on data on individuals' resource use (Zaccarelli *et al*., 2013). It implements many ecological niche metrics to measure individual and population niche width to measure individual specialization such as the proportional similarity index (PS_i) (Zaccarelli *et al*., 2013). For some of these

indices, Monte Carlo re-sampling procedures for testing significance are provided (Zaccarelli *et al*., 2013).

The workflow was based on a recent study conducted by Balme *et al*. (2020). As different indices tend to consistently yield very similar results when applied to the same data (Bolnick *et al*., 2002), we quantified the extent of overlap between an individual *i*'s diet and overall population diet using the PS_i (Bolnick *et al*., 2002; Robertson *et al*., 2015; Balme *et al*., 2020). This index measures individual specialization based on the average pairwise overlap of the niche distribution of individuals and population. Generalists (that exhibit extensive overlap with the population) will have their PS_i scores tending to 1, while specialists (that exhibit little overlap with the population) will have their PS_i scores tending to 0 (Balme *et al*., 2020). The population-wide prevalence of individual specialization (IS) - the degree of intrapopulation specialization - was calculated by averaging the PS_i scores for all individuals (Bolnick *et al*., 2002; Balme *et al*., 2020).

Results

From 2017-2019, a total of 140 leopard scat samples were collected from the BNP for DNA extraction, and species and individual identification. The DNA from these 140 samples was available and has been used in this thesis. 46 scats were collected in 2017, 28 in 2018, and 66 in 2019. In general, 100 were collected in the dry season and 40 in the wet season. Additionally, only 69 (49%) of these samples were individually identified, resulting in 14 different leopards in 140 samples (mean of 10 samples per individual), of which 8 (57%) were females and 6 (43%) were males.

3.1 Taxonomic Resolution

After sequencing and initial filtering steps, a total of 3,121,078 reads passed the filtering process corresponding to 1390 different MOTUs. MOTUs with less than 50 reads were discarded to avoid possible sequencing errors. After this step, a total of 3,064,400 reads were maintained corresponding to 516 different MOTUs. Of these MOTUs, 47 were assigned to leopard with a total of 2,082,901 (68%) reads, and 64 were assigned to *Homo sapiens* or to Iberian endemic species and were discarded. The remaining 405 different MOTUs corresponded to 914,134 reads and were used for the assessment of the taxonomic resolution of diet species. From these, 85.1% of reads were determined to the Order level, 70.5% to the Family level, 50.4% to the Genus level and 42.6% to the Species level. 14.9% of reads were not identified.

The average raw output per sample after initial filtering was 21,888 reads, with a minimum and maximum of 425 and 127,177 reads, respectively. However, the average number of leopard reads per sample was 14,877 with a minimum and maximum of 116 and 119,968 reads, respectively. Furthermore, after assessing the number of reads present in the negative controls ($N \leq 50$), it was established that diet items with the same or a smaller number of reads would not be considered. Additionally, diet items were only considered when observed in both replicates with more than 50 reads each. After these filtering steps, 50 samples, and the corresponding 261 MOTUs which were only detected in those samples, were discarded from the analysis. The remaining 90 samples were represented by a total of 638,500 prey reads corresponding to 144 MOTUs. These MOTUs were converted to 16 prey taxa identified in the diet of leopard, of which 15 were identified up to the species level. A maximum of 2 prey items were identified within the same faecal sample (N=9), whereas 81 samples (90%) had only DNA of one prey. The results of the different filtering steps are present in Figure 10.

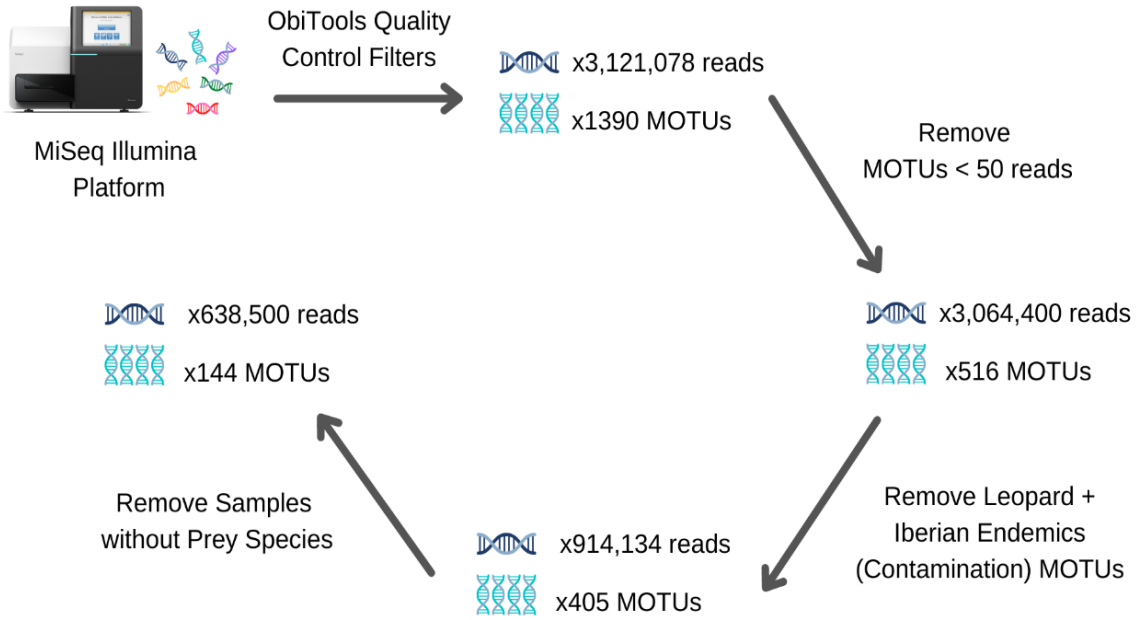


Figure 10. – Results of filtering steps performed–Results of the filtering steps performed from MiSeq sequencing until the detection of prey. Each row indicates a filtering step.

3.2 Leopard Diet

Sixteen different prey taxa were identified from 90 leopard faecal samples in BNP (Table 3) and there were significant differences in leopards diet in the different years and seasons. The species detected in the leopard diet were from 5 ungulates - the common duiker, greater kudu, roan antelope, bushpig and common warthog -, 5 carnivores - the wild cat, honey badger, common genet, slender mongoose (*Galerella sanguinea*) and white-tailed mongoose (*Ichneumia albicauda*) -, one primate - the malbrouck (*Chlorocebus cynosuros*) -, one rodent - the cape porcupine (*Hystrix africaeaustralis*) -, and four other taxa comprehending 3 birds - the black-chested snake eagle (*Circateus pectoralis),* red-crested bustard (*Lophotis ruficrista),* crested francolin (*Francolinus sephaena*) - and one reptile - the python (Python sp.; Table 4).

Table 4. – List of Prey Detected in Leopard Diet – This list contains the group and scientific name of the species, the number of reads in the final filter process and the number of times they were detected for the 90 samples. Weight assessed in Kingdon, 2015.

Group and Scientific Name	Number of Reads	Counts of Occurrence	Biomass (Kg)
Ungulates	412,042	78	
Sylvicapra grimmia	373,910	62	10 - 26
Tragelaphus strepsiceros	9,355	5	120 - 315
Hippotragus equinus	9,042	1	223 - 300
Potamochoerus larvatus	13,969	9	45 - 150

Phacochoerus africanus	5,766	1	45 - 150
Carnivores	109,318	9	
Felis silvestris	264	1	2.4 – 6.5
Mellivora capensis	4,610	1	5.2 – 14.5
Genetta genetta	70,781	3	1.3 – 2.3
Galerella sanguinea	29,106	1	0.35 - 0.80
Ichneumia albicauda	4,557	3	2 – 5.2
Primates	79,494	6	
Chlorocebus cynosuros	79,494	6	3.4 - 8
Rodents	25,670	2	
Hystrix africaeaustralis	25,670	2	10 - 24
Others	11,976	4	
Circateus pectoralis	9,362	1	1.2 – 2.3
Lophotis ruficrista	1,042	1	0.4 – 0.68
Francolinus sephaena	1,332	1	0.22 – 0.42
Python sp.	240	1	30 – 60

The common duiker was the predominant species, presenting a frequency of occurrence of 68.9%, followed by the bushpig (FO = 10.0%), the malbrouck (FO = 6.7%) and the greater kudu (FO = 5.6%). The remaining species were represented in the samples with a FO < 5.0%, with 9 species only appearing in a single faecal sample each (Figure 11a). Overall, ungulates dominated the diet of leopards in BNP appearing in 86.7% of the faeces, followed by carnivores (FO = 10.0%), primates (FO = 6.7%), birds (FO = 3.3%), rodents (FO = 2.2%) and reptiles (FO = 1.1%) (Figure 11b).

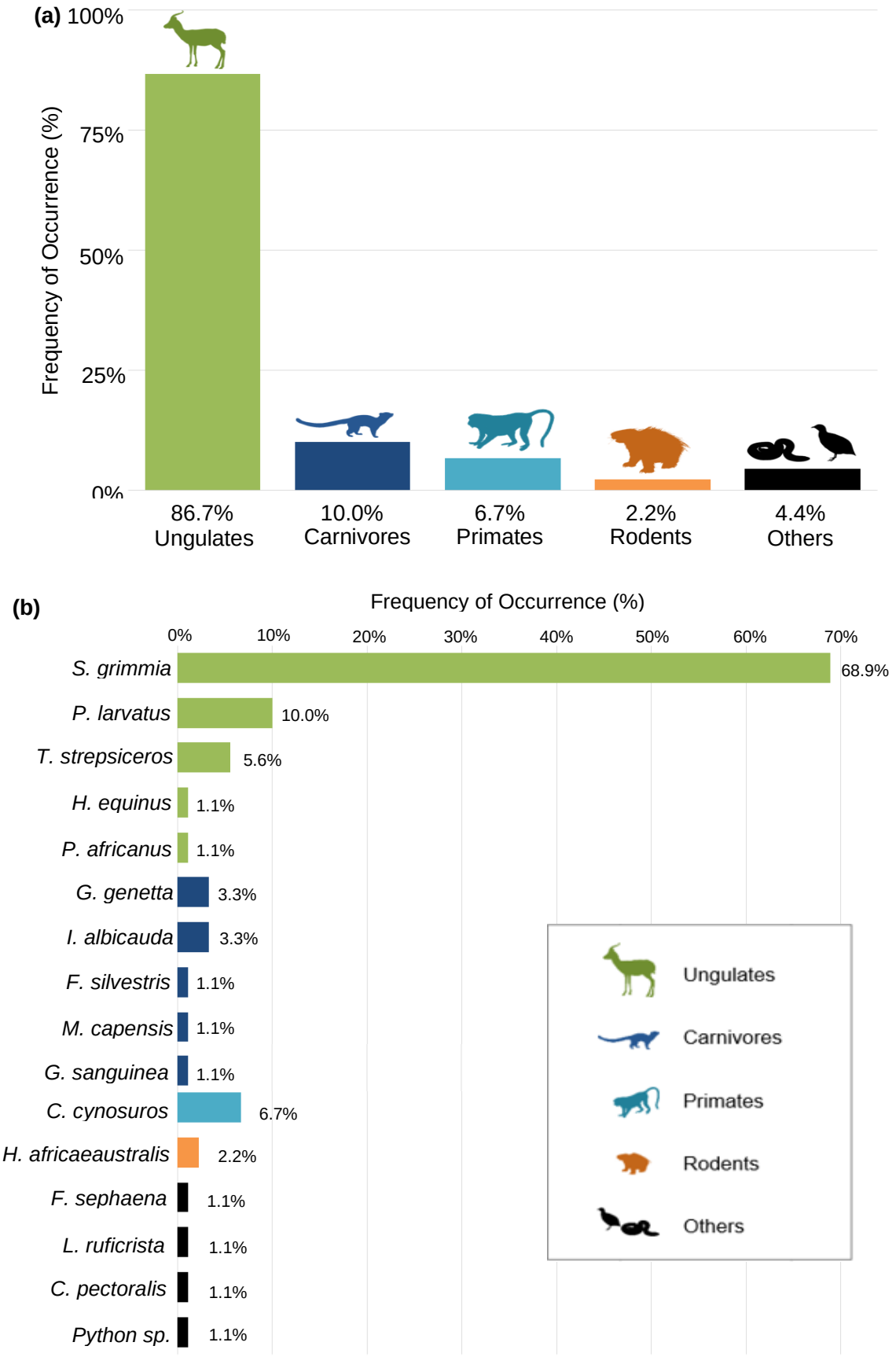


Figure 11. Frequency of Occurrence (%) of each detected prey item – (a) Frequency of Occurrence (%) of the 5 taxonomic groups detected in leopard diet; (b) Frequency of Occurrence (%) of the 16 different species detected in leopard diet. Green bars represent Ungulates, Dark Blue represent Carnivores, Light Blue represent Primates, Orange represents Rodents and Black represents the remaining detected species.

The cumulative curve of the Shannon diversity index illustrated that the approximate minimum number of samples that can be representative of the overall diet of the leopard inhabiting the study area is 37, though with large confidence intervals (Figure 12). This result provides statistical support to perform and interpret further analyses. The dietary diversity for the overall leopard population in Bicuar NP calculated using the Shannon's index, was estimated to be *H* = 1.45 +/- 0.15.

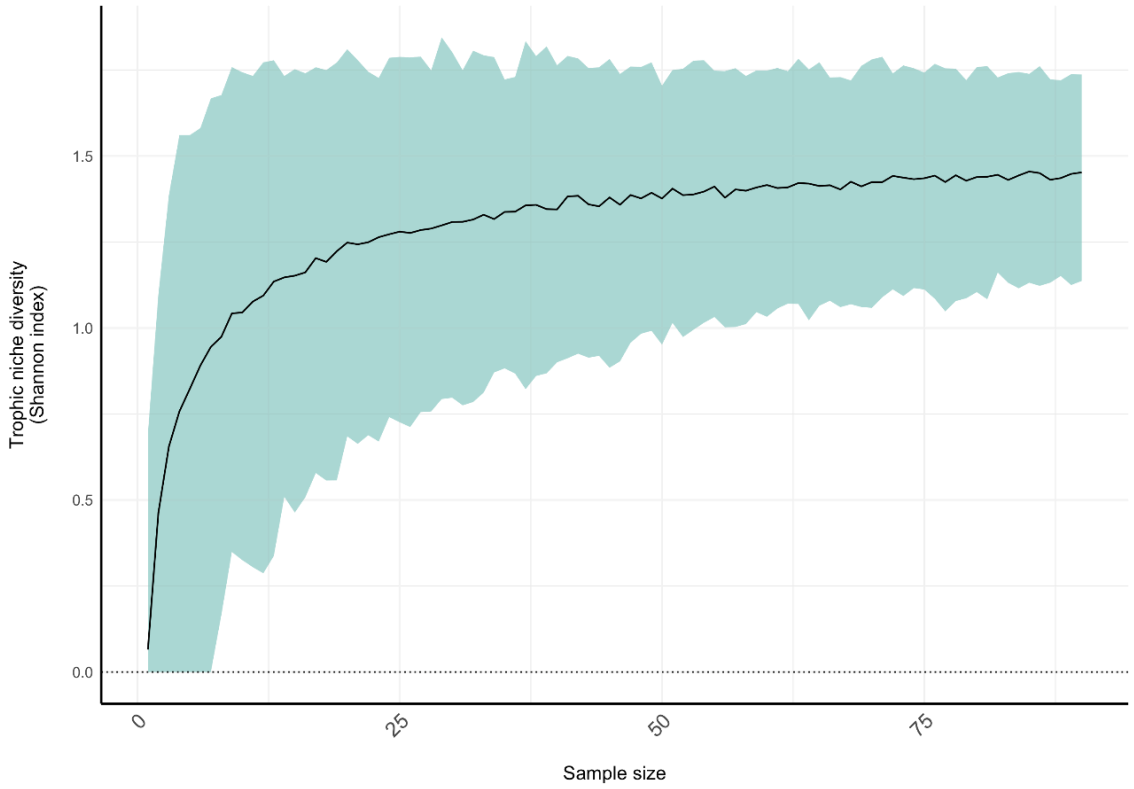


Figure 12. Cumulative dietary diversity indexed by the Shannon index diversity for leopards against an increasing number of sample size - The curve reaches an asymptotic value around 37 samples meaning that this is the minimum number of samples that can be representative of the overall leopard diet .

