Universiteit Leiden
The Netherlands

What's in the diet? DNA-based analysis for qualitative and quantitative assessment of animal diet

Groen, K.

Citation

Groen, K. (2024, October 9). *What's in the diet?: DNA-based analysis for qualitative and quantitative assessment of animal diet*. Retrieved from https://hdl.handle.net/1887/4094106

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/4094106

Note: To cite this publication please use the final published version (if applicable).

What's in the diet?

DNA-based analysis for qualitative and quantitative assessment of animal diet

Kevin Groen

Institute of Environmental Sciences

Leiden University

2024

© Kevin Groen (2024).

Title: What’s in the diet?

Subtitle: *DNA-based analysis for qualitative and quantitative assessment of animal diet.*

PhD thesis at Leiden University, the Netherlands.
ALL RIGHTS RESERVED.

ISBN: 978-94-6473-576-5

Cover Design by Suzanne Heesbeen & Kevin Groen

Layout by Kevin Groen

Printed by Ipskamp Printing

This research was carried out at the Institute of Environmental Sciences of Leiden University, The Netherlands. The described work was supported by Bayer AF, the Royal Netherlands Academy of Arts and Sciences (KNAW) and the National Geographic Society (grant number EC-51438R-18).

What’s in the diet?

DNA-based analysis for qualitative and quantitative assessment of animal diet

Proefschrift

ter verkrijging van

de graad van doctor aan de Universiteit Leiden,

op gezag van rector magnificus prof.dr.ir. H. Bijl,

volgens besluit van het college voor promoties

te verdedigen op woensdag 9 oktober 2024

klokke 16:00 uur

door

Kevin Groen

geboren te Schiedam, Nederland

in 1991

Promotor:

Prof. dr. P. M. van Bodegom

Co-promotor:

Dr. K. B. Trimbos

Promotiecommissie:

Prof. dr. ing. M. G. Vijver

Prof. dr. G. R. de Snoo (NIOO-KNAW)

Prof. dr. B. Gravendeel (Naturalis & Radboud University Nijmegen)

Dr. M. P. Veldhuis

Dr. R. Brys (Instituut Natuur- en Bosonderzoek)

It’s not what you look at that matters,

it’s what you see |

Henry David Thoreau

Table of Contents

Chapter 1 | **11**

General Introduction

Chapter 2 | **29**

Establishment of a fecal DNA quantification technique
for rare and cryptic diet constituents in small mammals

Chapter 3 | **51**

DNA-based seed intake quantification for
enhanced ecological risk assessment of small mammals

Chapter 4 | **71**

DNA metabarcoding illuminates the contribution
of small and very small prey taxa to the diet of lions

Chapter 5 | **91**

Exploring the effect of fencing on the diet composition
and prey preference of lions using DNA-based diet analysis:
a case study in four Kenyan National Parks

Chapter 6 | **115**

General Discussion

References **130**

Summary **151**

Samenvatting **155**

Acknowledgements **159**

Curriculum Vitae **161**

List of publications **162**

1|

General Introduction

1.1 | Diet studies in ecology

For decades, biologists have been confronted with a deceptively simple question: *"What's in the diet?"* This question serves as the cornerstone for unraveling the trophic interactions that shape ecological communities and regulate the flow of energy and nutrients within food webs (McCann, 2007; Kartzinel et al., 2015; Meyer et al., 2020). Diet studies play a crucial role in ecology by providing insights into how animals specialize in their resource utilization within and between species (to minimize competition) and the intricacies of their nutritional physiology (Del Rio et al., 2009; Razgour et al., 2011; Kratina et al., 2012). When aggregated across entire ecological communities, records of dietary composition wield the power to illuminate the structure and function of complete ecosystems (Estes et al., 2011; Meyer et al., 2020); because trophic relationships serve as crucial regulators of community dynamics and ecosystem functionality, our comprehension of natural systems hinges on rigorous investigations into animal feeding habits (Nielsen et al., 2017). Understanding what mammals eat, both qualitatively and quantitatively, is thus a fundamental question in ecology.

Answering this fundamental question yields a ton of ecological applications which already helped to inform conservation strategies, support sustainable ecosystem management, and contribute to our understanding of the natural world (see Nielsen et al., 2017). For example, next to understanding food webs, trophic interactions (who eats who) and energy flows through ecosystems as already mentioned in the above paragraph, dietary information can aid in modeling energy budgets and nutrient cycling (Fernandes et al., 2014). Furthermore, insights into resource partitioning are essential for managing and maintaining biodiversity and coexistence among species (Schoener, 1974). Additionally, diet studies contribute to our understanding of the dynamics of predator-prey relationships, competition, and predation, which are crucial for population regulation and management (Traugott et al., 2020). Identifying the behavior of keystone species and their impact on their ecosystems, which is disproportionately large, often relies on diet studies and conserving these species is critical for maintaining ecosystem integrity (Libralato et al., 2006). Moreover, by examining diet preferences, researchers can gain insights into the preferred habitats and critical information about the dietary requirements of endangered species, helping conservation efforts and habitat management (Castle et al., 2020). Understanding the diets of such endangered or keystone species can also inform ecosystem management practices, such as predator control, reforestation and even controlled burns (Ward et al., 2011; Boukhdoud et al., 2021; Wanniarachchi et al., 2022). For instance, Ward et al. (2011) used data on the distribution, movement, and diet of a generalist marine predator (harbor seals) to identify and map "hot spots" of predation risk for an endangered prey species (rockfish). Moreover, changes in climate, habitat, and land use can influence the availability of food resources for species. Herein, diet studies can help us track and understand how ecosystems respond to these changes. Additionally, the diets of organisms can serve as bioindicators for environmental health as changes in diet can signal environmental stress or contamination (Gül & Griffen, 2020). Furthermore, diet analysis can identify invasive species and infer their impact on native ecosystems, next to potential species that provide valuable ecosystem services by regulating pest or invasive species populations through predation, which is vital for managing invasive species and minimizing their effects on native flora and fauna (Egeter et al., 2019).

Unfortunately, as many animals exhibit diverse diets and engage in intricate spatial and temporal foraging patterns, and local and global environmental disturbances perpetually influence animal physiology and feeding behaviors, the empirical characterization of feeding interactions remains a formidable challenge (Tunney et al., 2014; McMeans et al., 2015). Additionally, the optimal methods and strategies for applying animal dietary analyses to answer ecological questions, have remained undetermined up till now (Klare et al., 2011).

1.2 | Methodologies in animal dietary studies

Ecologists have a variety of techniques for assessing the diets of consumers at their disposal. These methods encompass non-genetic approaches, such as visual examinations of guts, stomachs, feces, or scat contents, as well as utilizing organic macromolecules like fatty acids, and stable isotope analyses of bulk or specific compounds, to identify prey items and genetic approaches (Figure 1.1). While the primary goal of all these methods is to extract information about what animals (or even carnivorous plants) are consuming, these techniques vary significantly in their capacity to qualitatively and quantitatively determine the components of a diet (Traugott et al., 2013).

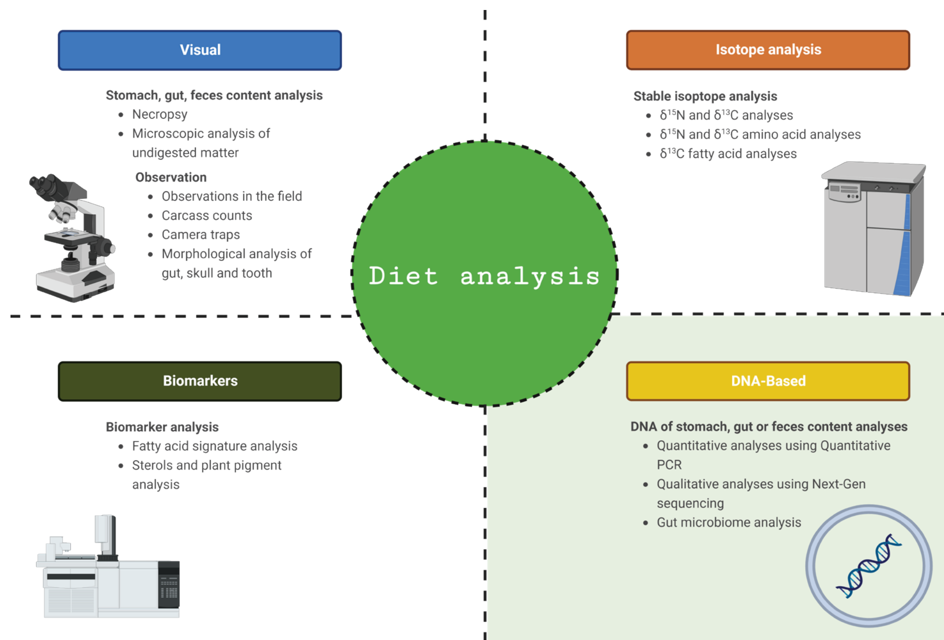

Figure 1.1 | Conceptual overview of common non-genetic and genetic techniques (shaded green) used in animal diet analysis.

1.2.1 | Non-genetic approaches

A common non-genetic method of assessing diet is by stomach, gut, pellet, regurgitated mass or scat content analysis (Klare et al., 2011). For mammals that have been captured or found dead, a necropsy can be performed to examine the contents of their stomachs. This visual method provides precise data but is invasive and limited to specific circumstances. Examining the undigested plant and animal matter in diet remains (mostly feces but also stomach content and regurgitated mass are used) is a non-invasive way to assess diets. It's commonly used for carnivores and herbivores alike but can be very time-consuming, biased by different digestion rates between prey species and tissues and often requires in-depth expertise in (species) identification of the undigested matter, which often involves microscopy (Spaulding et al., 2000).

Diet information can also be visually achieved by field observations. For example, researchers directly observe and record what mammals are eating by following carnivores or counting carcasses in the wild. This method can provide valuable insights into dietary preferences, but it can be extremely time-consuming and challenging for elusive or nocturnal species. Next to researchers going into the field, camera traps can capture images or videos of mammals feeding (including elusive and nocturnal species), allowing for non-invasive observations. Extra information regarding diet can also be obtained by visually assessing skull, tooth and gut morphology, as these can provide insights into a mammal's feeding habits, e.g., whether it's a herbivore, carnivore, or omnivore. Visual approaches are, to date, the only methods that consistently identify different life stages of prey and prey size (Nielsen et al., 2017).

Next to visual methods of assessing diet, isotope analysis is used as a non-genetic technique to infer feeding information. This technique relies on the assumption that a consumer's stable isotope ratios reflect that of its prey (Michener et al., 1994). As different preys often have distinct stable isotope ratios, the relationship between the stable isotope ratio of a consumer and its putative prey can be used to estimate the dietary use of the consumer (Nielsen et al., 2017). For example, one isotope (typically ^{13}C) shows considerable isotopic change during its fixation by primary producers, while another isotope (typically ^{15}N) shows considerable change as it is processed by consumers. Therefore, the combination of these two isotopes allows the investigation of different energy flow processes that shape the structure and function of food webs (Jardine et al., 2006). The offset between the isotope ratios of a consumer and its prey is termed the trophic discrimination factor (TDF). Commonly, for stable isotopes of carbon (^{13}C) TDFs are minimal, typically <1‰, but for stable isotopes of nitrogen (^{15}N) TDF is consistently higher, typically 2‰–4‰ (McCutchan et al., 2003). Hence, a consumer's ^{13}C isotope ratio is used to identify reliance on different primary producers, whereas ^{15}N is used to estimate the consumer's trophic position in the food chain as well as diet (Nielsen et al., 2017). For instance, differences in $\delta^{13}C$ or $\delta^{15}N$ (isotopic ratios are commonly expressed in delta (δ) notation) values can distinguish between a consumer's reliance on C3 and C4 plants, or between benthic and pelagic resources or phytoplankton (Hayden et al., 2014; Hayden et al., 2015).

One of the strengths of stable isotope analyses over alternative methods lies in its capacity to integrate information about an animal's diet over extended time frames (Nielsen et al., 2017). The temporal integration enables researchers to deduce dietary utilization over varying periods, from days (blood, plasma) to years (bone, fish otoliths) (see Vander Zanden et al., 2015). Nevertheless, these analyses might not always paint a complete picture of an organism's diet, as they're typically confined to tracking a limited number of diet resources with distinct isotopic signatures. Additionally, inputs of nutrients from exogenous sources, both natural and anthropogenic in origin, are common in many aquatic and terrestrial food webs. These nutrients often have distinct isotopic ratios, their influence can vary spatially and temporally, and as a result can cause primary producers to vary in their isotopic ratios both within and across systems and cause uncertainty regarding variations in the isotope baseline (Jardine et al., 2006; Nielsen et al., 2017).

A third approach to non-genetically assessing the diet of an animal is analyzing biomarkers, such as fatty acids, sterols and pigments. Fatty acid signature analysis is based on a particular diet item often having a unique fatty acid profile, allowing depiction and differentiation among dietary sources such as bacteria, fungi, terrestrial and aquatic plants. When an organism consumes these sources, these unique signatures are incorporated into its fatty acid composition (Dalsgaard et al., 2003). For instance, terrestrial plants often contain specific fatty acids, like high proportions of long-chain saturated fatty acids, while marine sources (e.g. algae) are normally rich in essential omega-3 and omega-6 fatty acids (Nielsen et al., 2017). By analyzing the fatty acid composition of an organism's tissues, scientists can deduce the types of fatty acids present and thereby infer the likely dietary sources. Similarly, trace elements such as cholesterols, phytosterols and algal pigments can function as alternative biomarkers. For instance, since pigment composition can be algae-specific, algal pigments have been used to estimate the diet of zooplankton (Letelier et al., 1993). As a major drawback, similar to the stable isotope approach, using the biomarker approach it is rarely possible to identify all items contributing to a consumer's diet (Traugott et al., 2013), although quantitative inferences can be drawn and trophic linkages can be inferred from both these techniques (Nielsen et al., 2017).

1.2.2 | Genetic approaches

DNA-Based diet analysis is based on DNA sequencing techniques that can identify the DNA of ingested species in scat or stomach contents. This provides highly specific and accurate dietary information, allowing for both qualitative assessments using Next Generation Sequencing (NGS) and quantitative assessments via quantitative PCR (qPCR) or droplet digital PCR (ddPCR) (Monterroso et al., 2018). Recent genetic diet research has also focused on analyzing the gut microbiome to understand dietary patterns in mammals as the microbial communities in the gut can be indicative of diet type (Angelakis et al., 2016). In the following paragraphs we will explore the advantages and disadvantages of genetic approaches in more depth.

1.3 | Challenges in non-genetic animal dietary studies

Traditionally, the study of animal diet has relied on non-genetic methods such as direct observation of foraging behavior, carcass counts and microscopic identification of prey remains in fecal and stomach contents. While these techniques have provided valuable insights into the diets of various species, they are not without limitations, often characterized by biases, incomplete data, and destructive sampling (Liu et al., 2021).

Field behavior observation (including carcass counts) yields results that are qualitatively descriptive but lack operational efficiency (i.e. time consuming and expensive) and (very) small prey are likely to be missed more often compared to large prey or carcasses (Pierce & Boyle, 1991). Stomach contents analysis, while informative, is a destructive approach and may not be widely accepted in mammalian studies (Amundsen & Sánchez-Hernández, 2019). Microscopic analysis demands expert knowledge and a high requirement of microscopic technology for identification and is labor-intensive, providing low resolution for diet items with similar micro-morphological tissues and highly digestible tissues (e.g. fungi or gastropoda) (Westoby et al., 1976; Egeter et al., 2014). Stable isotope analysis and biomarker analysis, which have their strengths in elucidating food web structures and analyzing energy flows, remain challenged when investigating the fine-scale diet patterns often desired in food web studies (Han et al., 2019).