3 .4 Dietary Diversity Between Males and Females

Thirty-eight leopard samples used for the diet analysis have been characterized for the sex, representing 20 female and 18 male samples. Notably, only two prey species were identified in male leopard scats, the common duiker (FO = 60.0%) and the bushpig (FO = 30.0%) (Table 5). In contrast, 9 prey species were identified in leopard females' diet, namely common duiker (FO = 75.0%), white-tailed mongoose (FO = 10.0%), malbrouck (FO = 5.0%), roan antelope (FO = 5.0%), cape porcupine (FO = 5.0%), common warthog (FO = 5.0%), crested francolin (FO = 5.0%), python (FO = 5.0%) and common genet (FO = 5.0%) (Table 5). The common duiker was the most predominant and the only common prey species in the diet of both sexes.

Table 5. Frequency of Occurrence (%) of the different prey species in Females and Males diet – Females have a higher diversity of prey (n=9) found in their diet when compared to males (n=2). Nonetheless, the common duiker was the most consumed prey in both sexes. "-" means no detection.

Mammal Group and Scientific Name	Frequency of Occurrence (%)	
	Females	Males
Ungulates		
Sylvicapra grimmia	75.0	60.0
Hippotragus equinus	5.0	-
Potamochoerus larvatus	-	30.0
Phacochoerus africanus	5.0	-
Carnivores		
Genetta genetta	5.0	-
Ichneumia albicauda	10.0	-
Primates		
Chlorocebus cynosuros	5.0	-
Rodents		
Hystrix africaeaustralis	5.0	-
Others		
Francolinus sephaena	5.0	-
Python sp.	5.0	-

Female leopards in Bicuar NP were found to have a higher dietary diversity (H = 1.22 ± 0.29) when compared to males (H = 0.60 ± 0.09), and both sexes show lower dietary diversity than the overall leopard population (H =1.45 ± 0.15; Figure 13). Accordingly, the Mann-Whitney U Test calculated for the dietary diversity between male and female leopards was statistically significant ($W = 732$, $p < 0.05$).

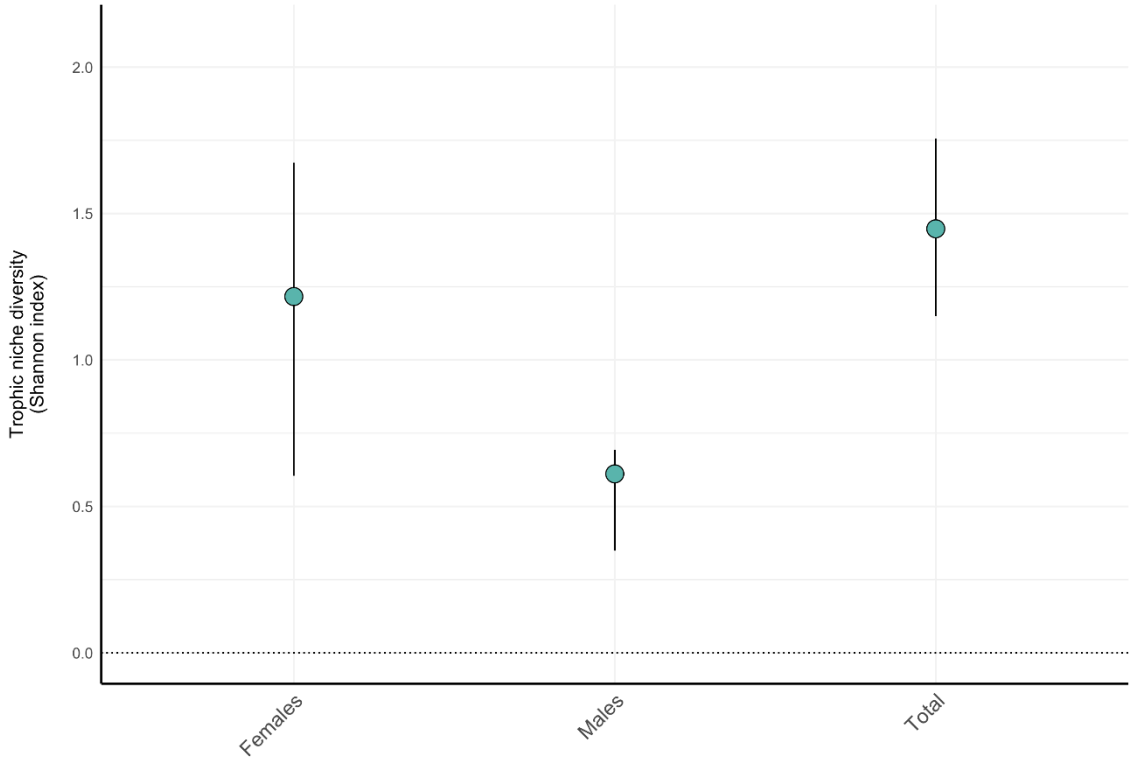


Figure 13. Trophic niche diversity calculated with the Shannon's index for females, males and the total population diet – The overall population diet (*H* = 1.45 +/- 0.15) has higher value of trophic niche diversity when compared to both females (*H = 1.22 ± 0.29)* and males (*H = 0.60 ± 0.09)* diet .However, females have significantly higher values when compared to males.

Regarding analysis of niche overlap between female and male leopards, the resulting value of the Pianka's index (0.84) is indicative of a strong dietary overlap between both sexes as both relied on the same main prey.

3.5 Individual Specialization

The same 38 leopard samples with information for sex have also been individually identified, resulting in 9 different individuals (5 males and 4 females) replicated between one and 12 samples each, and where the mean number of samples per individual was 4.1 (Table 6). The information for the different replicates (samples) of each individual was combined to examine whether individual specialization is present among leopards inhabiting the Bicuar NP. Common duiker was the only species present in the diet of all individuals. The ecological niche metrics implemented to measure individual specialization, the Proportional Similarity index, showed no evidence of significant individual dietary specialization among leopards in the BNP (IS = 0.674 ± 0.1; $p > 0.05$).

Nonetheless, this result should be taken carefully given the low sample size available for this analysis.

Table 6. Frequency of Occurrence of the different prey species in the diet of individuals identified – The number of samples corresponding to each individual, as well as their sex and prey species scientific name are present in the table. The most diverse individual was PP01 (number of prey detected (n) = 6 prey detected) followed by PP02 (n = 3), PP03, PP04 and PP09 (n=2). The remain individuals only had 1 prey detected. The number of samples corresponding to each individual was higher in PP01 and PP03 (12) followed by PP02 (5), PP04 (2) and PP09 (2). The remaining individuals only had 1 sample attributed to them.

Frequency of Occurrence Per Individual (%)

	Leopard Individual ID (Sex)								
	PP01 (F)	PP02 (F)	PP03 (M)	PP04 (F)	PP05 (M)	PP06 (F)	PP08 (M)	PP09 (M)	PP13 (M)
Nº of samples	12	5	12	2	1	1	1	2	1
Scientific name									
Ungulates									
H. equinus	-	20.0	-	-	-	-	-	-	-
P. africanus	-	20.0	-	-	-	-	-	-	-
P. larvatus	-	-	33.3	-	-	-	-	100.0	-
S. grimmia	83.3	60.0	66.6	50.0	100.0	100.0	100.0	50.0	100.0
Carnivores									
I. albicauda	16.7	-	-	-	-	-	-	-	-
G. genetta	8.3	-	-	-	-	-	-	-	-
Primates									
C. cynosuros	8.3	-	-	-	-	-	-	-	-
Rodents									
H. africaeaustralis	8.3	-	-	-	-	-	-	-	-
Others									
F. sephaena	-	-	-	50.0	-	-	-	-	-
Python sp.	8.3	-	-	-	-	-	-	-	-

Discussion

4.1 Metabarcoding Analysis

This study investigated the dietary habits of leopards in the Bicuar National Park, Angola, analysed through a metabarcoding approach applied to non-invasive DNA extracted from scats collected in the field between 2017 and 2019. Recently, many studies have shown the benefits of using DNA metabarcoding approaches for diet analysis in several species, owing to the ability of HTS technologies to identify simultaneously all the species present in the DNA sample, and furthermore, to the possibility of combining many samples in the same sequencing run (De Barba *et al*., 2014). Non-invasive samples are however prone to generate amplification errors due to the low quality and low quantity of DNA available per sample, and also to cross contaminations among samples or with extrinsic DNA (Pompanon *et al*., 2012). Additionally, HTS also generates a high rate of sequencing errors (Glenn, 2011; De Barba *et al*., 2014). These errors should be identified and filtered before diet analysis to secure accurate diet assessments. (De Barba *et al*., 2014). In this work, after quality control, we removed 80% of the sequencing reads that were not from prey DNA, for diet analysis, which is similar to other works performing similar analyses in mammal species (83% reads eliminated in Shi *et al*. 2021).

The non-use of blocking primers for leopard DNA in this work resulted in a high rate of leopard's reads among the filtered dataset (76.5%). It is expected that the DNA extracted from scat samples is highly enriched in intrinsic DNA rather than in prey DNA, triggering an overamplification in the final sequence dataset and eventually masking the detection of prey (Egeter *et al*., 2019; Ruppert *et al*., 2019; Piñol *et al*., 2015). Although not previously mentioned, leopard's blocking primers were designed and tested in different pools of leopard + prey species DNA. Nonetheless, the presence of the blocking primer in the amplification reaction has also inhibited the amplification of common duiker DNA, which is the most consumed prey of leopards in BNP, hampering its use in the final design of this study. Nevertheless, as leopards normally do not hunt more than one prey per week (Hayward *et al*., 2006), which was in part confirmed in this analysis by finding 90% of the scats exhibiting only sequence reads of one prey species, it was not expected that the overamplification of leopard DNA would hamper the detection of one or two additional species in the DNA extract. Further research is needed to understand the advantage of using blocking primers when the diet analysis refers to a predator species that do not kill prey frequently.

A limitation of metabarcoding diet analysis is the difficulty to distinguish prey species from secondary predation (the prey of the prey; Hardy *et al*., 2017). In this study, the uncertainty of this kind of limitation was less critical as leopard's prey are mostly herbivores. Regardless, we believe that the detection of secondary prey may have only occurred in the case of Angolan small rodents. Notably, those rodent sequencing reads (not considered in leopard diet) were only present in samples where other small carnivores were similarly detected. This may suggest that rodents were consumed by the mesocarnivores that were later hunted by leopards. The uncertainty of distinguishing between the predator's prey and the prey of its prey also depends on the previous knowledge of the feeding habits of the predator (Brassea-Pérez *et al*., 2019). Although leopards are opportunistic predators, the rate of energy intake by hunting, killing and feeding on a small species is unrewarding, and thus, the likelihood of these species be consumed by leopards when other more valuable resources are present, is significantly low (Hardy *et al*., 2017). Moreover, when using molecular techniques such as DNA metabarcoding, the detection of secondary predation is reduced by double digestion and only recent prey could be detected in a considerable frequency (Brassea-Pérez *et al*., 2019).

4.2 Taxonomic resolution

The taxonomic resolutions obtained in this study (70.5% Family level, 50.4% Genus level and 42.6% Species level) is similar to the one of Valsecchi *et al*. (2020) which used the same primers to identify mammals. The author identified more than 75%, more than 60% and around 50% of the sequences to the Family, Genus and Species, respectively. However, the study was conducted on marine mammals and therefore, results from terrestrial species may be different. Notwithstanding, according to Riaz *et al*. (2011) and Shehzad *et al*. (2012), the taxonomic resolution results presented in this study are low. They refer to a taxonomic resolution of 94% for Family, 88% for Genus and 73% for Species using the same primer pair, but with different target species such as domestic ungulates and small rodents. The lower taxonomic resolution in this study is possibly due to the reference database, at least for species (and perhaps genus) level since every single MOTU was double-checked when matching to the correspondent species. The reference database was built according to GenBank and other sequences that were not from Angolan populations. Even knowing that the mutation rate of the 12S rRNA fragment is low (King *et al*., 2008), different haplotypes are known for several of these species in the fragment, and we hypothesise that different haplotypes from those available in international databases for some of the species may be present in the BNP. Nonetheless, the lower taxonomic resolution at the Order and Family level could be

explained by the detection of DNA sequences of organisms that were not present in the reference database, such as insects, larvaceans or bacteria (and many others), which also reduces the taxonomic resolution at Genus and Species level.

4.3 The Leopard Diet

Despite leopards' eclectic diet, this study detected 16 different prey species consumed by leopards in the Bicuar National Park, Angola. The number of different prey detected is in accordance with other studies conducted in eastern and southern African dry savanna habitats where usually less diverse diets are reported (21 species, Grobler & Wilson, 1972; 12 species, Bailey, 1993) when compared with studies conducted in rainforests (32 species, Hoppe-Dominik, 1984; 31 species, Hart *et al*., 1996; 30 species, Henschel *et al*., 2005; 37 species, Bodendorfer *et al*., 2006). However, these comparisons must be carefully interpreted as prey species richness depends on the number of samples collected and analysed, as well as the number of prey present in the study area. The studies mentioned have more than 100 samples for diet analysis while this study only analysed the diet from 90 samples. Additionally, the civil war that happened in Angola until 2002, caused the disappearance of several species that were within the preference prey bodyweight of leopards, such as the impala in the BNP, and therefore, it may be expected that leopards were forced to constrain their diet due to the decrease of potential prey caused by human persecution. Also, the spotted hyena is another large carnivore species that is abundant in Bicuar (Rocha *et al.,* 2019) which is known to adopt a kleptoparasite and scavenging behaviour (Tarugara *et al.,* 2021). This means that they usually acquire their food resources by taking them from another species. It is a cost-effective way of obtaining food because it saves the energy that would be spent in a chase. In fact, several studies report hyenas kleptoparasiting on leopards' kills (Tarugara *et al.,* 2021; Havmøller *et al.,* 2020a). Therefore, this behaviour can negatively influence the amount of food that leopards eat possibly leading to less different prey being detected in this study.

In accordance with previous studies (Bodendorfer *et al*., 2006; Hayward *et al*., 2006), ungulates were found to be the staple prey of leopards in the Bicuar National Park. The three ungulates most consumed were the common duiker, the bushpig and the greater kudu comprising over four-fifths of their diet. Given the leopard's preferred prey weight range, it is not surprising that the common duiker was the most consumed prey. Common duikers are small (10 - 26 kg; O'Brien *et al*., 2020) solitary antelopes (Kigozi, 2000), that entail minimal risk of injury for a solitary hunter such as the leopard. The diel activity patterns of common duikers highly overlap the ones displayed by leopards, being more active at dusk, night and evening (Kigozi, 2000). According to

Overton *et al*. (2017), among the preferred prey weight range of leopards, common duikers are likely the most abundant species in BNP. This antelope's high availability its likely responsible for is high consumption rates by leopards. Notwithstanding, the steenbok, another small ungulate that also occurs in the BNP (Overton *et al.,* 2017), was not detected in the leopards' diet. This could be explained as a result of the availability of both prey, as common duiker may be more available over steenboks. The same happens with oribi (*Ourebia ourebi)* which was not detected in the leopard diet although it is present in low densities in Bicuar. Also, the same author did not distinguish between common duiker and steenbok when counting them and therefore, many steenbok identifications could be, in fact, common duikers underestimating its abundance.