Ultimately, the choice of method depends on factors such as the study species, research objectives, available resources, and the level of precision required. Often, a combination of methods may be used to obtain a more comprehensive understanding of mammal diets. Nonetheless, the quest for reliable methods to measure dietary intake in animals is hindered by the limitations inherent in current approaches, prompting the exploration of innovative solutions in diet research. In recent years, DNA-based analysis has gained popularity due to its non-invasive nature and high specificity in identifying consumed species (Ando et al., 2020). The following paragraphs will exclusively discuss the promise of DNA-based diet analyses.

1.4 | The promise of DNA-based diet studies

Advancements in molecular biology and genomics have introduced a transformative approach to the analysis of animal diets; DNA-based diet analysis. The first DNA-based diet studies utilized species group-specific (e.g. targeting all plant DNA or insect DNA) and species-specific PCR amplification to identify specific food sources (Höss et al., 1992; Kohn et al., 1995; Reed et al., 1997; Deagle et al., 2005). Subsequently, cloning and Sanger sequencing were employed to isolate food DNA sequences from a mixture of fecal DNA representing multiple species (Jarman et al., 2004; Deagle et al., 2007). The obtained sequences were identified using a reference sequence database based using a DNA barcoding approach—a system based on standardized DNA region sequences for species-specific identification (Hebert & Gregory, 2005). While DNA barcoding demonstrated higher resolution in taxon identification compared to PCR amplification with species group-specific or species-specific primers via the traditional process of cloning and DNA sequencing, it also proved to be both expensive and time-consuming for acquiring sufficient sequence data (Ando et al., 2020). This challenge was eventually overcome with the advent of a high-throughput sequencing (HTS) approach utilizing NGS, leading to the emergence of DNA metabarcoding. This method uses the process of DNA barcoding coupled with HTS to obtain sequencing data from environmental samples (e.g. scat samples). The onset of DNA metabarcoding has led to the emergence (and increase) of relevant dietary studies encompassing mammals, birds, amphibians, fish, and even invertebrates over the past decade (Figure 1.2).

The arrival of HTS has revolutionized molecular approaches and enabled the development of highly efficient protocols for analyzing low-quantity (trace amounts) and low-quality DNA samples extracted from fecal origin (Pompanon et al., 2012; De Barba et al., 2016; Liu et al., 2021). DNA metabarcoding allows positively identifying (with high taxonomic resolution) the prey taxa (or even specific genotypes) in the scat, even when remains are small or too decomposed for morphological analyses (Mumma et al., 2016). Recent molecular-based dietary assessments have already revealed errors in estimated diet composition based on morphology-based assessments from scats (Gosselin et al., 2017; Oja et al., 2017) showing the promise of DNA-based diet studies. Furthermore, DNA metabarcoding protocols, next to identifying the diet composition, also allow identification of the origin of the scat. Monterosso et al. (2018) have revealed, using genetic methods, that identifying scat based on scat morphology alone is prone to errors and can even lead to biased ecological inferences further illustrating the potential of genetics in diet analysis.

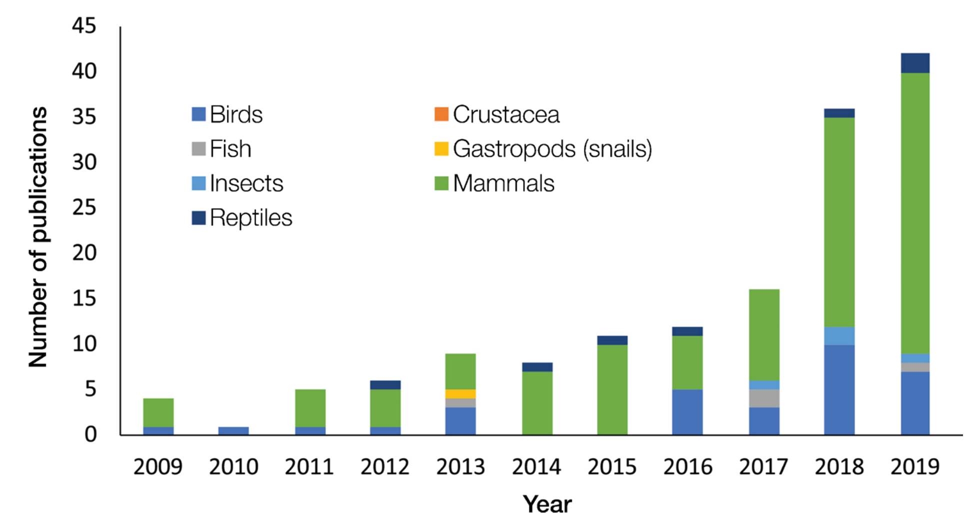

Figure 1.2 | The bar chart depicts the yearly count of original articles utilizing DNA metabarcoding of fecal samples for dietary assessment published in international journals up to 2019. A similar trend is expected for 2020 and onwards. Each distinct color on the chart corresponds to a specific target animal taxon employed in dietary studies. Adapted from Ando et al., 2020.

DNA-based diet studies are broadly applicable and can be applied to a wide range of animals, e.g. not only to large species like carnivores but also to small insectivores or omnivores and species that are difficult to observe directly, elusive, or rare (Shehzad et al., 2012; De Barba et al., 2014). Additionally, DNA-based

techniques allow researchers to study an animal's diet over time and space by analyzing fecal samples collected at different time points and locations with possibly less effort compared to non-genetic studies, and can provide insights into seasonal or dietary changes. Next, DNA-based methods are often more reliable for preserving samples over time. DNA degrades comparatively slower than traditional diet study materials, such as food remains in feces or stomach contents when preserved appropriately. Moreover, non-genetic diet studies often require the subjective judgment of observers in the field or behind the microscope, which can introduce bias and increase the potential for human error. Furthermore, DNA-based studies are often non-invasive, which means they do not require capturing, handling, or disturbing the animals being studied. This reduces stress and potential harm to the animals while collecting data. On top of this, DNA metabarcoding of sequences originating from scat samples offer opportunities for additional insights from understanding gut microbiome composition and disease dynamics to individual behavior (Monterroso et al., 2018).

1.4.1 | Qualitative DNA-based diet analysis

The general steps (Figure 1.3) that need to be taken to qualitatively analyze an animal's diet using genetics are, first; to collect dietary samples and subsequently extract the DNA out of these samples. The vast majority of dietary studies applying NGS use feces as the sample, because fecal samples contain the undigested feed materials, and are easily collected (Liu et al., 2021). It is important to correctly sample, by mixing the center, the middle and the outer layer of feces to improve the detection rate of prey DNA, especially for the rare item that animals consume less frequently. Furthermore, it's crucial to properly label, store, and preserve the samples, most often in ethanol, to stop or at least reduce DNA degradation. For DNA extraction, commercial kits, such as the QIAamp DNA stool mini kit, are most frequently used. However, the optimal DNA extraction method may depend on the feeding characteristics (e.g. feeding mode, meal size and physiology) of the animal that produces the feces and thorough optimization is recommended to efficiently yield trace amounts of prey DNA and minimize potential PCR inhibitors and contamination (Liu et al., 2021).

Second, the isolated DNA needs to be amplified with primers that target the right taxa. This can either be taxa-specific primers (i.e. universal primers targeting a

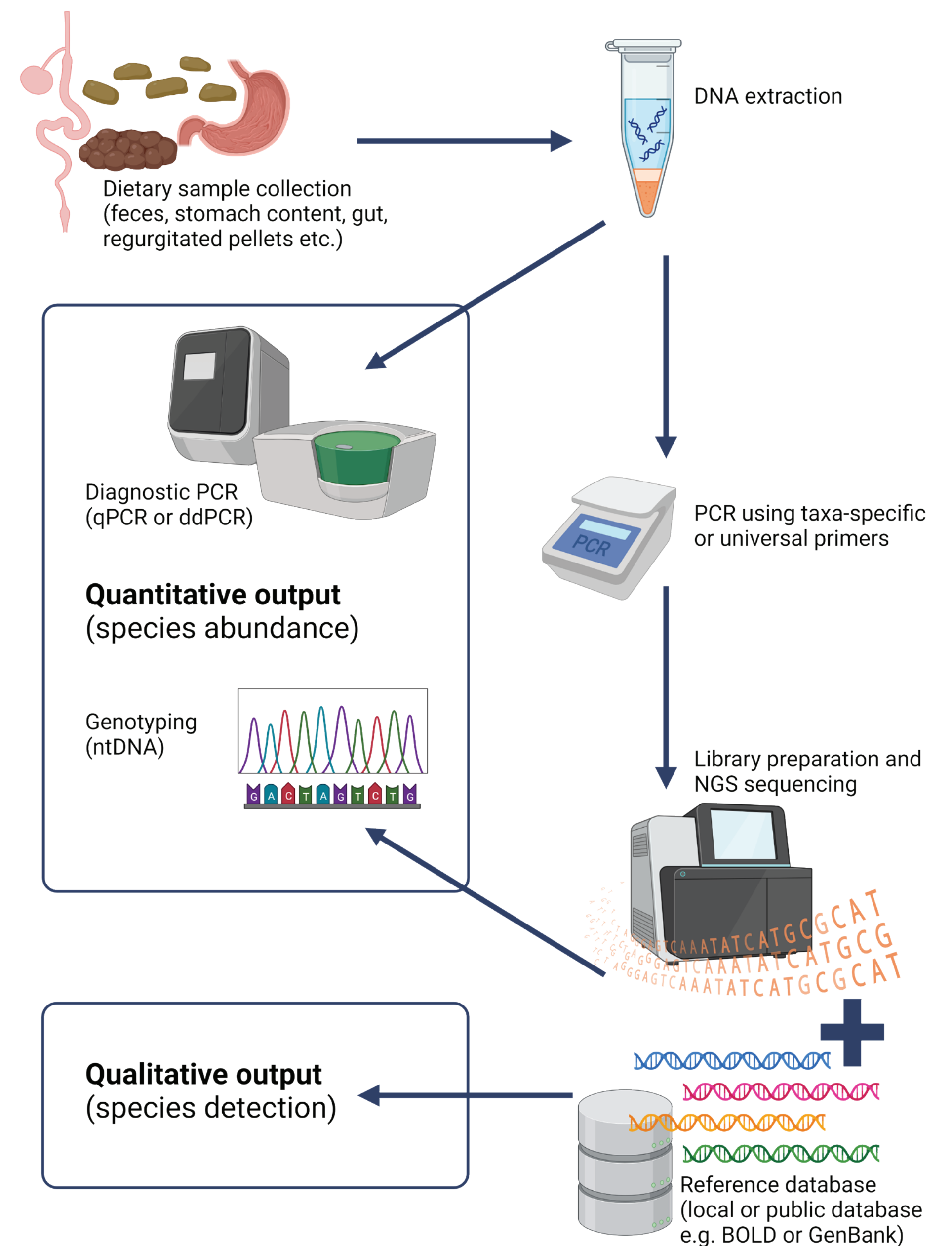

Figure 1.3 | A conceptual framework of DNA-based diet analysis using quantitative PCR (qPCR) and droplet digital PCR (ddPCR) or Next Generation Sequencing (NGS).

whole family or clade such as vertebrates) or species-specific primers and can either target nuclear DNA (ntDNA) but more often mitochondrial DNA (mtDNA) markers (e.g. COI) or plastid DNA since it is more abundant in animal cells. See Ando et al. (2020) or Liu et al. (2021) for overviews of DNA barcoding primers for specific prey taxa that have been frequently used in previous dietary studies. Additionally, a blocking primer might be used to prevent amplification of host DNA as suggested by Vestheim and Jarman (2008). Although, host DNA can be informative to test the correct origin of the feces, an overflow of host DNA might also cause a loss of sequencing depth causing rare species to be missed during sequencing (Vestheim and Jarman, 2008). Subsequently, the amplified PCR products, after library preparation (see Creer et al., 2016 for more details), can be sequenced on an NGS platform (usually Illumina Miseq or Novaseq).

Third, the obtained sequences are compared (blasted) to a local or public reference database using bioinformatics (see Creer et al., 2016 for further information on bioinformatic software) and assigned to prey species (or a higher taxonomic level). For common molecular markers as COI, public databases such as BOLD (the Barcode Of Life Database) contain for example more than 500,000 barcodes for roughly 65% (45,000 species) of the total estimated 70,000 recognized extant Chordata species in the world (accessed on April 15, 2019; Sousa et al., 2019; Slobodian et al., 2021) and is free to use. Otherwise, more than 35,000 COI sequences from vertebrate species are deposited in GenBank (accessed on April 13, 2019; Sousa et al., 2019), which could all be used for DNA-based diet analysis of vertebrates. However, when prey animals or plants are rare, cryptic or not frequently studied, barcodes in such public databases can be scarce and may result in a low-resolution taxa assignation and the classification accuracy of diet analysis will be reduced. This means that a local DNA barcode reference database should ideally be constructed containing barcodes of all local potential diet resources if a high-resolution species level result is needed. These resources should be collected and identified by both morphological and molecular methods (see Liu et al., 2021 for local reference database construction). After successful prey taxa assignment, the dietary information can then be used in further (semi)quantitative and qualitative data analyses, for example calculating the frequency of occurrence of prey in the predator's diet or exploring relationships between diet and ecological factors (Klare et al., 2011).

1.4.2 | Quantitative DNA-based diet analysis

Quantitative DNA-based diet analysis is an approach within DNA-based diet analysis that focuses on providing precise and quantitative information about the dietary composition of animals based on DNA analysis of their fecal samples (beyond the presence-absences obtained by qualitative DNA-based diet analysis). Quantitative methods offer a more detailed understanding of an animal's diet, allowing researchers to determine accurate abundance estimations and consumption patterns of various food items within the diet.

Currently, DNA-based diet studies that use NGS approaches, are mainly using two indicators to quantify the diet results (RRA: relative read abundance and FOO: relative frequency of occurrence). However, these indicators are semiquantitative at most, due to biases that get introduced along the steps of the DNA-based diet analysis. An important issue is that multiple prey taxa are simultaneously amplified with a universal primer; during this process some taxa can be preferentially amplified as universal primers have different binding affinities to different prey sequences depending on the number of primer mismatches it has with these different prey sequences and this results in biased DNA copy numbers (termed amplification bias, see Pompanon et al., 2012 for an extensive review). Next, DNA copy numbers, originating from mitochondria which can be very different in numbers depending on the tissue that was degraded, and DNA degradation (which will always be present in samples that pass the animals' gut), vary among prey species and prey parts and can subsequentially affect the number of sequence reads for each food item (Pompanon et al., 2012). Examinations using food-controlled fecal samples indicated that RRA does not reflect actual food proportions fed to target species (Nakahara et al., 2015; Deagle et al., 2018), although some studies did indicate a positive correlation between RRA and the mass percentage of tissue mixture (Willerslev et al., 2014) or observed percentages of feeding behavior (Mallott et al., 2018) or used correction factors to correct for these biases in RRA (Thomas et al., 2015).

Quantitative approaches to obtain species abundance or biomass estimates using NGS involve targeting nuclear microsatellite DNA (e.g. genotyping) instead of mitochondrial DNA, but are still limited to obtain abundance counts for only a single or few prey species. For example, Andres et al. (2021) counted the number of round goby (***Neogobius melanostomus***) by analyzing intraspecific genetic

variation in mixtures of DNA from aquatic eDNA samples. Also SNP (Single Nucleotide Polymorphism) panels might be used in the same fashion (Pujolar et al., 2022). Additionally, so-called ultra-deep sequencing techniques might pose a way of preventing amplification bias, as no PCR amplification step is used and sequencing is done directly on enriched mitochondrial DNA mixtures (Zhou et al., 2013).