The second most consumed ungulate was the bushpig (10%). Alongside the common warthog (1%), these two Suidae are also among the known preferred prey of leopards (Hayward *et al*., 2006). The difference in the frequency of occurrence between these two species could be related to their availability and distribution within BNP. The bushpig is more abundant and is widely distributed across the BNP whilst the common warthog has a more discontinuous distribution (Overton *et al*., 2017). Additionally, bushpigs are associated with dense vegetation types (Kingdom & Hoffman, 2013a) which makes them more suitable for predation by leopards since their hunting strategies rely on good cover (Balme *et al*., 2007). Contrastingly, common warthog usually occurs in open areas where there is no cover for leopards to hide (Kingdom & Hoffman, 2013a). Nevertheless, the contribution of these two species for leopards' diet in BNP could be lower because their body mass exceeds the upper limit of the leopard's preferred weight (40 kg). Also, the aggressive behaviour and defensive weaponry (tusks) have the potential to inflict significant injuries on predators (Hayward *et al*., 2006; Havmøller *et al*., 2020b). Another reason could be that prey that pose low risk of injury are still sufficient to fulfil leopard's energetic demands in the BNP (Havmøller *et al*., 2020b). Other studies have shown that the loss of leopards preferred prey changed their behaviour as they were forced to prey upon more dangerous species (Havmøller *et al*., 2020b; Ghoddousi *et al*., 2017), highlighting the importance of protecting ungulate species in the BNP.

The remaining ungulates [greater kudu (5.6%) and roan antelope (1.0%)], are considered large prey species as they greatly exceed the weight range of leopards preferred prey. Despite being the most common large ungulate in BNP, roan antelope's body mass (>200 kg; Kingdom & Hoffman, 2013a) is a major concern for predators that hunt alone such as leopards. The same reasoning applies to the greater kudu, which is also common in BNP (Overton *et al*., 2017). These results are in agreement with studies elsewhere (Havmøller *et al*., 2020b) demonstrating that leopards tend to avoid large prey species. Regardless, the greater kudu was considered to be frequently consumed (FO

> 5%) in BNP. Contrary to bushpig and common duiker, labelling greater kudu as a preferred prey by only taking into consideration the frequency of occurrence, is not ideal. One of the reasons, already mentioned before, has to do with their body mass which is at least 5x higher than the preferred by leopards (Kingdom & Hoffman, 2013b). Additionally, it is hard to believe that leopards could drag such heavy prey and hide them from other species like hyenas which also occur in the BNP. Therefore, leopards should consume those heavy prey as much as they can (Hayward *et al*., 2006) increasing the likelihood of producing more faeces corresponding to a single predation event. Other good possibilities could be related to the preference of juveniles of larger species such as the greater kudu (Hunter, 2015), scavenging events between leopards or specialization of some individuals on killing greater kudus.

Carnivore species were the second most consumed (10%) group by leopards in the BNP. This group is comprised only of medium to small-sized carnivores, being in accordance with other traditional and molecular studies which found that this group is a significant part of the leopards' diet (De Luca & Mpunga, 2018; Hayward *et al*., 2006). However, the appearance of these species in leopards diet could also be related to scent-marking which is a common behaviour in carnivore species to communicate with each other (Allen *et al.,* 2017; Rafiq *et al.,* 2020). Nonetheless, according to Hayward *et al*. (2006), leopards frequently kill smaller competitors although these prey species mostly focus their diet on invertebrates or smaller vertebrates. Similarly, Palomares & Caro (1999) revealed that Felids (as killers) are the most involved in interspecific interactions. This event also occurs in other carnivore species because they are negatively influenced by other members of the guild (Caro & Stoner, 2001). This is important because interspecific killing and intraguild predation, can either reduce the population size of an endangered carnivore or affect species at lower trophic levels (Caro & Stoner, 2001). In theory, these behaviours can occur to free up potential prey that would be consumed by the victims or due to the energetic value of the prey (Palomares & Caro, 1999). However, when applied to leopards and prey consumed, specifically in this study, these events remain unclear especially because those smaller carnivores do not frequently prey on the same species as leopards and therefore, it could be related to opportunistic events for alternative food resources or aggressive behaviour which is frequently observed in large carnivores, specially in leopards as shown by Curveira-Santos *et al.* (2021).

The only primate present in the diet of leopards was the malbrouck monkey (FO=6.7%). Although Overton *et al*. (2017) did not record the presence of this primate species in the BNP, these authors refer to the presence of the vervet monkey (*Chlorocebus pygerythrus)*, which is a reference to the same entity once there is still a

debate regarding the name of this species (Groves, 2001; Hunter, 2015). As *C. pygerythrus* and *C. cynosuros* are morphologically very similar, it could result from a misidentification as it was only based on morphology. Conversely, in this study, we undoubtedly detected the malbrouck in our genetic analysis and the species was also identified morphologically by CIBIO researchers during ongoing fieldwork in Bicuar NP (Filipe Rocha, pers. comm.) which highlights the importance of metabarcoding studies. Thus, we assume that this is the primate species present in the BNP. Primates are also common prey of leopards (Hayward *et al*., 2006; Havmøller *et al*., 2020b). Nonetheless, their arboreal refuge and group vigilance with vocalizations allow them more protection against large terrestrial predators when compared to other terrestrial prey. Hunter (2015) in the *Wild Cats of the World* book refers that "leopard's reputation for preferring primates is exaggerated" and so, leopard killing primates may be an opportunistic kill.

The remaining species that occurred less frequently in the leopard's diet comprehend the rodent (FO=2.2%), bird (FO=3.3%) and reptile (FO=1.1%) groups. These are potentially opportunistic or secondary predation events as the weight of these species are very low compared to the leopard's preferred weight. The exception would be the python (30 - 60 kg) and the cape porcupine (10 - 24 kg). However, this large rodent poses a serious risk of injuries for leopards. This predator was previously recorded killing several birds and very large pythons (up to 4m) (Hunter, 2015). They will frequently and opportunistically kill anything that they can catch or overpower including rodents, birds and reptiles which are often locally important (Hunter, 2015).

Overall, the most consumed prey were within the preferred weight range of leopards (10 – 40 kg) although very large ungulates (> 40 kg) and other smaller species (< 10 kg) were also detected in the diet of leopards.

The Shannon's diversity index (H = 1.45 +/- 0.15) is lower than the one reported by Joseph *et al*., 2007 in India (H = 2.11) , Akrim *et al.,* 2018 in Pakistan (H_{winter} = 1.85 & H_{summer} = 2.27), and Zehra, 2013 in India (H = 1.95). Although less prey was detected in *Joseph et al*., 2007 study, the proportion of each species in leopards' diet is less divergent when compared to this study. Also, another factor could be related to a higher prey richness, evenness, or a more equal distribution of each consumed prey item, encountered in the other studies. With more prey available for leopards, as well as more evenness in the number of individuals of different prey, the prey detected in leopards' diet may be higher increasing their dietary diversity (Balme *et al*., 2020). The BNP availability of common duikers, when compared to other prey species, is much higher which may reduce the probability of leopards preying on other prey, reducing their dietary diversity (Rocha et al., 2019; Balme *et al*., 2020; Overton *et al*., 2020).

4.4 Dietary Diversity Between Males and Females

The differences in the diversity of the dietary niche between males and females of leopard species are still a matter of debate. For example, Balme *et al*. (2020) found that male leopards have a broader dietary niche than females, possibly due to their larger size allowing them to sub-due a wider range of prey. Additionally, male's larger home range could facilitate encounters with a wider diversity of prey, providing greater choice upon which to base foraging decisions (Balme *et al*. 2020). These choices are the basis for individual specialization (Araújo *et al*., 2011). To corroborate these theories, Hayward *et al*. (2006) refer that the marked sexual dimorphism of leopards, especially in skull morphology, might be an adaptation for different food habits among sexes. Interestingly, among samples for which sex could be assessed, bushpig was only consumed by male leopards in BNP. The size and aggressiveness of this Suidae likely entail higher risks for female leopards given their smaller body size and more fragile build.

Contrastingly, based on an isotopic dietary niche approach, Voigt *et al*. (2018) found that females exhibited a wider dietary niche than males, and highlighted that females are more generalist consumers, although specializing in smaller prey than males. These results are coherent with those found here for BNP. Indeed, females were found to consume 7 prey species more than males, and 5 (56.0%) of the 9 prey species are small-sized prey (<10 kg). This could be explained by two main reasons. Firstly, the body size - being smaller than males, females could be more efficient predators of smaller-sized prey increasing the diversity of prey species found in females' diet. Additionally, the energetic value of smaller prey is lower when compared to larger ones and consequently, females may need to consume more small-sized prey to maintain their nutritional demands possibly leading to a higher spectrum of different prey consumed. Furthermore, these species can be easily dragged to trees to avoid kleptoparasitism, mainly by hyenas. Secondly, the parental care – if females are accompanied by cubs their energetic demands should be higher (Bothma & Coertze, 2004) and consequently, they should have more flexibility regarding which species to prey upon depending on the age of the young (Voigt *et al*., 2018). According to Bothma & Coertze (2004), females with cubs increase their hunting success by feeding on smaller prey and moving shorter distances. On the other hand, females also consumed large and aggressive species which was not so expectable since they are smaller than males. Therefore, those occurrences in female's diet can be related to juveniles of those large species or even scavenging events.

Although the dietary niche of leopards differs, an important dietary niche overlap between males and females was found. Both male and female leopards showed a strong

preference for the common duikers which is the most consumed and should be the most preferred prey in the BNP, as discussed earlier. Because most of the diet of both sexes include mainly the common duiker, it was expected that the overlap between males and females was high. Therefore, the differences between males and females are essentially related to their secondary prey, with females having a more diverse dietary niche than males.

4.5 Individual Dietary Specialization

There are studies in the literature that provide evidence of individual dietary specialization in carnivore species, namely in leopards based on direct observations, isotopic analysis, GPS cluster analysis, and morphological identification of prey in predator scats (Balme *et al*., 2020; Voigt *et al*., 2018; Pitman *et al*., 2013; Kittle *et al*., 2014). Notwithstanding, there was no statistical support for individual specialization among leopards in the Bicuar National Park although some differences in the diet of individuals can be stated, especially in females. However, the number of samples of each individual can influence the number of species detected, and therefore, we assumed that there was no individual specialization due to the lack of statistical support. This can be explained by the reduced number of scats with individual identification available for this analysis. Only 42% of the scats containing prey DNA had leopard individual identification. Furthermore, 6 samples representing those individuals only had one identifiable prey species. Because of the low number of samples with individual identification, the statistical tests were not powerful enough to be applied to this sample size. Hence, to assess leopard individual dietary specialization in further studies, it is suggested a higher sample size with identified individuals and additional prey detected per individual. Notwithstanding, overall, female individuals showed to have a broader dietary diversity diet, with their number of prey varying between 1 and 6, when compared to males, 1-2, and that all the individuals consumed the common duiker which was the most consumed prey.

Conclusion

To the best of our knowledge, this is the first study using faecal DNA and DNA metabarcoding to investigate the diet of a large carnivore in Angola. This research constitutes additional evidence that DNA metabarcoding methods are suitable for investigating the dietary patterns of carnivore species, in particular for leopards. Moreover, this study contributed to the body of knowledge of Angolan biodiversity and predator-prey relationship as these remain understudied in the country. Despite the limitations of the molecular approach followed, namely the overamplification of predator's DNA, and the detection of secondary prey and potential contaminations, it was possible to reliably reconstruct the diet of leopards in the Bicuar National Park, identifying 16 different prey species and correctly identify the primate living species in Bicuar which is the malbrouck.

The most important prey species for leopards in the BNP ecosystem were the common duiker, the bushpig, the malbrouck and the greater kudu. This study is in agreement with previous research analysing the leopard diet by identifying the common duiker and the bushpig leopard's most consumed prey. Additionally, leopard scats revealed that the predator consumed both large and medium-sized mammalian prey species in BNP, including mesocarnivores. Similarly, leopards were found to prey on small species like birds and reptiles which would be less expectable as they do not fulfil the energy requirements for this predator probably due to opportunistic events or a system with depleted prey.

The dietary differences between males and females were significant, with females having a broader dietary niche than males, possibly because they hunt more small-sized prey than males although larger species also being detected in females' diet (Bothma & Coertze, 2004; Vougt *et al*., 2018). Females were found to prey on 9 different prey species whereas only 2 species were found in males' diet. Regardless, there was an important overlap between the diet of both sexes, as both relied on the same staple prey - common duikers. Furthermore, no statistical support for individual dietary specialization on leopards in the BNP was found due to reduced sample size.

This study has also some limitations which are important to mention. Although a good taxonomic resolution was obtained in this study, it could eventually be increased if blocking-primers have been used to reduce the overamplification of leopards' DNA. Secondly, the reference database for 12S haplotypes of prey species was constructed using sequences of prey species from geographical regions different from Angola. Because the 12S fragment used is very short and differentiation between a few species is not high, the use of local haplotypes could have increased the accuracy of prey species

identification. Thirdly, the sample size for the analysis of dietary diversity between males and females and for individual specialization was low. Low sample sizes may introduce bias in the estimation of parameters and therefore, these same analyses should be repeated using a higher number of samples identified for sex and for the individual.

Further analysis on BNP leopards' diet is needed to understand the differences between males and females' diet, since their dimorphic morphology, both in size and skull, can lead to different types of diet specialization (Voigt *et al*., 2018). Males should be more specialised in medium- to large-sized prey whilst females should be more suited for preying upon small- to medium-size prey although our findings report that females also consumed large species (Bothma & Coertze, 2004). Moreover, a spatial and temporal analysis between predator and prey abundance/occurrence could shed light on which part of their niche is exploited and if it is correlated with prey type, prey abundance or, for instance, different types of habitats as well as evaluating the segregation activity between both sexes.

Implications for Wildlife Management and Conservation in Bicuar

Carnivores have received a disproportional attention of wildlife biologists and managers due to the position they occupy in ecosystems (Mills, 1991). Large carnivores are usually on the top of the food chain and therefore, any perturbance in their ecosystem makes them vulnerable to possible threats. Additionally, the impact they have on their prey is important in the management of those prey species (Mills, 1991). However, obtaining information about which species are present in a protected area is often challenging. The diet analysis of leopards allowed the detection of several prey species that are considered important for leopard populations (Hayward *et al*., 2006). These species occur through the leopard range and hence, they are a vital resource as prey. Therefore, protecting and conserving not only the predator species but also their prey, especially the ones weighing between 10 and 40 kg, is a crucial step in the conservation of leopards, a species that is considered "Vulnerable" and with their numbers decreasing worldwide (IUCN, 2021).

The Bicuar National Park biodiversity has been gaining attention due to the lack of studies regarding their biology, management, and conservation especially after the civil war that extirpated several animal species from the park. Although several management actions have been implemented, such as dedicated staff and some designed infrastructures, animal populations are still slowly recovering and some threats are still present in the park due to proximity to several villages in the perimeter of the park. Although human populations are not present in the core area of the BNP, they still apply forces in the park's perimeter, such as intensive agriculture, logging, and illegal poaching, which may have implications in wildlife populations. We recommend that these activities should be controlled by increasing the number of staff in order to protect Bicuar biodiversity. However, more staff implies more money expenditure which can be obtained with more efficient programmes for tourists such as the creation of camping facilities and low-cost promotion, since Bicuar has also been attracting some tourism activity (Overton *et al.,* 2017).

Another management strategy could include the re-introduction of prey species that were extirpated from Bicuar such as buffalos, cheetahs, or impalas. Notwithstanding, this type of measures requires significant amounts of funding that, while they can be obtained from international funding sources, are challenging to secure.