Other quantitative DNA-based (diet) analysis methods are qPCR and ddPCR, so called diagnostic PCR. Here, PCR products are not sequenced but DNA concentrations (or copy numbers) are obtained during the actual amplification step by measuring fluorescent signals that are released during (qPCR) or at the end of the PCR (ddPCR). These results can be calibrated against known DNA concentrations (controls), then estimates of the individuals consumed can be obtained. However, these methods do not allow the use of universal primers that target diverse species groups as species-specific primers are needed to exactly quantify the number of sequences of a prey taxon. Furthermore, despite promising results in other fields (e.g. in food science: Floren et al., 2015 or forensics: Coyle, 2014), for diet studies diagnostic PCR has not yet been used to our knowledge.

1.5 | Challenges in DNA-based diet studies

Qualitative and quantitative DNA-based animal diet analysis has made significant advancements in recent years, yet there are still several challenges and areas where improvements or further research are needed. An important limitation within the realm of DNA-based diet studies is the lack of standardized protocols for DNA-based diet analysis (Deagle et al., 2018). Different studies may use varying DNA extraction, amplification, sequencing methods and bioinformatic analyses which can make it difficult to compare results across studies (Deagle et al., 2018). Additionally, the accuracy of diet analysis relies heavily on reference DNA databases that contain sequences of known species. These databases are often incomplete or biased toward certain taxonomic groups or geographical areas making it challenging to identify less common or newly discovered species in the diet. For example, Sousa et al. (2019) point out that in the Southern Hemisphere terrestrial realms only a small fraction of all known metazoan species has been barcoded. Furthermore, the complexity of a diet (e.g. omnivorous animals have a high number of potential prey items) asks for more complex DNA analyses, where differentiating between similar items or quantifying mixtures of prey based on short DNA markers is an ongoing challenge (but will still outcompete non-genetic methods) (De Barba et al., 2014).

Fecal samples are commonly used as non-invasive starting material for DNA-based diet analysis but suffer from being highly degraded (due to digestive processes) and PCR inhibition. Furthermore, environmental factors, such as UV radiation, microbial degradation, or exposure to air, can cause further DNA degradation of fecal samples in the field. This begs the need for very short DNA markers (<150 bp), although causing a trade-off with taxonomic resolution (Vamos et al., 2017). In any case, it is difficult to obtain reliable qualitative data from highly degraded or PCR-inhibited samples, let alone quantitative data. Therefore, strategies related to sampling, sample preservation, extraction, and PCR for minimizing degradation and inhibition are essential but remain scarce (Ando et al., 2020).

While quantitative DNA-based diet analysis can estimate the relative abundance of dietary items, accurately quantifying the precise quantity of multiple food items consumed is still a complex problem largely caused by differential digestibility and preferential amplification. In terms of qualitative diet assessments, understanding how diet composition varies across different spatial and temporal scales is an ongoing challenge. Environmental factors, seasonal changes, and habitat variability can all influence an animal's diet. The development of HTS technologies grants us the opportunity to analyze a large number of samples which allows us to conduct wide-range (spatially varying) or long-term (over time) monitoring of complex food webs, including those of multiple species. However, collecting fecal (or other diet) samples can be challenging, especially for elusive or rare species and ensuring an adequate sample size and spatial coverage can be difficult. Furthermore, contamination can be a significant issue, particularly in samples collected from the environment. Further studies are required to evaluate contamination (i.e. during sampling) in the field and secondary consumption (i.e. when a predator consumes another type of (meso)predator), which are important for establishing general rules to avoid such contamination or remove contaminant sequences from dietary data to minimize the risk of false positives (Ando et al., 2020). Ultimately, overcoming these limitations will help not only understanding who is eating what, but also where and why, by linking diet information to ecological situations and questions.

Out of the challenges discussed above, I choose to focus on a few important quantitative and qualitative issues in diet analysis to explore in this thesis. Quantitatively, strategies related to sampling, sample preservation, extraction, and PCR for minimizing degradation and inhibition are essential but remain scarce plus standardized protocols are lacking. Combined with the fact that (quantitative) diagnostic PCR is promising but not used in diet analysis, I chose to focus on this issue and tried to establish a standardized protocol for quantifying fecal DNA for (rare and cryptic) diet constituents using ddPCR. Qualitatively, further studies are required to evaluate field contamination and secondary consumption to be able to fully understand how diet composition varies across different spatial and temporal scales and avoid such contamination or remove contaminant sequences from dietary data. I chose to focus on the diet of lions, a keystone and endangered species that has a disproportionately large impact on its ecosystem (as discussed in detail in section 1.1). Gaining better qualitative insights into their diet is crucial for conservation efforts, habitat management, and maintaining ecosystem integrity.

1.6 | Research aim and questions

This thesis aims to investigate some of the critical challenges associated with DNA-based analysis by qualitative and quantitative assessment of animal diet. It explores the wealth of opportunities and challenges presented by this innovative approach, offering a comprehensive investigation into its merits, limitations, and applications for ecological research. The central objectives of this research are to (1) quantitatively address the challenges of DNA extraction, marker choice, PCR inhibition and degradation (due to digestive processes) in DNA-based dietary analysis using droplet digital PCR and (2) subsequently develop and validate a fecal DNA quantification technique to harness DNA data to provide accurate and reliable estimates of dietary intake. (3) Qualitatively, I aim to use metabarcoding to investigate the contribution of small and very small prey taxa to the diet of a key ecological species (lion) and simultaneously explore the problem of ecological contaminations. Furthermore, (4) I use these qualitative inferred diet data to test an application of DNA-based dietary analysis in real ecological scenarios by exploring the effect of fencing on the diet composition and prey preference of lions.

1.7 | Thesis outline

Generally, this thesis is structured as follows; Chapters 2 to 5 describe scientific studies in which I try to achieve the research aims of this thesis, while Chapters 1 and 6 provide the general introduction and discussion of the subject of this thesis. The general introduction starts with providing a background on the multitude of applications of diet studies in different ecological research fields. Then, different methodologies of diet analysis are discussed split between genetic and non-genetic approaches. Next, challenges of non-genetic approaches are presented and are framed in light of the promise of using qualitative and quantitative genetic (i.e. DNA-based) methods instead to overcome these challenges. I end the introduction with issues and forthcoming knowledge gaps in DNA-based diet studies and my research aims. In Chapters 2 and 3, diagnostic PCR is used to establish a fecal DNA quantification technique for small vegetable seeds in wood mice (*Apodemus sylvaticus*) to estimate intake estimates and enhance ecological risk assessment of small mammals. For this, we manipulated the diet of wood mice by feeding them small vegetable seeds in known portions and relating these to DNA quantities in their feces. Chapters 4 and 5 center on qualitative DNA-based analyses of diets. In these chapters, I delve into the examination of the diet composition of African lions (*Panthera leo melanochaita*) by employing metabarcoding. In Chapter 4, by leveraging extensive sample sizes across various temporal and spatial dimensions, I investigate the significance of small and very small prey taxa in the diet of this crucial ecological species, serving as an apex predator that shapes trophic interactions. Furthermore, the discussion explores the challenges posed by ecological contamination. Subsequently, the qualitative diet data inferred from these analyses are utilized to probe the impact of fencing on the diet composition and prey preferences of lions in Chapter 5. Finally, in the general discussion (Chapter 6), I propose a path in which quantitative and qualitative, genetic and non-genetic diet analyses could be combined and, moreover, I discuss when and where these approaches could be complementary. Finally, I aim to give a brief overview of the future of DNA-based diet analysis and its implications for ecological research. Overall, this thesis represents a comprehensive exploration of the power of DNA-based analysis for animal diet assessment. And hopefully aids in unlocking the potential of these approaches to study the impact of humans on ecosystems and biodiversity and striving to contribute significantly to our understanding of the natural world.

2|

Establishment of a fecal DNA quantification technique for rare and cryptic diet constituents in small mammals

Kevin Groen | Krijn B. Trimbos | Susanne Hein | Astrid I. Blaauw |
Peter M. van Bodegom | Joerg Hahne | Jens Jacob |

Published in **Molecular Ecology Resources**, 2022, *00*, 1-12.
DOI: 10.1111/1755-0998.13609

2.1 | Abstract

DNA-based approaches have highly improved the applicability of dietary studies aimed at investigating ecological processes. These studies have provided direct insights into, otherwise difficult to measure, interactions between species and trophic levels, food web structure and ecosystem functioning. However, despite these advances, DNA-based methods have been struggling to accurately quantify the whole breadth of diet constituents because of methodological biases, such as amplification bias and digestive processes. This study is, to our knowledge, the first diet study that used droplet digital PCR to quantify diet constituents. We manipulated the diet of wild caught wood mice (*Apodemus sylvaticus*) by feeding them with a known amount of small vegetable seeds (onion and carrot) and quantified the DNA traces of these diet constituents in fecal samples. The sensitivity of the technique combined with the control on the experimental design allowed mitigation of methodological bias. We were able to accurately determine DNA concentrations of small vegetable seeds in the diet of wood mice. Quantification of target DNA demonstrated significant differences in DNA content when one vs. five seeds were consumed. These differences remained significant when the age, sex, and other diet constituents of the mice were altered. Different DNA markers, targeting different parts of the chloroplast, influenced onion DNA detectability. However, all onion and carrot markers showed higher DNA content for higher seed numbers. Overall, the sensitive DNA based approach developed in this study allows for minimally-invasive quantification of small diet constituents in feces, which would otherwise be undetectable with traditional methods.

2.2 | Introduction

Diet studies have been an integral component of research related to the biology and ecology of animals for decades. Traditionally, morphological and visual approaches, such as gut, stomach, fecal and scat content analyses, are used to determine what a consumer has eaten (Miller & McEwen, 1995; Montague & Cullen, 1985). In this way, a qualitative list of dietary items can be obtained (Miller & McEwen, 1995; Montague & Cullen, 1985) and further ecological relationships can theoretically be determined. However, morphological methods are restricted to what is not digested at the time of sampling and therefore lack resolution (Casper, Jarman, Deagle, Gales, & Hindell, 1997; Mumma et al., 2016). Additionally, partly digested diet remains are difficult to identify and the process is time-consuming (Pompanon et al., 2012). The result is, at best, a biased picture of diet choice (Symondson, 2002). Furthermore, studying animal diets using morphological methods has undesirable effects on the sampled individuals and potentially on populations because of invasiveness of the techniques (Murray et al., 2011).

Recently, DNA-based approaches have received attention because of their use in dietary studies (for example using feces as a source of DNA of diet constituents) (Creer et al., 2016). These approaches are now extensively used by ecologists for (qualitatively and semi-quantitatively) assessing the diet of herbivores (Hibert et al., 2013; Soininen et al. 2015), carnivores (Alberdi et al., 2020; Deagle, Kirkwood & Jarman, 2009; Shehzad et al., 2012) and omnivores (De Barba et al., 2014; Robeson et al., 2017). These studies have utilized the outcome of DNA analyses for investigating ecological processes such as interactions between trophic levels (see Clare, 2014) and species interactions (e.g. predator-prey impacts, see Zarzoso-Lacoste et al., 2016), food web structure (Roslin & Majaneva, 2016) and ecosystem functioning (Nielsen, Clare, Hayden, Brett, & Kratina, 2017) which are otherwise difficult to measure.

Despite these advances, quantitative determination of diet constituents has remained a major challenge, while this is critical for assessing trophic interactions, predator-prey relationships and dynamics of animal populations (Bowles, Schulte, Tollit, Deagle, & Trites, 2011). Although the advent of DNA-based studies has greatly increased the resolution of dietary breadth (Deagle et al., 2005; Galan et al., 2017; King, Read, Traugott, & Symondson, 2008; Morisset, Štebih, Milavec, Gruden, & Žel, 2013; Murray et al., 2011; Piñol et al., 2014; Pompanon et al.,

2012; Zeale, Butlin, Barker, Lees, & Jones, 2011, and see Creer et al., 2016), DNA-based methods struggled to accurately quantify the whole breadth of diet constituents because of current technical and methodological bias (Clare, 2014; Deagle, Thomas, Shaffer, Trites, & Jarman, 2013; Deagle et al., 2019; Jusino et al., 2019; King et al., 2008; Mata et al., 2019; Pompanon et al., 2012; Symondson, 2012). According to Clare (2014), tissue-specific differences in DNA content, primer or marker choice, copy number variation, digestive processes, sample pooling and DNA purification are all steps in the quantification process that introduce biases (see Deagle et al., 2013; Deagle et al., 2019; Jusino et al., 2019; Mata et al., 2019; Pompanon et al., 2012). Nevertheless, several studies did show positive correlations between proportions of diet constituents consumed and the amount of DNA of the respective diet constituents in scat samples (using High-Throughput Sequencing (HTS): Deagle, Chiaradia, McInnes, & Jarman, 2010; using quantitative PCR (qPCR): Bowles et al., 2011; McCracken et al., 2012, for a comparison between HTS and qPCR see Murray et al., 2011). Additionally, Thomas, Jarman, Haman, Trites and Deagle (2014) used HTS and diet-specific correction factors to account for the above-mentioned biases and found promising improvements regarding the accuracy of DNA diet estimates using these corrections.

Real-time PCR (RT-PCR) techniques, as droplet digital PCR (ddPCR), are less prone to methodological biases (such as amplification bias) compared to HTS techniques and allow quantitative measurements when analyzing diet components of fecal samples (Bowles et al., 2011; Morisset et al., 2013). Bowles et al. (2011) demonstrated that proportions of diet constituents can be accurately determined from fecal DNA using RT-PCR. Additionally, Murray et al. (2011) compared the two methods for DNA-based fecal dietary analysis and concluded that species-specific qPCR assays have higher sensitivity than HTS approaches. Furthermore, ddPCR is favored over quantitative PCR (qPCR) for DNA quantification since it does not need reference material, is less susceptible to PCR inhibition and is more sensitive towards very small quantities of DNA (Floren, Wiedemann, Brenig, Schütz, & Beck, 2015; Miotke, Lau, Rumma, & Ji, 2014; Morisset et al., 2013; Verhaegen et al., 2016). To illustrate the sensitivity, ddPCR has already been proven to reliably quantify minimal traces of contamination in assays of food and feed samples (Floren et al., 2015; Morisset et al., 2013), plant remains at crime scenes (Coyle, 2014) and malaria parasite densities in human blood (Koepfli et al., 2016). However, despite promising results in other fields, for diet studies ddPCR has not yet been used and quantitative relationships with small quantities of cryptic diet constituents have yet to be established.

This study specifically aimed for absolute quantification, using species-specific primers, of small vegetable seeds that are hard to quantify by visual observations or stomach content analysis. We investigate the detectability of seed DNA in fecal samples of wood mice fed with one or five small vegetable seeds and the relationship between seed intake and seed DNA content in fecal samples of wood mice. Furthermore, we explore the effect of digestive biases, by looking at the effect that may occur through sex differences (Asarian & Geary, 2013), age differences (Karasov & Douglas, 2013) and diet composition, on establishing quantitative relationships. Additionally, the effect of marker choice on quantification is examined.

For this, we manipulated the diet of wood mice (*Apodemus sylvaticus*) by feeding them small vegetable seeds. Wood mice are distributed across most of Europe and parts of North Africa (Wilson & Reeder, 2005). They prefer forests and other habitats with well-developed woody plants but can also inhabit arable fields if food and shelter is present (Mitchell-Jones et al., 1999), where mainly seeds are consumed (Abt & Bock, 1998). Additionally, wood mice are used as an animal model and a focal species for ecological risk assessment studies (European Food Safety Authority, 2009).

Insights into the effect of methodological biases when quantifying small diet constituents can improve fecal DNA quantification techniques for small diet constituents for use in realistic field scenarios and ecological research. Additionally, a proof of concept is needed for studies where quantification of other small dietary constituents matters. The application of a sensitive, robust and validated technique is desirable for research in the field of animal ecology (resource use, trophic interaction, competition) and applied research alike (exposure to environmental contaminants, risk assessment).