Nonetheless, several periodic evaluations of human activities should be performed in the park to monitor its effects on Bicuar's biodiversity. Also, regular wildlife surveys allowing to assess Bicuar's animal populations and how they respond to management actions are required to allow assessing if updating or adjusting the management plan is needed since as wildlife recover, more conflicts can arise.

The creation of ecological corridors between the Bicuar and Mupa National Parks would allow the movement of species from one park to another, with downstream increase in the levels of biodiversity of both parks.

References

Akrim, F., Mahmood, T., Nadeem, M.S., Andleeb, S., & Qasim, S. (2018). Spatial distribution and dietary niche breadth of the leopard *Panthera pardus* (Carnivora: Felidae) in the northeastern Himalayan region of Pakistan. *Turkish Journal of Zoology,* **42**, 585-595. https://doi.org/10.3906/zoo-1803-2

Aksöyek, E., İbiş, O., Özcan, S., Moradi, M., & Tez, C. (2017). DNA barcoding of three species (*Canis aureus, Canis lupus* and *Vulpes vulpes*) of Canidae. *Mitochondrial DNA Part A*, **28**, 747–755. https://doi.org/10.1080/24701394.2016.1180512

Alberdi, A., Aizpurua, O., Bohmann, K., Gopalakrishnan, S., Lynggaard, C., Nielsen, M., & Gilbert, M. T. P. (2019). Promises and pitfalls of using high-throughput sequencing for diet analysis. *Molecular Ecology Resources*, **19**, 327–348. https://doi.org/10.1111/1755-0998.12960

Alberdi, A., Aizpurua, O., Gilbert, M. T. P., & Bohmann, K. (2017). Scrutinizing key steps for reliable metabarcoding of environmental samples. *Methods in Ecology and Evolution*, **9**, 134–147. https://doi.org/10.1111/2041-210X.12849

Allen, M. L., Gunther, M. S., & Wilmers, C. C. (2017). The scent of your enemy is my friend? The acquisition of large carnivore scent by a smaller carnivore. *Journal of Ethology*, ***35***, 13-19. https://doi.org/10.1007/s10164-016-0492-6

Araújo, M. S., Bolnick, D. I., & Layman, C. A. (2011). The ecological causes of individual specialisation. *Ecology Letters*, **14**, 948–958. https://doi.org/10.1111/j.1461-0248.2011.01662.x

Araújo, M. S., Guimarães, P. R., Svanbäck, R., Pinheiro, A., Guimarães, P., Dos Reis, S. F., & Bolnick, D. I. (2008). Network analysis reveals contrasting effects of intraspecific competition on individual vs. population diets. *Ecology*, **89**, 1981–1993. https://doi.org/10.1890/07-0630.1

Athreya, V., Odden, M., Linnell, J. D. C., Krishnaswamy, J., & Karanth, K. U. (2016). A cat among the dogs: leopard *Panthera pardus* diet in a human-dominated landscape in western Maharashtra, India. *Oryx*, **50**, 156–162. https://doi.org/10.1017/S0030605314000106

Atkins, J. L., Long, R. A., Pansu, J., Daskin, J. H., Potter, A. B., Stalmans, M. E., Tarnita, C. E., & Pringle, R. M. (2019). Cascading impacts of large-carnivore extirpation in an African ecosystem. *Science*, **364**, 173–177. https://doi.org/10.1126/science.aau3561

Authier, M., Dragon, A. C., Richard, P., Cherel, Y., & Guinet, C. (2012). O' mother where wert thou? Maternal strategies in the southern elephant seal: A stable isotope investigation. *Proceedings of the Royal Society B*, **279**, 2681–2690. https://doi.org/10.1098/rspb.2012.0199

Azevedo, F. C. C., Lester, V., Gorsuch, W., Larivière, S., Wirsing, A. J., & Murray, D. L. (2006). Dietary breadth and overlap among five sympatric prairie carnivores. *Journal of Zoology*, **269**, 127–135. https://doi.org/10.1111/j.1469-7998.2006.00075.x

Bagchi, S., & Mishra, C. (2006). Living with large carnivores: Predation on livestock by the snow leopard (*Uncia uncia*). *Journal of Zoology*, **268**, 217–224. https://doi.org/10.1111/j.1469-7998.2005.00030.x

Bailey, T.N. (1993). *The African leopard. Ecology and behavior of a solitary felid*. Columbia University Press, New York

Balme, G. A., Slotow, R., & Hunter, L. T. B. (2010). Edge effects and the impact of non-protected areas in carnivore conservation: Leopards in the Phinda-Mkhuze Complex, South Africa. *Animal Conservation*, **13**, 315–323. https://doi.org/10.1111/j.1469-1795.2009.00342.x

Balme, Guy A., le Roex, N., Rogan, M. S., & Hunter, L. T. B. (2020). Ecological opportunity drives individual dietary specialization in leopards. *Journal of Animal Ecology*, **89**, 589–600. https://doi.org/10.1111/1365-2656.13109

Barabás, G., & D'Andrea, R. (2016). The effect of intraspecific variation and heritability on community pattern and robustness. *Ecology Letters*, **19**, 977–986. https://doi.org/10.1111/ele.12636

Barea-Azcón, J. M., Virgós, E., Ballesteros-Duperón, E., Moleón, M., & Chirosa, M. (2007). Surveying carnivores at large spatial scales: A comparison of four broad-applied methods. *Biodiversity and Conservation*, **16**, 1213–1230. https://doi.org/10.1007/s10531-006-9114-x

Beja, P., Pinto, P.V., Veríssimo, L., Bersacola, E., Faabiano, E., Palmeirim, J.M., Monadjem, A., Monterroso, P., Svensson, M.S., & Taylor, J. (2019). The Mammals of Angola. In: Huntley BJ, Russo V, Lages F, Ferrand N (Eds.), *Biodiversity of Angola: Science & Conservation: a modern synthesis.* (pp. 357-443). Springer Nature, Cham, Switezerland. https://doi.org/10.1007/978-3-030-03083-4_15

Beja-Pereira, A., Oliveira, R., Alves, P. C., Schwartz, M. K., & Luikart, G. (2009). Advancing ecological understandings through technological transformations in noninvasive genetics. *Molecular Ecology Resources*, **9**, 1279–1301. https://doi.org/10.1111/j.1755-0998.2009.02699.x

Bellemain, E., Carlsen, T., Brochmann, C., Coissac, E., Taberlet, P., & Kauserud, H. (2010). ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiology*, **10**, 189. https://doi.org/10.1186/1471-2180-10-189

Bitanyi, S., Bjørnstad, G., Ernest, E. M., Nesje, M., Kusiluka, L. J., Keyyu, J. D., Mdegela, R. H., & Røed, K. H. (2011). Species identification of Tanzanian antelopes using DNA barcoding. *Molecular Ecology Resources*, **11**, 442–449. https://doi.org/10.1111/j.1755-0998.2011.02980.x

Bodendorfer, T., Hoppe-Dominik, B., Fischer, F., & Linsenmair, K. E. (2006). Prey of the leopard (*Panthera pardus*) and the lion (*Panthera leo*) in the Comoé and Marahoué National Parks, Côte d'Ivoire, West Africa. *Mammalia*, **70**, 231–246. https://doi.org/10.1515/MAMM.2006.037

Boitani, L., & Powell, R. A. (Eds.). (2012). *Carnivore ecology and conservation: a handbook of techniques*. Oxford University Press, New York, USA.

Bolnick, D. I., Amarasekare, P., Araújo, M. S., Bürger, R., Levine, J. M., Novak, M., Rudolf, V.H.W, Schreiber, S.J., Urban, M.C., & Vasseur, D. A. (2011). Why intraspecific trait variation matters in community ecology. *Trends in Ecology & Evolution*, **26**, 183–192. https://doi.org/10.1016/j.tree.2011.01.009

Bolnick, D. I., Svanbäck, R., Fordyce, J. A., Yang, L. H., & Davis, J. D. (2003). The Ecology of Individuals : Incidence and Implications of Individual Specialization. *The American Naturalist*, **161**, 1–28. https://doi.org/10.1086/343878

Bolnick, D. I., Yang, L. H., Fordyce, J. A., Davis, J. M., & Svanbäck, R. (2002). Measuring individual-level resource specialization. *Ecology*, **83**, 2936–2941. https://doi.org/10.1890/0012-9658(2002)083[2936:MILRS]2.0.CO;2

Bothma, J. D. P., & Coertze, R. J. (2004). Motherhood increases hunting success in southern Kalahari leopards. *Journal of Mammalogy*, **85**, 756–760. https://doi.org/10.1644/BNS-010

Bothma, J.d.P. & le Riche, E.A.N. (1984). Aspects of the ecology and the behaviour of the leopard Panthera pardus in the Kalahari desert. *Koedoe,* **84**, 259–279.

Bourlat, S. J., Haenel, Q., Finnman, J., & Leray, M. (2016). Preparation of amplicon libraries for metabarcoding of marine eukaryotes using illumina MiSeq: The Dual-PCR Method. *Methods in molecular biology* (Clifton, N.J.), **1452**, 197–207. https://doi.org/10.1007/978-1-4939-3774-5_13

Bowen, W. D., & Iverson, S. J. (2013). Methods of estimating marine mammal diets: A review of validation experiments and sources of bias and uncertainty. *Marine Mammal Science*, **29**, 719–754. https://doi.org/10.1111/j.1748-7692.2012.00604.x

Bowles, E., Schulte, P. M., Tollit, D. J., Deagle, B. E., & Trites, A. W. (2011). Proportion of prey consumed can be determined from faecal DNA using real-time PCR. *Molecular Ecology Resources*, **11**, 530–540. https://doi.org/10.1111/j.1755-0998.2010.02974.x

Bowman, W. D., Hacker, S.D., & Cain, M.L. (2017). *Ecology, Fourth Edition*. Sunderland, Massachusetts: Sinauer Associates, Inc., 2017. Print.

Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., & Coissac, E. (2016). obitools: A unix-inspired software package for DNA metabarcoding. *Molecular Ecology Resources*, **16**, 176–182. https://doi.org/10.1111/1755-0998.12428

Boyer, S., Yeates, G. W., Wratten, S. D., Holyoake, A., & Cruickshank, R. H. (2011). Molecular and morphological analyses of faeces to investigate the diet of earthworm predators: Example of a carnivorous land snail endemic to New Zealand. *Pedobiologia*, **54**, S153–S158. https://doi.org/10.1016/j.pedobi.2011.08.002

Brassea-Pérez, E., Schramm, Y., Heckel, G., Chong-Robles, J., & Lago-Lestón, A. (2019). Metabarcoding analysis of the Pacific harbor seal diet in Mexico. *Marine Biology*, **166**, 1–14. https://doi.org/10.1007/s00227-019-3555-8

Butler, B. O., Ceríaco, L. M. P., Marques, Bandeira, S., Júlio, T., Heinicke, M.P., & Bauer, A. M. (2019). Herpetological Survey of Huíla Province, Southwest Angola, Including First Records from Bicuar National Park. *Herpetological Review*, **50**, 225–240.

Carbone, C., Mace, G. M., Roberts, S. C., & Macdonald, D. W. (1999). Energetic constraints on the diet of terrestrial carnivores. *Nature*, **402**, 286–288. https://doi.org/10.1038/46266

Caro, T. M., & Stoner, C. J. (2003). The potential for interspecific competition among African carnivores. *Biological Conservation*, **110**, 67–75. https://doi.org/10.1016/S0006-3207(02)00177-5

Carreon-Martinez, L., Johnson, T. B., Ludsin, S. A., & Heath, D. D. (2011). Utilization of stomach content DNA to determine diet diversity in piscivorous fishes. *Journal of Fish Biology*, **78**, 1170–1182. https://doi.org/10.1111/j.1095-8649.2011.02925.x

Casper, R. M., Jarman, S. N., Deagle, B. E., Gales, N. J., & Hindell, M. A. (2007a). Detecting prey from DNA in predator scats: A comparison with morphological analysis, using Arctocephalus seals fed a known diet. *Journal of Experimental Marine Biology and Ecology*, **347**, 144–154. https://doi.org/10.1016/j.jembe.2007.04.002

Casper, R. M., Jarman, S. N., Gales, N. J., & Hindell, M. A. (2007b). Combining DNA and morphological analyses of faecal samples improves insight into trophic interactions: A case study using a generalist predator. *Marine Biology*, **152**, 815–825. https://doi.org/10.1007/s00227-007-0732-y

Chakrabarti, S., Jhala, Y. V., Dutta, S., Qureshi, Q., Kadivar, R. F., & Rana, V. J. (2016). Adding constraints to predation through allometric relation of scats to consumption. *Journal of Animal Ecology*, **85**, 660–670. https://doi.org/10.1111/1365-2656.12508

Chaves, P. B., Graeff, V. G., Lion, M. B., Oliveira, L. R., & Eizirik, E. (2012). DNA barcoding meets molecular scatology: Short mtDNA sequences for standardized species assignment of carnivore noninvasive samples. *Molecular Ecology Resources*, **12**, 18–35. https://doi.org/10.1111/j.1755-0998.2011.03056.x

Clarke, L. J., Soubrier, J., Weyrich, L. S., & Cooper, A. (2014). Environmental metabarcodes for insects: In silico PCR reveals potential for taxonomic bias. *Molecular Ecology Resources*, **14**, 1160–1170. https://doi.org/10.1111/1755-0998.12265

Crawford-Cabral, J. (1991). Zoogeografia dos vertebrados de Angola. Programa de Investigação. *Unpublished Report, Instituto de Investigação Científica Tropical*, Lisboa, Portugal, 59.

Curveira-Santos, G., Gigliotti, L., Silva, A. P., Sutherland, C., Foord, S., Santos-Reis, M., & Swanepoel, L. H. (2021). Broad aggressive interactions among African carnivores suggest intraguild killing is driven by more than competition. *Ecology*, **0**, e03600. https://doi.org/10.1002/ecy.3600

Dalerum, F., & Angerbjörn, A. (2005). Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia*, **144**, 647–658. https://doi.org/10.1007/s00442-005-0118-0

Darimont, C. T., Paquet, P. C., & Reimchen, T. E. (2007). Stable isotopic niche predicts fitness of prey in a wolf-deer system. *Biological Journal of the Linnean Society*, **90**, 125–137. https://doi.org/10.1111/j.1095-8312.2007.00716.x

Davison, A., Birks, J. D. S., Brookes, R. C., Braithwaite, T. C., & Messenger, J. E. (2002). On the origin of faeces: Morphological versus molecular methods for surveying rare carnivores from their scats. *Journal of Zoology*, **257**, 141–143. https://doi.org/10.1017/S0952836902000730

Dawson, S. J., Crawford, H. M., Huston, R. M., Adams, P. J., & Fleming, P. A. (2016). How to catch red foxes red handed: Identifying predation of freshwater turtles and nests. *Wildlife Research*, **43**, 615–622. https://doi.org/10.1071/WR16066

De Barba, M., Miquel, C., Boyer, F., Mercier, C., Rioux, D., Coissac, E., & Taberlet, P. (2014). DNA metabarcoding multiplexing and validation of data accuracy for diet assessment: Application to omnivorous diet. *Molecular Ecology Resources*, **14**, 306–323. https://doi.org/10.1111/1755-0998.12188

De Barba, M., Miquel, C., Lobréaux, S., Quenette, P. Y., Swenson, J. E., & Taberlet, P. (2017). High-throughput microsatellite genotyping in ecology: improved accuracy, efficiency, standardization and success with low-quantity and degraded DNA. *Molecular Ecology Resources*, **17**, 492–507. https://doi.org/10.1111/1755-0998.12594

De Luca, D. W., & Mpunga, N. E. (2018). Leopard abundance, distribution and food habits in the Mt. Rungwe–Kitulo landscape, Southern Tanzania. *African Journal of Ecology*, **56**, 358–367. https://doi.org/10.1111/aje.12464

Deagle, Bruce E., & Tollit, D. J. (2007). Quantitative analysis of prey DNA in pinniped faeces: Potential to estimate diet composition? *Conservation Genetics*, **8**, 743–747. https://doi.org/10.1007/s10592-006-9197-7

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: Not a perfect match. *Biology Letters*, **10**(9), 2–5. https://doi.org/10.1098/rsbl.2014.0562

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., Kartzinel, T. R., & Eveson, J. P. (2019). Counting with DNA in metabarcoding

studies: How should we convert sequence reads to dietary data? *Molecular Ecology*, **28**, 391–406. https://doi.org/10.1111/mec.14734

Deagle, B. E., Thomas, A. C., Shaffer, A. K., Trites, A. W., & Jarman, S. N. (2013). Quantifying sequence proportions in a DNA-based diet study using Ion Torrent amplicon sequencing: Which counts count? *Molecular Ecology Resources*, **13**, 620–633. https://doi.org/10.1111/1755-0998.12103

Di Minin, E., Slotow, R., Hunter, L. T. B., Montesino Pouzols, F., Toivonen, T., Verburg, P. H., Leader-Williams, N., Petracca, L., & Moilanen, A. (2016). Global priorities for national carnivore conservation under land use change. *Scientific Reports*, **6**, 1–9. https://doi.org/10.1038/srep23814

Dobrovolny, S., Blaschitz, M., Weinmaier, T., Pechatschek, J., Cichna-Markl, M., Indra, A., Hufnagl, P., & Hochegger, R. (2019). Development of a DNA metabarcoding method for the identification of fifteen mammalian and six poultry species in food. *Food Chemistry*, **272**, 354–361. https://doi.org/10.1016/j.foodchem.2018.08.032

Egeter, B., Roe, C., Peixoto, S., Puppo, P., Easton, L. J., Pinto, J., Bishop, P. J., & Robertson, B. C. (2019). Using molecular diet analysis to inform invasive species management: A case study of introduced rats consuming endemic New Zealand frogs. *Ecology and Evolution*, **9**, 5032–5048. https://doi.org/10.1002/ece3.4903

Eklöf, A., & Allesina, S. (2012). Networks, Ecological. In Hastings, A., Gross L.J. (Eds.) *Encyclopedia of Theoretical Ecology* (pp. 470-478). University of California Press

Farrell, L. E., Roman, J., & Sunquist, M. E. (2000). Dietary separation of sympatric carnivores identified by molecular analysis of scats. *Molecular Ecology*, **9**, 1583–1590. https://doi.org/10.1046/j.1365-294X.2000.01037.x

Fedriani, J. M., Fuller, T. K., Sauvajot, R. M., & York, E. C. (2000). Competition and intraguild predation among three sympatric carnivores. *Oecologia*, **125**, 258–270. https://doi.org/10.1007/s004420000448

Ficetola, G. F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., & Pompanon, F. (2010). An In silico approach for the evaluation of DNA barcodes. *BMC Genomics*, **11,** 1-10. https://doi.org/10.1186/1471-2164-11-434

Flather, C. H., & Sieg, C. H. (2007). Species rarity: definition, causes, and classification. In Raphael, M. G.; Molina, R. (Eds.). *Conservation of Rare or Little-Known Species: Biological, Social, and Economic Considerations*, (pp. 40–66). Washington: Island Press.

Forsman, A., & Wennersten, L. (2016). Inter-individual variation promotes ecological success of populations and species: evidence from experimental and comparative studies. *Ecography*, **9**, 630–648. https://doi.org/10.1111/ecog.01357

Galan, M., Pagès, M., & Cosson, J. F. (2012). Next-Generation Sequencing for Rodent Barcoding: Species Identification from Fresh, Degraded and Environmental Samples. *PLoS ONE*, **7**. https://doi.org/10.1371/journal.pone.0048374

Geyle, H. M., Stevens, M., Duffy, R., Greenwood, L., Nimmo, D. G., Sandow, D., Thomas, B., White, J., & Ritchie, E. G. (2020). Evaluation of camera placement for detection of free-ranging carnivores; implications for assessing population changes. *Ecological Solutions and Evidence*, **1**, 1–13. https://doi.org/10.1002/2688-8319.12018

Ghoddousi, A., Soofi, M., Kh. Hamidi, A., Lumetsberger, T., Egli, L., Ashayeri, S., Khorozyan, I., Kiabi, B., & Waltert, M. (2017). When pork is not on the menu: Assessing trophic competition between large carnivores and poachers. *Biological Conservation*, **209**, 223–229. https://doi.org/10.1016/j.biocon.2017.02.032

Gillet, F., Tiouchichine, M. L., Galan, M., Blanc, F., Némoz, M., Aulagnier, S., & Michaux, J. R. (2015). A new method to identify the endangered Pyrenean desman (Galemys pyrenaicus) and to study its diet, using next generation sequencing from faeces. *Mammalian Biology*, **80**, 505–509. https://doi.org/10.1016/j.mambio.2015.08.002

Gitari, M. M. (2018). *Assessment of African Leopard and Spotted Hyena Diet and Prey Base Along Burguret Trail in Mt. Kenya Forest, Kenya* [Master Thesis, The University of Nairobi].

Glenn, T. C. (2011). Field guide to next-generation DNA sequencers. *Molecular Ecology Resources*, **11**, 759–769. https://doi.org/10.1111/j.1755-0998.2011.03024.x

Gosselin, E. N., Lonsinger, R. C., & Waits, L. P. (2017). Comparing morphological and molecular diet analyses and fecal DNA sampling protocols for a terrestrial carnivore. *Wildlife Society Bulletin*, **41**, 362–369. https://doi.org/10.1002/wsb.749

Grobler, J.H. & Wilson, V.J. (1972). Food of the leopard *Panthera pardus* (Linn.) in the Rhodes Matopos National Park, Rhodesia, as determined by faecal analysis. *Arnoldia,* **5**, 1–10.

Groves, C.P. (2001). *Primate taxonomy*. Smithsonian Institution Press, Washington, DC.

Hall, T.A. (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.

Hardy, N., Berry, T., Kelaher, B. P., Goldsworthy, S. D., Bunce, M., Coleman, M. A., Gillanders, B. M., Connell, S. D., Blewitt, M., & Figueira, W. (2017). Assessing the trophic ecology of top predators across a recolonisation frontier using DNA metabarcoding of diets. *Marine Ecology Progress Series*, **573**, 237–254. https://doi.org/10.3354/meps12165

Hart, J. A., Katembo, M., & Punga, K. (1996). Diet, prey selection and ecological relations of leopard and golden cat in the Ituri Forest, Zaire. *African Journal of Ecology*, **34**, 364–379. https://doi.org/10.1111/j.1365-2028.1996.tb00632.x

Hatton, I. A., McCann, K. S., Fryxell, J. M., Davies, T. J., Smerlak, M., Sinclair, A. R. E., & Loreau, M. (2015). The predator-prey power law: Biomass scaling across terrestrial and aquatic biomes. *Science*, **349**. https://doi.org/10.1126/science.aac6284

Havmøller, R. W., Jacobsen, N. S., Scharff, N., Rovero, F., & Zimmermann, F. (2020a). Assessing the activity pattern overlap among leopards (*Panthera pardus*), potential prey and competitors in a complex landscape in Tanzania. *Journal of Zoology*, **311**, 175–182. https://doi.org/10.1111/jzo.12774

Havmøller, Rasmus Worsøe, Jacobsen, N. S., Havmøller, L. W., Rovero, F., Scharff, N., & Bohmann, K. (2020b). DNA metabarcoding reveals that African leopard diet varies between habitats. *African Journal of Ecology*, **59,** 37-50. https://doi.org/10.1111/aje.12817

Hawlitschek, O., Fernández-González, A., Balmori-de la Puente, A., & Castresana, J. (2018). A pipeline for metabarcoding and diet analysis from fecal samples developed for a small semi-aquatic mammal. *PLoS ONE*, **13**, 1–19. https://doi.org/10.1371/journal.pone.0201763

Hayssen, V., & Stein, A. B. (2013). *Panthera pardus* (Carnivora : Felidae). *Mammalian Species,* **45**, 30–48.

Hayward, M. W., Henschel, P., O'Brien, J., Hofmeyr, M., Balme, G., & Kerley, G. I. H. (2006). Prey preferences of the leopard (*Panthera pardus*). *Journal of Zoology*, **270**, 298–313. https://doi.org/10.1111/j.1469-7998.2006.00139.x

Hayward, Matt W., & Kerley, G. I. H. (2008). Prey preferences and dietary overlap amongst Africa's large predators. *African Journal of Wildlife Research*, **38**, 93–108. https://doi.org/10.3957/0379-4369-38.2.93

Hebblewhite, M., Merrill, E. H., & McDonald, T. L. (2005). Spatial decomposition of predation risk using resource selection functions: An example in a wolf-elk predator-prey system. *Oikos*, **111**, 101–111. https://doi.org/10.1111/j.0030-1299.2005.13858.x

Hebert, P. D. N., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 313–321. https://doi.org/10.1098/rspb.2002.2218

Henschel, P., Abernethy, K. A., & White, L. J. T. (2005). Leopard food habits in the Lopé National Park, Gabon, Central Africa. *African Journal of Ecology*, **43**, 21–28. https://doi.org/10.1111/j.1365-2028.2004.00518.x

Holt, R. & Kimbrell, T. (2008). 11. Foraging and Population Dynamics. In D. Stephens, J. Brown & R. Ydenberg (Ed.), *Foraging: Behavior and Ecology* (pp. 364-396). University of Chicago Press, Chicago.

Hopcraft, G. C., Sinclair, A. R. E., & Packer, C. (2005). Planning for success: Serengeti lions seek prey accessibility rather than abundance. *Journal of Animal Ecology*, **74**, 559–566. https://doi.org/10.1111/j.1365-2656.2005.00955.x

Hoppe-Dominik, B. 1984. Etude du spectre des proies de la panthère, Panthera pardus, dans le Parc National de Taï en Côte d'Ivoire. *Mammalia,* **48**, 477–487.

Hunter, L. (2015). *Wild Cats of the world*. Bloomsbury Publishing, London, UK.

Hunter, L., Henschel, P. & Ray, J.C. (2013) Panthera pardus. In Kingdon, J.S. & Hoffmann, M. (Eds) *The Mammals of Africa* (pp. 159–168). Amsterdam: Academic Press.

Huntley, B.J. (1973). Distribution and Status of the Larger Mammals of Angola, with particular reference to Rare and Endangered species: First Progress Report. December 1973. Repartição Técnica da Fauna, Serviços de Veterinária, Luanda, Mimeograph report, 14 pp

Huntley, B.J. (1974). Ecosystem Conservation Priorities in Angola. Ecologist's Report 26. Repartição Técnica da Fauna, Serviços de Veterinária, Luanda, Mimeograph report

Huntley, B. J. (1971) Ecological Survey of the National Parks of Angola. Direcção Provincial dos Serviços de Veterinária, nº 1, Luanda, Angola

Huntley, B. J., Russo, V., Lages, F., & Ferrand, N. (2019). Angolan biodiversity: Towards a modern synthesis. In : Huntley, B.J., Russo, V., Lages, F., Ferrand, N. (eds). *Biodiversity of Angola: Science and Conservation: A Modern Synthesis*. Springer Nature, Cham, Switezerland. https://doi.org/10.1007/978-3-030-03083-4_1

Husseman, J. S., Murray, D. L., Power, G., Mack, C., Wenger, C. R., & Quigley, H. (2003). Assessing differential prey selection patterns between two sympatric large carnivores. *Oikos*, **101**, 591–601. https://doi.org/10.1034/j.1600-0706.2003.12230.x

Inskip, C., & Zimmermann, A. (2009). Human-felid conflict: A review of patterns and priorities worldwide. *Oryx*, **43**, 18–34. https://doi.org/10.1017/S003060530899030X

IUCN (2021). *The IUCN Red List of Threatened Species.* Version 2021-2. https://www.iucnredlist.org. Accessed 26 June 2021

Iverson, S. J., Field, C., Bowen, W. D., & Blanchard, W. (2004). Quantitative fatty acid signature analysis: A new method of estimating predator diets. *Ecological Monographs*, **74**, 211–235. https://doi.org/10.1890/02-4105

Jacobson, A. P., Gerngross, P., Lemeris, J. R., Schoonover, R. F., Anco, C., Breitenmoser-Würsten, C., Durant, S. M., Farhadinia, M. S., Henschel, P., Kamler, J. F., Laguardia, A., Rostro-García, S., Stein, A. B., & Dollar, L. (2016). Leopard (*Panthera pardus*) status, distribution, and the research efforts across its range. *PeerJ*, **2016**, 1–28. https://doi.org/10.7717/peerj.1974

Jones, M. E., & Barmuta, L. A. (1998). Diet overlap and relative abundance of sympatric dasyurid carnivores: A hypothesis of competition. *Journal of Animal Ecology*, **67**, 410–421. https://doi.org/10.1046/j.1365-2656.1998.00203.x

Joseph, S., Thomas, A. P., Sathessh, R., & Suyathan, R. (2007). Foraging ecology and relative abundance of large carnivores in Parambikulam Wildlife Sanctuary, Southern India. *Zoos' Print Journal*, **22**, 2667–2670. https://doi.org/10.11609/jott.zpj.1491.2667-70

Karanth, K. U., & Nichols, J. D. (1998). Estimation of tiger densities in India using photographic captures and recaptures. *Ecology*, **79**, 2852–2862. https://doi.org/10.1890/0012-9658(1998)079[2852:EOTDII]2.0.CO;2

Karanth, K. U., & Sunquist, M. E. (1995). Prey Selection by Tiger, Leopard and Dhole in Tropical Forests. *The Journal of Animal Ecology*, **64**, 439. https://doi.org/10.2307/5647

Karanth, K. U., Nichols, J. D., Kumar, N. S., Link, W. A., & Hines, J. E. (2004). Tigers and their prey: Predicting carnivore densities from prey abundance. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 4854–4858. https://doi.org/10.1073/pnas.0306210101

Kartzinel, T. R., Chen, P. A., Coverdale, T. C., Erickson, D. L., Kress, W. J., Kuzmina, M. L., Rubenstein, D. I., Wang, W., & Pringle, R. M. (2015). DNA metabarcoding illuminates dietary niche partitioning by African large herbivores. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 8019–8024. https://doi.org/10.1073/pnas.1503283112

Kasper, M. L., Reeson, A. F., Cooper, S. J. B., Perry, K. D., & Austin, A. D. (2004). Assessment of prey overlap between a native (*Polistes humilis*) and an introduced (*Vespula germanica*) social wasp using morphology and phylogenetic analyses of 16S rDNA. *Molecular Ecology*, **13**, 2037–2048. https://doi.org/10.1111/j.1365-294X.2004.02193.x

Keim, J. L., DeWitt, P. D., & Lele, S. R. (2011). Predators choose prey over prey habitats: evidence from a lynx-hare systeam. *Ecological Applications*, **21**, 1011–1016.

Kelly, M. J., Betsch, J., Wultsch, C., Mesa, B., & Mills, L. S. (2012). Noninvasive sampling for carnivores. *Carnivore ecology and conservation: a handbook of techniques*, 47-69.

Kigozi, F. (2000). *Feeding biology of common and blue duiker* [Doctoral dissertation, Rhodes University]

King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. C. (2008). Molecular analysis of predation: A review of best practice for DNA-based approaches. *Molecular Ecology*, **17**, 947–963. https://doi.org/10.1111/j.1365-294X.2007.03613.x

Kingdon, J. (2015). The Kingdon Field Guide to African Mammals. Second Edition. Bloomsbury Publishing, London, UK.