2.3 | Materials and Methods

2.3.1 | *Live trapping*

Wood mice were live-trapped with Ugglan traps in the surroundings of Muenster, Germany (51.97 N, 7.55 W). Traps were set in forest habitat or along hedgerows and/or tree rows. The trapping regime was identical to previous studies (Chiron et al., 2018, Hein & Jacob, 2018). In brief, traps were pre-baited with rolled oats for 3 nights before restocking with apple chunks, peanut curls and rolled oats as bait and wood wool for nesting material. Traps were activated for three to five nights and checked about every 12 hours. Live trapping was conducted several times from April to November 2017. After capture, the individuals were individually marked with a passive integrated transponder (PIT) tag (LUX-IDent, Lanškroun, Czech Republic), sexed and weighed with a spring scale (Pesola, Schindellegi, Switzerland) to the nearest gram. Mice were housed individually in standard rodent cages with wood shavings, a turned over clay flower pot for nesting, standard pellets (Altromin 1324; Altromin, Lage, Germany) as food source and tap water *ad libitum* at standard holding conditions at reversed day-night cycle. Subadults were younger than 4 weeks (F_1 generation were captive-born offspring from wild parents). All procedures involving animals were conducted according to relevant legislation and by permission of the authorities of the German federal state of North Rhine-Westphalia under permit 84-02.04.2016.A540.

2.3.2 | *Food pellet preparation*

We produced custom-made food pellets (FPs) to deliver known proportions of known diet components to the wood mice (see Appendix S2.1 for details). Components of FPs were wheat kernels (non-target seeds), mealworms (invertebrates) and wheat leaves (foliage). These three ingredients were selected to represent the main food categories at relevant composition - seeds 50%, invertebrates 25%, foliage 25% - for wood mice reported in natural habitats (Abt & Bock, 1998). Matrix pellets (MPs) were produced; these were FPs spiked with a given number of target seeds. These were onion (*Allium cepa*) and carrot (*Daucus carota sativus*) seeds (mean seed weight for onion 3.8 mg ± 0.3 mg (SD), and for carrot 1.8 mg ± 0.3 mg (SD)) at varying composition of components (Table 2.1).

Table 2.1 | Composition of matrix pellets (MP) and the number of wood mice tested per feeding trial. MPs main constituents were wheat kernels (as non-target seeds), mealworms (as invertebrates) and wheat leaves (as foliage). O = onion; C = carrot. MP 1.1 and MP 1.2 were used for testing the effect of sex and age on DNA quantification. MP 1.1 - 1.2 and 2.1 - 2.6 were used for testing the effect of varying diet composition on DNA quantification and MP 3.1 - 3.2 for testing the effect of a DNA marker on quantification. Note that the absence of subadults in MP 2.1 – 3.2 was based on the results gained in the MP 1.1 and 1.2 trials (see section 2.4).

MP	Non-target seeds	Inverte-brates	Foliage	Target seeds	♂ adult	♂ subadult	♀ adult	♀ subadult
1.1	50%	25%	25%	5O + 5C	5	8	5	6
1.2	50%	25%	25%	1O + 1C	5	9	5	5
2.1	50%	50%	0%	1O + 1C	5	0	5	0
2.2	50%	0%	50%	1O + 1C	5	0	5	0
2.3	0%	50%	50%	1O + 1C	5	0	5	0
2.4	50%	50%	0%	5O + 5C	5	0	5	0
2.5	50%	0%	50%	5O + 5C	5	0	5	0
2.6	0%	50%	50%	5O + 5C	5	0	5	0
3.1	50%	25%	25%	1O + 1C	5	0	5	0
3.2	50%	25%	25%	5O + 5C	5	0	5	0

2.3.3 | *Gut passage time*

To acquire as much target DNA as possible for accurate quantification of seed intake, we determined the time it took for the target seed DNA to completely pass through the gastrointestinal tract (GIT) of a wood mouse. For this purpose, we fed 3 adult male and 3 adult female wood mice MPs with five onion and five carrot seeds. Fecal samples were collected after t = 8, 24, 36 and 48 hours and analysed for their DNA content. Results showed that >97% of all target seed DNA passed the GIT after t=24 hours (see Results, Fig. 2.1) and therefore further analyses were done using t=8 plus t=24 hours after feeding only.

2.3.4 | Feeding trials

The feeding trial routine was conducted according to the schedule depicted in Table S2.1. Each feeding trial started with placing randomly selected mice in fresh cages with filter paper as bedding and a turned over clay flower pot for shelter for 3.5 days. After the first day in the new cage, food was switched from the standard pellet diet to a FP diet to let the GIT get used to the different feed. After 2.5 days, FPs leftovers were removed and a 12-hour fasting phase was conducted overnight (reversed day-night cycle). On day 4, all individuals were fed one MP (t = 0) which was provided in a small glass Petri dish (see Table 2.1 for the different MPs fed and the replication). MPs were usually consumed within 1-2 hours. All fecal samples during 8 hours after MP consumption were collected (t = 8, average number of droppings 59 ± 17 (1 SD) , average sample weight 265 ± 108 mg (1 SD)) and individuals were placed in fresh cages with filter paper until the next morning, when another fecal sample was collected (t = 24, average number of droppings 50 ± 29 (1 SD), average sample weight 376 ± 129 mg (1 SD)). Droppings per individual were collected in a collection tube, labelled and dried in a drying cabinet for 2-4 hours at 35 °C. Twenty-four hours after pellet consumption (= day 5), individuals were returned to cages with wood shavings and a flower pot. All individuals were weighed before each trial. Captured individuals were re-used in trials after a resting period of at least 3 days. Wood mice, not used anymore for ongoing feeding trials, or after all feeding trials were finished, were released at place of capture.

2.3.5 | Primer design

Major DNA fragmentation occurs in the GIT and the fragment size of dietary DNA decreases along the GIT (Rizzi et al., 2012). Different positions on the chloroplast DNA (cpDNA) might therefore be influenced differently by digestion. In order to investigate quantification variation between primer sets, two species-specific primers were designed on different positions on the chloroplast DNA (see Figure S2.1) to specifically quantify the DNA content of onion and carrot (Table 2.2). Each primer set was designed to only amplify one copy (without mismatches) to prevent copy number variation. In addition, to enhance the specificity of the species-specific primer combinations, TaqMan Hydrolysis probes (FAM & HEX dyes) were constructed (see Table 2.2). A PCR protocol for a multiplex reaction with both primer sets was constructed and optimized using a thermal gradient (Table S2.2). To minimize cross amplification, specificity testing was done *in silico* using Geneious 10.2.6 (https://www.geneious.com) and Primer-BLAST (Ye et al., 2020) (Table S2.3). Additionally, specificity tests were performed *in vitro* using DNA extracts of the MPs raw components (wheat: *Triticum aestivum* and mealworm: *Tenebrio molitor*), wood mouse (ear) tissue, droppings and several plants closely related to onion and carrot (*Allium sativum*, *Anethum graveolens*, *Petroselinum crispum* and *Coriandrum sativum*; see Table S2.4). Each of these tests suggested high specificity of the primer sets for the target taxa.

Table 2.2 | Specifications of primers and probes designed and used in this study. All oligonucleotides were designed to target chloroplast DNA of *A. cepa* (accession NC_024813) and *D. carota sat.* (accession CM004358).