Kingdon, J. & Hoffmann, M. (eds) (2013a). *Mammals of Africa. Volume VI: Pigs, Hippopotamuses, Chevrotain, Giraffes, Deer and Bovids*. Bloomsbury Publishing, London, UK.

Kitano, T., Umetsu, K., Tian, W., & Osawa, M. (2007). Two universal primer sets for species identification among vertebrates. *International Journal of Legal Medicine*, **121**, 423–427. https://doi.org/10.1007/s00414-006-0113-y

Kittle, A. M., Watson, A. C., Kumara, P. H. S. C., Sandanayake, S. D. K. C., Sanjeewani, H. K. N., & Fernando, T. S. P. (2014). Notes on the diet and habitat selection of the Sri Lankan Leopard *Panthera pardus kotiya* (Mammalia: Felidae) in the central highlands of Sri Lanka. *Journal of Threatened Taxa*, **6**, 6214–6221. https://doi.org/10.11609/jott.o3731.6214-21

Kocher, A., de Thoisy, B., Catzeflis, F., Huguin, M., Valière, S., Zinger, L., Bañuls, A. L., & Murienne, J. (2017). Evaluation of short mitochondrial metabarcodes for the identification of Amazonian mammals. *Methods in Ecology and Evolution*, **8**, 1276–1283. https://doi.org/10.1111/2041-210X.12729

Kotler B.P., & Brown J.S. (2007). Community ecology. In: Stephens DW, Brown JS, Ydenberg RC (eds). *Foraging: Behavior and Ecology* (pp. 397-434). University of Chicago Press, Chicago.

Larson, R. N., Morin, D. J., Wierzbowska, I. A., & Crooks, K. R. (2015). Food Habits of Coyotes, Gray Foxes, and Bobcats in a Coastal Southern California Urban Landscape. *Western North American Naturalist*, **75**, 339–347. https://doi.org/10.3398/064.075.0311

Latinne, A., Galan, M., Waengsothorn, S., Rojanadilok, P., Eiamampai, K., Sribuarod, K., & Michaux, J. R. (2014). Diet analysis of *Leopoldamys neilli*, a cave-dwelling rodent in Southeast Asia, using next-generation sequencing from feces. *Journal of Cave and Karst Studies*, **76**, 139–145. https://doi.org/10.4311/2013LSC0100

Leaché, A. D., Wagner, P., Linkem, C. W., Böhme, W., Papenfuss, T. J., Chong, R. A., Lavin, B. R., Bauer, A. M., Nielsen, S. V., Greenbaum, E., Rödel, M. O., Schmitz, A., LeBreton, M., Ineich, I., Chirio, L., Ofori-Boateng, C., Eniang, E. A., Baha El Din, S., Lemmon, A. R., & Burbrink, F. T. (2014). A hybrid phylogenetic-phylogenomic approach for species tree estimation in african agama lizards with applications to biogeography, character evolution, and diversification. *Molecular Phylogenetics and Evolution*, **79**, 215–230. https://doi.org/10.1016/j.ympev.2014.06.013

Lumetsberger, T., Ghoddousi, A., Appel, A., Khorozyan, I., Waltert, M., & Kiffner, C. (2017). Re-evaluating models for estimating prey consumption by leopards. *Journal of Zoology*, **302**, 201–210. https://doi.org/10.1111/jzo.12449

MacFarland, T. W., & Yates, J. M. (2016). *Introduction to nonparametric statistics for the biological sciences using R* (pp. 103-132). Springer, Cham.

Marker, L. L., & Dickman, A. J. (2005). Factors affecting leopard (*Panthera pardus*) spatial ecology, with particular reference to Namibian farmlands*. African Journal of Wildlife Research*, **35**, 105–115.

Martínez-Gutiérrez, P. G., Palomares, F., & Fernández, N. (2015). Predator identification methods in diet studies: Uncertain assignment produces biased results? *Ecography*, **38**, 922–929.

Mata, V. A., Rebelo, H., Amorim, F., McCracken, G. F., Jarman, S., & Beja, P. (2019). How much is enough? Effects of technical and biological replication on metabarcoding dietary analysis. *Molecular Ecology*, **28**, 165–175. https://doi.org/10.1111/mec.14779

Menotti-Raymond, M., David, V. A., Lyons, L. A., Schäffer, A. A., Tomlin, J. F., Hutton, M. K., & O'Brien, S. J. (1999). A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics*, **57**, 9-23.

Mhuulu, L. (2015). *Identification of Individual Cheetahs (Acinonyx jubatus) represented in a sample collection, combining non-invasive genetic and camera-trapping techniques*. [Master Thesis, University of Namibia].

Mills, M. G. L. (1991). Conservation management of large carnivores in Africa. *Koedoe*, **34**, 81–90. https://doi.org/10.4102/koedoe.v34i1.417

Mondol, S., Navya, R., Athreya, V., Sunagar, K., Selvaraj, V. M., & Ramakrishnan, U. (2009). A panel of microsatellites to individually identify leopards and its application to leopard monitoring in human dominated landscapes. *BMC genetics*, **10**, 1-7.

Monterroso, P., Castro, D., Silva, T. L., Ferreras, P., Godinho, R., & Alves, P. C. (2013). Factors affecting the (in)accuracy of mammalian mesocarnivore scat identification in South-western Europe. *Journal of Zoology*, **289**, 243–250. https://doi.org/10.1111/jzo.12000

Monterroso, P., Godinho, R., Oliveira, T., Ferreras, P., Kelly, M. J., Morin, D. J., Waits, L. P., Alves, P. C., & Mills, L. S. (2019). Feeding ecological knowledge: the underutilised power of faecal DNA approaches for carnivore diet analysis. *Mammal Review*, **49**, 97–112. https://doi.org/10.1111/mam.12144

Monterroso, P., Rocha, F., Van Wyk, S., António, T., Chicomo, M., Kosmas, S., Lages, F., Fabiano, E., & Godinho, R. (2020). Updated ranges of the Vulnerable cheetah and Endangered African wild dog in Angola. *Oryx*, **54**, 851–853. https://doi.org/10.1017/S0030605319000966

Morin, D. J., Higdon, S. D., Holub, J. L., Montague, D. M., Fies, M. L., Waits, L. P., & Kelly, M. J. (2016). Bias in carnivore diet analysis resulting from misclassification of predator scats based on field identification. *Wildlife Society Bulletin*, **40**, 669–677. https://doi.org/10.1002/wsb.723

Morin, D. J., Higdon, S. D., Lonsinger, R. C., Gosselin, E. N., Kelly, M. J., & Waits, L. P. (2019). Comparing methods of estimating carnivore diets with uncertainty and imperfect detection. *Wildlife Society Bulletin*, **43**, 651–660. https://doi.org/10.1002/wsb.1021

Mukherjee, S., & Heithaus, M. R. (2013). Dangerous prey and daring predators: A review. *Biological Reviews*, **88**, 550–563. https://doi.org/10.1111/brv.12014

Newman, J. (2007). 6. Herbivory. In: Stephens DW, Brown JS, Ydenberg RC (eds). *Foraging: Behavior and Ecology* (pp. 175-218) University of Chicago Press, Chicago.

Nilsen, E. B., Christianson, D., Gaillard, J.-M., Halley, D., Linnel, J. D. C., Odden, M., Panzacchi, M., Toigo, C., & Zimmermann, B. (2012). Describing food habits and predation: field methods and statistical considerations. *Carnivore ecology and conservation: A handbook of techniques*, 256-272. https://doi.org/10.1093/acprof

Novaro, A. J., Funes, M. C., & Walker, R. S. (2000). Ecological extinction of native prey of a carnivore assemblage in Argentina Patagonia. *Biological Conservation*, **92**, 25–33. www.elsevier.com/locate/biocon

Nowak, C., Büntjen, M., Steyer, K., & Frosch, C. (2014). Testing mitochondrial markers for noninvasive genetic species identification in European mammals. *Conservation Genetics Resources*, **6**, 41–44. https://doi.org/10.1007/s12686-013-0068-5

O'Brien, T. G., Ahumada, J., Akampurila, E., Beaudrot, L., Boekee, K., Brncic, T., Hickey, J., Jansen, P. A., Kayijamahe, C., Moore, J., Mugerwa, B., Mulindahabi, F., Ndoundou-Hockemba, M., Niyigaba, P., Nyiratuza, M., Opepa, C. K., Rovero, F., Uzabaho, E., & Strindberg, S. (2020). Camera trapping reveals trends in forest duiker populations in African National Parks. *Remote Sensing in Ecology and Conservation*, **6**, 168–180. https://doi.org/10.1002/rse2.132

Oksanen J, Blanchet FG, Kindt R, Legendre P and others (2020) vegan: community ecology package. R package, version 23-2. https: //cran.r-project. org/ web/ packages/ vegan/

Ordiz, A., Bischof, R., & Swenson, J. E. (2013). Saving large carnivores, but losing the apex predator? *Biological Conservation*, **168**, 128–133. https://doi.org/10.1016/j.biocon.2013.09.024

Østby, N. (2019). DNA Metabarcoding Diet Analysis of Water Vole (*Arvicola amphibius*) in Northern Norway. [Master's Thesis, Norwegian University of Science and Technology].

Otterbeck, A., Lindén, A., & Roualet, É. (2015). Advantage of specialism: reproductive output is related to prey choice in a small raptor. *Oecologia*, **179**, 129–137. https://doi.org/10.1007/s00442-015-3320-8

Overton, J. M. C., Elizalde Castells, D., Figueira Fernandes Elizalde, S. R., Valério, H. M., Alexandre Zumbo, M. N., Groom, R. J., & Durant, S. M. (2020). Endangered African wild dogs (Lycaon pictus Temm.) in Angola: Filling a 50-year gap of knowledge with findings from two National Parks. *African Journal of Ecology*, **58**, 582–587. https://doi.org/10.1111/aje.12715

Overton, J., Fernandes, S., Elizalde, D., Groom, R., & Funston, P. (2017). A Large Mammal Survey of Bicuar and Mupa National Parks, Angola.

Palomares, F., & Caro, T. M. (1999). Interspecific Killing among Mammalian Carnivores. 153(5), 492–508.

Palomares, F., Delibes, M., Revilla, E., Calzada, J., & Fedriani, J. M. (2001). Spatial ecology of Iberian lynx and abundance of European rabbits in southwestern Spain. *Wildlife Monographs*, **148**, 1–36.

Perez, I., Geffen, E., & Mokady, O. (2006). Critically Endangered Arabian leopards Panthera pardus nimr in Israel: Estimating population parameters using molecular scatology. *Oryx*, **40**, 295–301. https://doi.org/10.1017/S0030605306000846

Phillips, R. B., Winchell, C. S., & Schmidt, R. H. (2007). Dietary overlap of an alien and native carnivore on San Clemente Island, California. *Journal of Mammalogy*, **88**, 173–180. https://doi.org/10.1644/06-MAMM-A-015R2.1

Pianka, E. R. (1974). Niche Overlap and Diffuse Competition. *Proceedings of the National Academy of Sciences of the United States of America*, **71**, 2141–2145.

Piñol, J., San Andrés, V., Clare, E. L., Mir, G., & Symondson, W. O. . (2014). A pragmatic approach to the analysis of diets of generalist predators: The use of next generation sequencing with no blocking probes. *Molecular Ecology Resources*, **14**, 18–26. https://doi.org/10.1111/1755-0998.12156

Piñol, Josep, Mir, G., Gomez-Polo, P., & Agustí, N. (2015). Universal and blocking primer mismatches limit the use of high-throughput DNA sequencing for the quantitative metabarcoding of arthropods. *Molecular Ecology Resources*, **15**, 819–830. https://doi.org/10.1111/1755-0998.12355

Pitman, R. T., Kilian, P. J., Ramsay, P. M., & Swanepoel, L. H. (2013). Foraging and habitat specialization by female leopards (Panthera pardus) in the Waterberg Mountains of South Africa. *South African Journal of Wildlife Research*, **43**, 167–176. https://doi.org/10.3957/056.043.0204

Pompanon, François, & Samadi, S. (2015). Next generation sequencing for characterizing biodiversity: promises and challenges. *Genetica*, **143**, 133–138. https://doi.org/10.1007/s10709-015-9816-7

Pompanon, Francois, Deagle, B. E., Symondson, W. O. C., Brown, D. S., Jarman, S. N., & Taberlet, P. (2012). Who is eating what: Diet assessment using next generation sequencing. *Molecular Ecology*, **21**, 1931–1950. https://doi.org/10.1111/j.1365-294X.2011.05403.x

Purcell, M., Mackey, G., LaHood, E., Huber, H., & Park, L. (2004). Molecular methods for the genetic identification of salmonid prey from Pacific harbor seal (*Phoca vitulina richardsi*) scat. *Fishery Bulletin*, **102**, 213–220.

Putman, R. J. (1984). Facts from faeces. *Mammal Review*, **14**, 79–97. https://doi.org/10.1111/j.1365-2907.1984.tb00341.x

Pyke, G. H. (2019). Animal movements - an optimal foraging theory approach. In *Encyclopedia of Animal Behavior* (pp. 149-156). Elsevier Academic Press. https://doi.org/10.1016/B978-0-12-809633-8.90160-2

Rafiq, K., Jordan, N. R., Meloro, C., Wilson, A. M., Hayward, M. W., Wich, S. A., & McNutt, J. W. (2020). Scent-marking strategies of a solitary carnivore: Boundary and road scent marking in the leopard. *Animal Behaviour*, **161**, 115-126. https://doi.org/10.1016/j.anbehav.2019.12.016

Ray, J. ., Hunter, L., & Zigouris, J. (2005). *Setting Conservation and Research Priorities for Larger African Carnivores*. WCS Working Paper No. 24. Wildlife Conservation Society, New York.

Riaz, T., Shehzad, W., Viari, A., Pompanon, F., Taberlet, P., & Coissac, E. (2011). EcoPrimers: Inference of new DNA barcode markers from whole genome sequence analysis. *Nucleic Acids Research*, **39**, 1–11. https://doi.org/10.1093/nar/gkr732

Risbey, D. A., Calver, M. C., & Short, J. (1999). The impact of cats and foxes on the small vertebrate fauna of Heirisson Prong, Western Australia. I. Exploring potential impact using diet analysis. *Wildlife Research*, **26**, 621–630. https://doi.org/10.1038/261637a0

Robertson, A., Mcdonald, R. A., Delahay, R. J., Kelly, S. D., & Bearhop, S. (2015). Resource availability affects individual niche variation and its consequences in group-living European badgers *Meles meles*. *Oecologia*, **178**, 31–43. https://doi.org/10.1007/s00442-014-3202-5

Rocha, F., Kosmas, S., Chicomo, M., António, T., Lages, F., Godinho, R. & Monterroso, P. (2019). Monitorização de mamíferos no Parque Nacional do Bicuar – Relatório do Período 2017-2018.

CIBIO/InBIO-UP & ISCED-Huíla. Lubango, Angola

Ron, T. (2015). Preliminary Assessment of eight National Parks and one Strict Nature Reserve for planning further Project and Government Interventions. *Ministério do Ambiente–Projecto Nacional da Biodiversidade: Conservação do Parque Nacional do Iona.*

Ruppert, K. M., Kline, R. J., & Rahman, M. S. (2019). Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. *Global Ecology and Conservation*, **17**, e00547. https://doi.org/10.1016/j.gecco.2019.e00547

Scheepers, J. L., & Gilchrist, D. (1991). Leopard predation on giraffe calves in the Etosha National Park. *Madoqua*, **18**, 49.

Schoener, T. W. (1986). *Resource partitioning. Community ecology pattern and process* (eds Kikkawa, J. & Anderson, D.J.). Blackwell Scientific, Boston, pp. 91– 126.

Searle, C. E., Bauer, D. T., Kesch, M. K., Hunt, J. E., Mandisodza-Chikerema, R., Flyman, M. V., Macdonald, D. W., Dickman, A. J., & Loveridge, A. J. (2020).

Drivers of leopard (*Panthera pardus*) habitat use and relative abundance in Africa's largest transfrontier conservation area. *Biological Conservation*, **248**, 108649. https://doi.org/10.1016/j.biocon.2020.108649

Shehzad W, Nawaz MA, Pompanon F, Coissac E, Riaz T, Shah SA, Taberlet P. 2015. Forest without prey: livestock sustain a leopard Panthera pardus population in Pakistan. *Oryx,* **49,** 248–253. https://doi.org/10.1017/S0030605313001026.

Shehzad, W., Riaz, T., Nawaz, M. A., Miquel, C., Poillot, C., Shah, S. A., Pompanon, F., Coissac, E., & Taberlet, P. (2012). Carnivore diet analysis based on next-generation sequencing: Application to the leopard cat (Prionailurus bengalensis) in Pakistan. *Molecular Ecology*, **21**, 1951–1965. https://doi.org/10.1111/j.1365-294X.2011.05424.x

Sheppard, S. K., & Harwood, J. D. (2005). Advances in molecular ecology: Tracking trophic links through predator-prey food-webs. *Functional Ecology*, **19**, 751–762. https://doi.org/10.1111/j.1365-2435.2005.01041.x

Shi, Y., Hoareau, Y., Reese, E. M., & Wasser, S. K. (2021). Prey partitioning between sympatric wild carnivores revealed by DNA metabarcoding : a case study on wolf (*Canis lupus*) and coyote (*Canis latrans*) in northeastern Washington. *Conservation Genetics*, **22**, 293–305. https://doi.org/10.1007/s10592-021-01337-2

Shokralla, S., Spall, J. L., Gibson, J. F., & Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology*, **21**, 1794–1805. https://doi.org/10.1111/j.1365-294X.2012.05538.x

Soininen, E. M., Valentini, A., Coissac, E., Miquel, C., Gielly, L., Brochmann, C., Brysting, A. K., Sønstebø, J. H., Ims, R. A., Yoccoz, N. G., & Taberlet, P. (2009). Analysing diet of small herbivores: The efficiency of DNA barcoding coupled with high-throughput pyrosequencing for deciphering the composition of complex plant mixtures*. Frontiers in Zoology*, **6**, 1–9. https://doi.org/10.1186/1742-9994-6-16

Spong, G. (2002). Space use in lions, Panthera leo, in the Selous Game Reserve: Social and ecological factors. *Behavioral Ecology and Sociobiology*, **52**, 303–307. https://doi.org/10.1007/s00265-002-0515-x

Stein, A. B. (2008). *Ecology and Conservations of the Leopard (Panthera pardus Linnaeus 1758) in Northcentral Namibia*. University of Massachusetts Amherst.

Stein, A. B., Fuller, T. K., Destefano, S., & Marker, L. L. (2011). Leopard population and home range estimates in north-central Namibia. *African Journal of Ecology*, **49**, 383–387.

Stephens, D. W. & Krebs, J. R. 1986. *Foraging Theory*. Princeton, New Jersey: Princeton University Press.

Steyn, V., & Funston, P. J. (2009). Land-use and socio-spatial organization of female leopards in a semi-arid wooded savanna, botswana. *African Journal of Wildlife Research*, **39**, 126–132. https://doi.org/10.3957/056.039.0203

Sudhir Kumar, Glen Stecher, Michael Li, Christina Knyaz, and Koichiro Tamura (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution,* **35,** 1547-1549

Svanbäck, R., & Bolnick, D. I. (2005). Intraspecific competition affects the strength of individual specialization: An optimal diet theory method. *Evolutionary Ecology Research*, **7**, 993–1012.

Taberlet P, Bonin A, Zinger L, Coissac E (2018) *Environmental DNA: For Biodiversity Research and Monitoring*. Oxford University Press, Oxford. doi: 10.2093/oso/9780198767220.001.0001

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., & Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. *Molecular Ecology*, **21**, 2045–2050. https://doi.org/10.1111/j.1365-294X.2012.05470.x

Tarugara, A., Clegg, B.W., Gandiwa, E., & Muposhi, V.K. (2020). The effect of competing carnivores on the feeding behaviour of leopards (*Panthera pardus*) in an African savanna. *Ecology and Evolution,* **11,** 7743-7753. https://doi.org/10.1002/ece3.7608

Thomas, A. C., Deagle, B. E., Eveson, J. P., Harsch, C. H., & Trites, A. W. (2016). Quantitative DNA metabarcoding: Improved estimates of species proportional biomass using correction factors derived from control material. *Molecular Ecology Resources*, **16**, 714–726. https://doi.org/10.1111/1755-0998.12490

Thomas, A. C., Jarman, S. N., Haman, K. H., Trites, A. W., & Deagle, B. E. (2014). Improving accuracy of DNA diet estimates using food tissue control materials and an evaluation of proxies for digestion bias. *Molecular Ecology*, **23**, 3706–3718. https://doi.org/10.1111/mec.12523

Thukral, A. K. (2017). A review on measurement of Alpha diversity in biology. *Agricultural Research Journal,* **54**, 1-10. https://doi.org/10.5958/2395-146X.2017.00001.1

Tinker, M. T., Bentall, G., & Estes, J. A. (2008). Food limitation leads to behavioral diversification and dietary specialization in sea otters. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 560–565. https://doi.org/10.1073/pnas.0709263105

Tollit, D. J., Schulze, A. D., Trites, A. W., Olesiuk, P. F., Crockford, S. J., Gelatt, T. S., Ream, R. R., & Miller, K. M. (2009). Development and application of DNA techniques for validating and improving pinniped diet estimates. *Ecological Applications*, **19**, 889–905. https://doi.org/10.1890/07-1701.1

Uphyrkina, O., Johnson, W. E., Quigley, H., Miquelle, D., Marker, L., Bush, M., & O'Brien, S. J. (2001). Phylogenetics, genome diversity and origin of modern leopard, Panthera pardus. *Molecular ecology*, **10**, 2617-2633.

Valsecchi, E., Bylemans, J., Goodman, S. J., Lombardi, R., Carr, I., Castellano, L., Galimberti, A., & Galli, P. (2020). Novel universal primers for metabarcoding environmental DNA surveys of marine mammals and other marine vertebrates. *Environmental DNA*, **2**, 460–476. https://doi.org/10.1002/edn3.72

van de Pol, M., Brouwer, L., Ens, B. J., Oosterbeek, K., & Tinbergen, J. M. (2010). Fluctuating selection and the maintenance of individual and sex-specific diet specialization in free-living oystercatchers. *Evolution*, **64**, 836–851. https://doi.org/10.1111/j.1558-5646.2009.00859.x

Vestheim, H., & Jarman, S. N. (2008). Blocking primers to enhance PCR amplification of rare sequences in mixed samples - A case study on prey DNA in Antarctic krill stomachs. *Frontiers in Zoology*, **5**, 1–11. https://doi.org/10.1186/1742-9994-5-12

Voigt, C. C., Krofel, M., Menges, V., Wachter, B., & Melzheimer, J. (2018). Sex-specific dietary specialization in a terrestrial apex predator, the leopard, revealed by stable isotope analysis. *Journal of Zoology*, **306**, 1–7. https://doi.org/10.1111/jzo.12566

Volume: Kingdon, J. & Hoffmann, M. (eds) (2013b). *Mammals of Africa. Volume V: Carnivores, Pangolins, Equids and Rhinoceroses*. Bloomsbury Publishing, London, UK.

Votier, S. C., Fayet, A. L., Bearhop, S., Bodey, T. W., Clark, B. L., Grecian, J., Guilford, T., Hamer, K. C., Jeglinski, J. W. E., Morgan, G., Wakefield, E., & Patrick, S. C.

(2017). Effects of age and reproductive status on individual foraging site fidelity in a long-lived marine predator. *Proceedings of the Royal Society B: Biological Sciences*, **284**, 20171068. https://doi.org/10.1098/rspb.2017.1068

Watanabe, Y. Y., Ito, M., & Takahashi, A. (2014). Testing optimal foraging theory in a penguin-krill system. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 20132376. https://doi.org/10.1098/rspb.2013.2376

Wegge, P., Odden, M., Pokharel, C. P., & Storaas, T. (2009). Predator-prey relationships and responses of ungulates and their predators to the establishment of protected areas: A case study of tigers, leopards and their prey in Bardia National Park, Nepal. *Biological Conservation*, **142**, 189–202. https://doi.org/10.1016/j.biocon.2008.10.020

Weiskopf, S. R., Kachel, S. M., & McCarthy, K. P. (2016). What are snow leopards really eating? Identifying bias in food-habit studies. *Wildlife Society Bulletin*, **40**, 233–240. https://doi.org/10.1002/wsb.640

Wolf, C., & Ripple, W. J. (2016). Prey depletion as a threat to the world's large carnivores. *Royal Society Open Science*, **3,** 160252. https://doi.org/10.1098/rsos.160252

Woo, K. J., Elliott, K. H., Davidson, M., Gaston, A. J., & Davoren, G. K. (2008). Individual specialization in diet by a generalist marine predator reflects specialization in foraging behaviour. *Journal of Animal Ecology*, **77**, 1082–1091. https://doi.org/10.1111/j.1365-2656.2008.01429.x

Xiong, M., Wang, D., Bu, H., Shao, X., Zhang, D., Li, S., Wang, R., & Yao, M. (2017). Molecular dietary analysis of two sympatric felids in the Mountains of Southwest China biodiversity hotspot and conservation implications. *Scientific Reports*, **7**, 1–12. https://doi.org/10.1038/srep41909

Yahnke, C. J. (2006). Optimal foraging theory using bird predation on goldenrod calls. *American Biology Teacher*, **68**, 471–475. https://doi.org/10.1662/0002-7685(2006)68[471:TOFTUB]2.0.CO;2

Zaccarelli, N., Bolnick, D. I., & Mancinelli, G. (2013). RInSp: An r package for the analysis of individual specialization in resource use. *Methods in Ecology and Evolution*, **4**, 1018–1023. https://doi.org/10.1111/2041-210X.12079

Zeale, M. R. K., Butlin, R. K., Barker, G. L. A., Lees, D. C., & Jones, G. (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. *Molecular Ecology Resources*, **11**, 236–244. https://doi.org/10.1111/j.1755-0998.2010.02920.x

Zehra, N. (2013). *Study of large mammalian prey-predators of Gir Lion Sanctuary with special reference to ecology of Leopard (Panthera pardus fusca)* [Doctoral dissertation, Aligarh Muslim University].

Supplementary Information - Tables

Table 1_SI. Information about the collection of each leopard scat sample - Information about laboratory ID, Date, who found the scat, the individual identification and sex.

Lab Sample-ID	Date	Found-by	Individual	Sex
2B003	12/06/2018	SD team		
2B008	13/06/2018	SD team		
2B010	13/06/2018	Filipe		
2B014	14/06/2018	SD team		
2B015	14/06/2018	Filipe		
2B030	17/06/2018	SD team		
2B031	18/06/2018	SD team	PP01	Female
2B032	18/06/2018	SD team	PP01	Female
2B033	18/06/2018	SD team	PP01	Female
2B034	18/06/2018	SD team	PP01	Female
2B035	19/06/2018	SD team	PP01	Female
2B036	19/06/2018	SD team	PP01	Female
2B038	19/06/2018	SD team		
2B040	19/06/2018	SD team		
2B041	19/06/2018	SD team		
2B044	19/06/2018	SD team	PP02	Female
2B045	19/06/2018	SD team		
2B063	21/06/2018	SD team	PP06	Female
2B066	22/06/2018	SD team		
2B069	22/06/2018	SD team		
2B070	22/06/2018	SD team	PP02	Female
2B071	22/06/2018	Filipe	PP01	Female
2B103	17/11/2018	Filipe		
2B114	20/11/2018	Filipe		
2B125	22/11/2018	Milcíades		
2B126	22/11/2018	Milcíades		
2B128	23/11/2018	Manuel		
2B144	24/11/2018	Filipe		
ANG10	12/06/2019	N/A		
ANG109	25/06/2019	dog	PP03	Male
ANG112	26/06/2019	Dog	PP01	Female
ANG113	26/06/2019	Human	PP01	Female
ANG116	26/06/2019	Dog		
ANG117	26/06/2019	Dog		
ANG118	26/06/2019	Human	PP09	Male
ANG124	26/06/2019	Dog	PP03	Male
ANG125	27/06/2019	Human	PP02	Female
ANG126	27/06/2019	Dog	PP02	Female
ANG131	27/06/2019	Dog	PP09	Male
ANG133	27/06/2019	Dog		
ANG135	28/06/2019	Dog	PP02	Female

ANG136	28/06/2019	Human		
ANG15	13/06/2019	N/A		
ANG158	01/07/2019	Dog		
ANG161	01/07/2019	Dog		
ANG162	01/07/2019	N/A	PP11	Male
ANG169	02/07/2019	Dog	PP12	Female
ANG204	05/11/2019	Dog	PP03	Male
ANG218	07/11/2019	Human		
ANG22	16/06/2019	N/A	PP01	Female
ANG239	07/11/2019	N/A	PP13	Male
ANG24	16/06/2019	N/A	PP01	Female
ANG248	08/11/2019	Human		
ANG265	09/11/2019	Human	PP09	Male
ANG275	09/11/2019	Human		
ANG28	17/06/2019	N/A	PP10	Female
ANG283	07/11/2019	Human		
ANG286	07/11/2019	N/A		
ANG287	08/11/2019	N/A		
ANG288	08/11/2019	N/A		
ANG289	08/11/2019	N/A		
ANG29	17/06/2019	N/A	PP10	Female
ANG293	12/11/2019	N/A		
ANG295	12/11/2019	Human		
ANG297	11/11/2019	Human		
ANG299	13/11/2019	Dog		
ANG302	13/11/2019	Human		
ANG304	13/11/2019	Human		
ANG305	13/11/2019	Human		
ANG307	14/11/2019	Human		
ANG313	15/11/2019	Human		
ANG315	15/11/2019	Dog	PP09	Male
ANG331	12/11/2019	N/A		
ANG332	12/11/2019	N/A		
ANG334	12/11/2019	N/A		
ANG339	11/11/2019	N/A		
ANG346	19/11/2019	Human		
ANG355	24/11/2019	Human		
ANG356	24/11/2019	dog	PP14	Female
ANG42	18/06/2019	N/A		
ANG43	18/06/2019	N/A	PP10	Female
ANG48	19/06/2019	N/A		
ANG51	23/06/2019	Dog	PP03	Male
ANG52	23/06/2019	Dog		
ANG56	24/06/2019	Dog		
ANG69	24/06/2019	Dog		
ANG70	24/06/2019	Dog	PP06	Female
ANG74	24/06/2019	Dog	PP06	Female

ANG8	12/06/2019	N/A		
ANG84	24/06/2019	Filipe		
ANG91	24/06/2019	Dog	PP03	Male
B011	22/03/2019	Dog-Levi		
B012	23/03/2019	Dog-Levi		
BNPS01F	23/03/2019	Filipe		
EXC12_R	01/07/2017	Pedro and Raquel	PP06	Female
EXC13_R	01/07/2017	Pedro and Raquel	PP02	Female
Tch_002	12/07/2017	Tchacaca	PP01	Female
Tch_003	13/07/2017	Tchacaca	PP04	Female
Tch_004	14/07/2017	Tchacaca	PP01	Female
Tch_016	13/07/2017	Tchacaca		
Tch_017	13/07/2017	Tchacaca	PP01	Female
Tch_018	13/07/2017	Tchacaca		
Tch_019	18/07/2017	Tchacaca	PP04	Female
Tch_021	20/07/2017	Tchacaca		
Tch_027	21/07/2017	Tchacaca		
Tch_028	21/07/2017	Tchacaca	PP01	Female
Tch_029	23/07/2017	Tchacaca	PP01	Female
Tch_030	23/07/2017	Tchacaca	PP01	Female
Tch_031	23/07/2017	Tchacaca		
Tch_032	23/07/2017	Tchacaca		
Tch_033	23/07/2017	Tchacaca	PP01	Female
Tch_034	23/07/2017	Tchacaca	PP05	Male
Tch_035	23/07/2017	Tchacaca		
Tch_036	23/07/2017	Tchacaca	PP05	Male
Tch_038	23/07/2017	Tchacaca		
Tch_039	23/07/2017	Tchacaca	PP02	Female
Tch_041	25/07/2017	Tchacaca	PP03	Male
Tch_042	25/07/2017	Tchacaca	PP07	Female
Tch_043	31/07/2017	Tchacaca	PP08	Male
Tch_044	28/07/2017	Tchacaca	PP03	Male
Tch_045	28/07/2017	Tchacaca		
Tch_046	28/07/2017	Tchacaca		
Tch_047	28/07/2017	Tchacaca	PP03	Male
Tch_048	28/07/2017	Tchacaca	PP03	Male
Tch_049	28/07/2017	Tchacaca	PP01	Female
Tch_050	28/07/2017	Tchacaca	PP03	Male
Tch_052	28/07/2017	Tchacaca	PP03	Male
Tch_055	28/07/2017	Tchacaca	PP03	Male
Tch_056	28/07/2017	Tchacaca		
Tch_057	28/07/2017	Tchacaca	PP01	Female
Tch_058	28/07/2017	Tchacaca	PP03	Male
Tch_060	28/07/2017	Tchacaca	PP03	Male
Tch_061	30/07/2017	Tchacaca	PP03	Male
Tch_063	08/11/2017	Tchacaca	PP01	Female

Tch_064	08/12/2017	Tchacaca	PP01	Female
Tch_065	08/12/2017	Tchacaca	PP01	Female
Tch_066	13/08/2017	Tchacaca	PP01	Female
Tch_067	15/08/2017	Tchacaca		
Tch_068	15/08/2017	Tchacaca	PP03	Male
Tch_070	15/08/2017	Tchacaca	PP03	Male

Table 2_SI. List of elutions used, dilutions perfmoed and amount of DNA used for PCR for the testing of 12S fragment and 12S-V5 primers – This list comprises the sample names and respective elution used, dilution performed and DNA amount of DNA used for PCR testing of primers and 12S fragment. Sample name is matched to the species in Table 2. * samples kindly offered by Professor Bettine van Vuuren (Centre for Ecological Genomics & Wildlife Conservation, University of Johannesburg); [&]samples available at CIBIO tissue collection.

List of Elutions used, Dilutions performed, and Amount of DNA used for PCR

Sample Name	Elution used	Dilution performed	DNA (µL)
8*	I elution	1:4	1.2
6595*	I elution	1:5	1.2
Fli307*	I elution	1:2	1.5
26 HN150*	I elution	-	3
A5842*	I elution	-	1.5
C7097*	I elution	1:2	2
HN565*	I elution	1:2	2
Sax1a*	I elution	-	3
Sax4a*	I elution	1:2	2
UJ2418[&]	I elution	1:2	2
UJ1850[&]	I elution	-	3
UJ2833[&]	I elution	-	3
UJ1865[&]	I elution	-	3
UJ2628[&]	II elution	1:2	2
UJ1862[&]	I elution	-	3
UJ1866[&]	I elution	-	3
UJ1868[&]	I elution	-	3
UJ1869[&]	I elution	-	3
UJ1888[&]	I elution	-	3
UJ1891[&]	I elution	-	3
UJ1964[&]	I elution	-	3
UJ2630[&]	II elution	1:2	2
UJ2742[&]	I elution	-	3
UJ2744[&]	I elution	-	3

Table 3_SI. PCR Mix used for 12S fragment and primer testing – The mix was prepared by adding 5 µL of Master Mix, 0.3 µL of both primers and 3.4 µL of H_2O per sample (1x).

PCR MIX

Reagents	Quantity per sample (1x)
Master Mix	5 µL
Primer Forward	0.3 µL
Primer Reverse	0.3 µL
H_2O	3.4 µL

Table 4_SI. Thermocycling conditions for the PCR for testing primers and the 12S fragment – The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95ºC for 15 minutes; then 40 cycles of denaturing, annealing and elongation at 95 ºC, 47 ºC and 72 ºC respectively, during 30, 20 and 30 seconds, respectively; and a final elongation at 60 ºC for 10 minutes

Thermocycling conditions PCR

Temperature (ºC)	Duration	Number of cycles
95ºC	15 min	1x
95ºC	30 sec	40x
47ºC	30 sec	
72ºC	30 sec	
60ºC	10 min	1x
12ºC	∞	

Table 5_SI. Sanger Sequencing Mix used for 12S fragment and primer testing – The mix was prepared by adding 0.5 µL of TRR Mix, 1 µL of TRR Buffer, 0.5 µL of one of the primers and 6 µL of H_2O per sample (1x).

SANGER SEQUENCING MIX

Reagents	Quantity per sample (1x)
TRR Mix	0.5 *µL*
TRR Buffer	1 *µL*
Primer Forward/Reverse (1 of them)	0.5 *µL*
H_2O	6 *µL*

Table 6_SI. INDEX PCR MIX - This mix was prepared by adding 7 µL of 2x Kappa HiFi Hot Start enzyme, 0.7 µL of each index and 2.8 µL of H_2O and DNA per sample (1x).

INDEX PCR MIX

Reagents	Quantity per sample (1x)
2x Kappa HiFi Hot Start enzyme	7 *µL*
Index N7xx	0.7 *µL*
Index S5xx	0.7 *µL*
H2O	2.8 *µL*
DNA	2.8 *µL*

Table 7_SI. NGS Library Prep Index PCR - The thermocycling conditions set were the following: Initial step of pre-denaturation of 1 cycle at 95ºC for 3 minutes; then 8 cycles of denaturing, annealing and elongation at 95 ºC, 55 ºC and 72 ºC respectively, during seconds each; and a final elongation at 72 ºC for 5 minutes.

Thermocycling Conditions INDEX PCR

Temperature (ºC)	Duration	Number of cycles
95ºC	3 min	1x
95ºC	30 sec	8x
55ºC	30 sec	
72ºC	30 sec	
72ºC	5 min	1x
10ºC	∞	

Table 8_SI . Results from Qubit Quantification – Table showing the results of the Qubit quantification for the pools of both replicates. Quantification was done two times (#1 and #2) and the mean was calculated.

			Qubit BR			
Sample Read#	Pool	Size (bp)	#1	#2	Média (ng/µL)	**nM**
#1	Leopardos Panthera pardus_12S_Rep1	314	4.89	4.64	4.77	23.0
#2	Leopardos Panthera pardus_12S_Rep2	318	6.04	5.96	6.00	28.6

Table 9_SI. Normalization for a final pool of 4nM

Sample Read#	Pool	Samples	%	Pool Conc. nM	Pool volume (Volume inicial da dil)	Tris + Tween Volume	µl final pool (= %)
#1	Leopardos Panthera pardus_12S_Rep1	144	19.3%	23.0	3.4	15.9	19.3
#2	Leopardos Panthera pardus_12S_Rep2	144	19.3%	28.6	2.7	16.6	19.3

Table 10_SI. Results from qPCR validation of the NGS library

Sample	**Cq**	**Cq Mean**	**Starting Quantity (SQ)**	**SQ Mean**	**Size (bp)**	**Dilution**	**SQ mean Av.**		**Conc (pM)**	**Conc (nM)**
Pool final d1000	17.13	17.11	0.13136	0.13371	295	10000	0.13371	2048.709	2230.50508	2.2
Pool final d1000	17.16	17.11	0.12931	0.13371	295	10000				
Pool final d2000	17.96	17.93	0.07709	0.07872	295	20000	0.07872	2412.301		
Pool final d2000	17.9	17.93	0.08036	0.07872	295	20000				

Supplementary Information - Figures

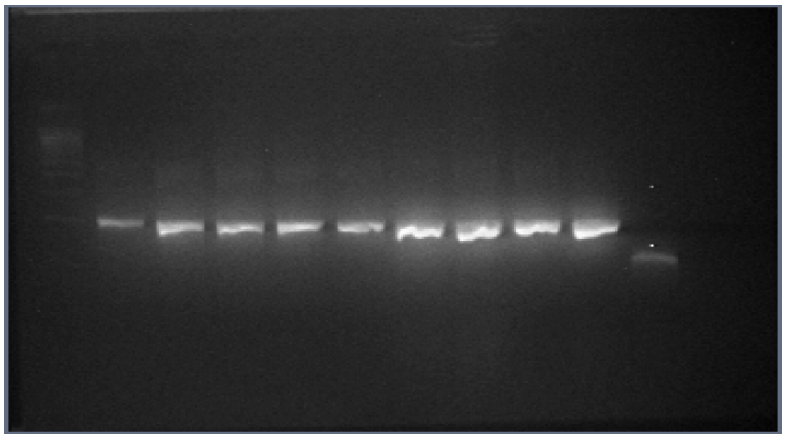


Figure 1_SI – Results of the PCR test of the 9 CIBIO samples (as listed in TABLE 2.1), with electrophoresis on agarose gel at 2%

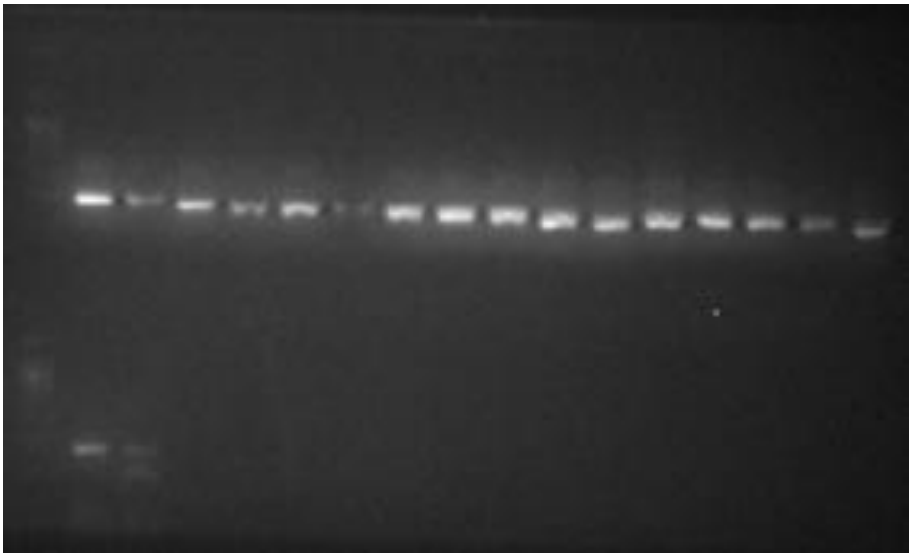


Figure 2_SI - Results of the PCR test of the samples from Centre for Ecological Genomics & Wildlife Conservation, University of Johannesburg, with electrophoresis on agarose gel at 2%

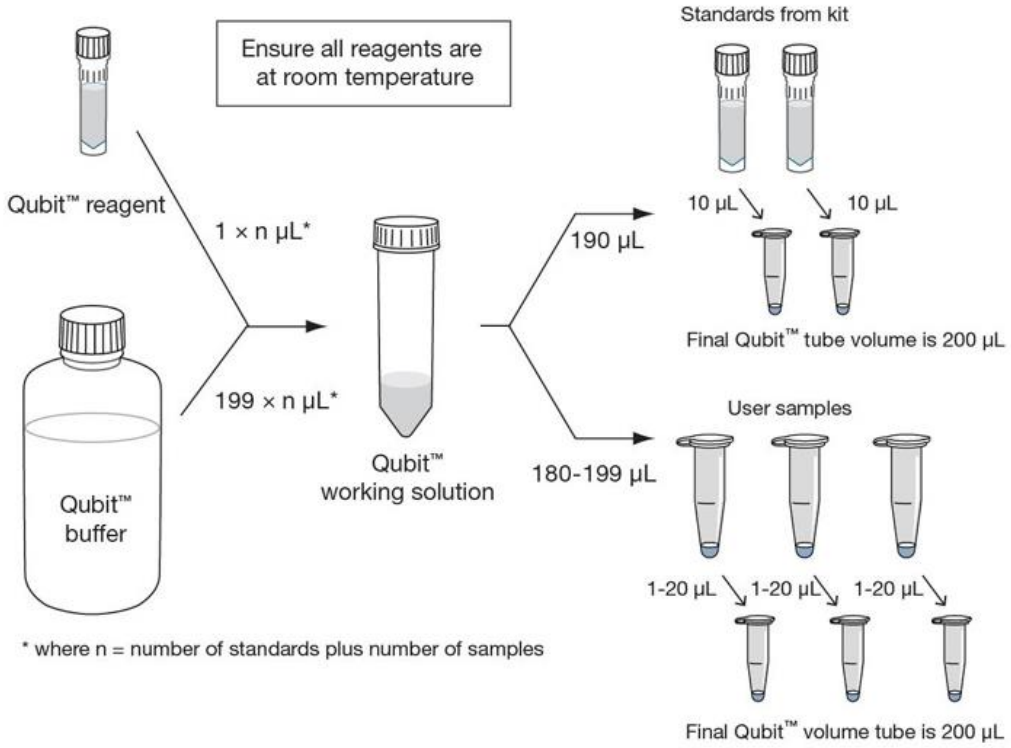


Figure 3_SI. Qubit standard/sample preparation workflow. Image retrieved from Quick Reference Qubit ASSAYS PDF file (Pub. No. MAN0017210), Thermo Fisher

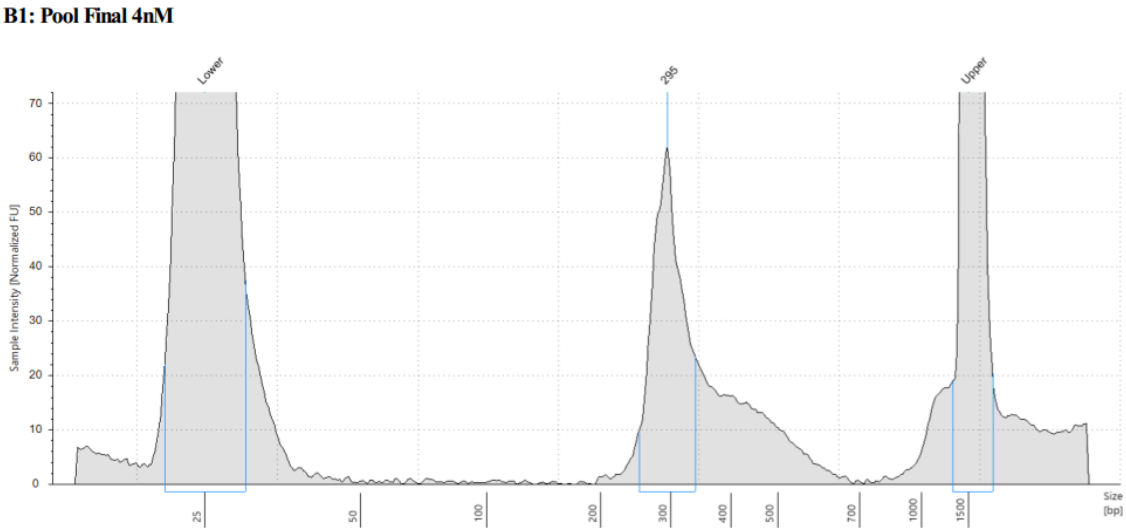


Figure 4_SI -> 10000 TapeStation Results – Library showed a clear peak in 295 bp