Species	Primer set (marker)	Name	Sequence (5'-3') †	Length (bp)	Amplicon length (bp)	Location on cpDNA (min. – max. bp)
Carrot Primers (*D. carota sat.*)	Dcsat1	Dcsat_F_1	GACGCACTCCTATGAACGTGA	21	127	50858 – 50984
		Dcsat_R_1	AGGAGACATGTCCTAAAGTTCG	22		
		Dcsat_probe_1	[HEX]AAATATACCGGATTAGTCGACTCGA[BHQ2]	25		
	Dcsat3	Dcsat_F_3	TCGGATTGACCCACTACTTT	20	129	49720 – 49848
		Dcsat_R_3	ATCAACCGACATCCACTTCG	20		
		Dcsat_probe_3	[HEX]GTTCAGTCTCTTCCCACGAA[BHQ2]	20		
Onion Primers (*A. cepa*)	AC1	AC_F_1	AAAGGTGAGGGGGACGTA	18	135	47212 – 47346
		AC_R_1	TTGGCTATCCCCTCTCGT	18		
		AC_probe_1	[FAM]CCACCTCTATTGTATGAATCATCTT[BHQ1]	25		
	AC3	AC_F_3	AATTGTTTCCGATTCACCAATTTTT	25	177	111375 – 111551
		AC_R_3	TCGAGCTATTTGGACAAAGCA	21		
		AC_probe_3	[FAM]TTGCTTTTCAAGAAAACAAT[BHQ1]	20		

† [HEX] and [FAM] are fluorophores, [BHQ2] and [BHQ1] are black hole quenchers.

2.3.6 | DNA extraction and quantification

Mouse droppings per individual were weighed, and each sample was divided into subsamples to not exceed the maximum starting amount (<220 mg) as stated in the protocol of the extraction kit. DNA extraction was performed using the DNeasy Plant Maxi kit (QIAGEN) with the introduction of a stool inhibitor removal step using an InhibitEX (QIAGEN) tablet. The Dneasy Blood & Tissue kit (QIAGEN) was used for DNA extraction of animal tissue samples and the Dneasy Plant Mini kit (QIAGEN) for plant tissue samples. Before sample lysis, the mouse droppings were homogenized in a bead mill using 5 mm stainless steel beads. After DNA extraction, the extract was quantified *in duplo* using ddPCR according to the protocol given in the manufacturer's protocol (ddPCR Supermix for Probes, Bio-Rad). In short, 1 or 5 µL DNA (depending on the MP, i.e. depending on whether 1 or 5 target seeds had been used in the feeding trial, to prevent overloading; 5 µL for MPs 1.2, 2.1-2.3 and 3.1 and 1 µL for all other MPs, see Table 2.1), 11 µL ddPCR supermix for probes (Bio-Rad), 1 µL target primers (10 µM) and 1 µL Taqman probes (5 µM) supplemented to 22 µL total volume with RNAse/DNAse free water were mixed and loaded on to a QX200 droplet generator. After droplet generation, the droplets were transferred to a thermal cycler machine. After PCR, the droplets were read on a QX200 droplet reader (Bio-Rad). Threshold values for determining positive droplets were determined using the Quantasoft software (v1.7, Bio-Rad). The threshold for a positive signal was set based on a positive control sample (*A. cepa* and *D. carota sat.* DNA only). Droplets above the threshold were counted as positive events. No-template controls were used as negative controls for the test samples. Count estimates for each sample were compared to the maximum confidence interval (95%) of the negative controls to determine if DNA concentrations were statistically different from zero. Raw DNA concentrations of droplet digital PCR were given in DNA copies/µL. These were recalculated to total DNA copies in the sample (DNA content) and used for further statistical analyses.

2.3.7 | Data analysis

To correct for varying body weight (BW) of mice and varying sample weight (SW), the total DNA copies per sample were divided by BW in grams and SW in grams. Ultimately, all steps taken in this laboratory study were performed to quantify seed intake in wood mice in a realistic field scenario. When zero copies of target DNA would be measured in the field, it might reflect two scenarios: 1) the wood mouse in question did not eat the target seed and therefore the DNA is not present in its feces; 2) the wood mouse did eat the target seed but too long ago for target DNA to be present (due to digestion) in its feces. In the field, it would be impossible to distinguish between the scenarios above. We argue that these measurements are not of any added value towards an application of this study in a realistic field scenario, where wood mice would be caught in the wild. On the other hand the zeros measured are true values and should be included. Therefore, we have run the analysis twice, first with the zeros omitted, second with the zero's included. The outcomes of both analysis were closely compared for any substantial differences between the two approaches (see Results). Distributions of DNA content were non-parametric and of a small sample size. Therefore, differences between DNA content were tested using non-paired Wilcoxon tests (Wilcoxon, 1945). Multiple comparisons were tested using Kruskal-Wallis rank sum tests and a post-hoc Holm adjusted Dunn test (Dunn, 1964) in the *FSA* package (Ogle, Wheeler & Dinno, 2021). Means and confidence intervals (95%) were determined by bootstrapping (R=10000, Bca method) (Carpenter & Bithell, 2000) using the *rcompanion* package (Mangiafico, 2021). The overall effect of a treatment (sex, age, diet composition and marker) on DNA quantification was analysed after correcting for the effect of increasing seed number. For this, linear regressions (DNA copies ~ number of seeds fed) were performed (model fits were evaluated based on visual representations of the residuals). The residuals of these models were then used to test for a significant overall effect of treatments on DNA content using the same non-parametric tests as specified above. When single comparisons did show significant differences for a treatment but were only applicable to a subset of the data (e.g. for mice fed 1 seed only or mice fed 5 seeds only) the result of the overall analysis (based on the full dataset) overruled these partial differences. This suggests namely that these differences were only applicable to a subset of the data. The markers used for quantification showed differences in detectability (see Results, Figure 2.5 and Figure S2.7). Therefore, we chose to base our results on AC1 and Dcsat1 only (see Discussion for further elaboration). If needed for appropriate visualization purposes data were log transformed. All figures were produced using the *ggpubr* package (Kassambara, 2020). All statistics were performed in R 3.6.3 (R Core team, 2014).

2.4 | Results

We detected an average of 606 onion DNA copies g^{-1} BW g^{-1} SW and 665 carrot DNA copies g^{-1} BW g^{-1} SW in the fecal samples collected within 8 hours after feeding five onion and carrot seeds to wood mice (Figure 2.1). Nine to 24 hours after feeding, we measured 34 onion DNA copies g^{-1} BW g^{-1} SW and 11 carrot DNA copies g^{-1} BW g^{-1} SW in the fecal samples collected within this time frame. Fecal samples collected 25-36 and 37-48 hours after feeding showed only marginal numbers of DNA copies g^{-1} BW g^{-1} SW (7 and 0 onion DNA copies g^{-1} BW g^{-1} SW, 9 and 7 carrot DNA copies g^{-1} BW g^{-1} SW, respectively). In percentages of the total amount of DNA copies collected within 48 hours, 98.9% of total onion DNA and 97.7% of total carrot DNA had been excreted within 24 hours after feeding.

Seed DNA was detectable in fecal samples even when only one onion and carrot seed was fed to a wood mouse although with large variation. Mean onion DNA copies in fecal samples of mice fed with one onion seed was 345 DNA copies g^{-1} BW g^{-1} SW (CI: 151 – 746 copies g^{-1} BW g^{-1} SW) and mean carrot DNA copies in fecal samples of mice fed with one carrot seed was $1.6*10^3$ DNA copies g^{-1} BW g^{-1} SW (CI: $0.8*10^3$ – $4.5*10^3$ copies g^{-1} BW g^{-1} SW). Moreover, a significant difference in total onion and carrot DNA copies in fecal samples was found between wood mice fed one onion and carrot seed and wood mice fed with five onion and carrot seeds (Figure 2.2). Droppings of mice fed with five onion seeds (mean = $18.1*10^3$ DNA copies g^{-1} BW g^{-1} SW, CI: $5.0*10^3$ – $61.1*10^3$) contained 52.5 times more onion DNA copies compared to droppings of mice fed with one onion seed ($W = 332$, $p < 0.0005$). Droppings of mice fed with five carrot seeds (mean = $9.3*10^3$ DNA copies g^{-1} BW g^{-1} SW, CI: $3.8*10^3$ – $21.3*10^3$) contained 5.8 times more carrot DNA copies compared to fecal samples of mice fed with one carrot seed ($W = 427$, $p < 0.005$).

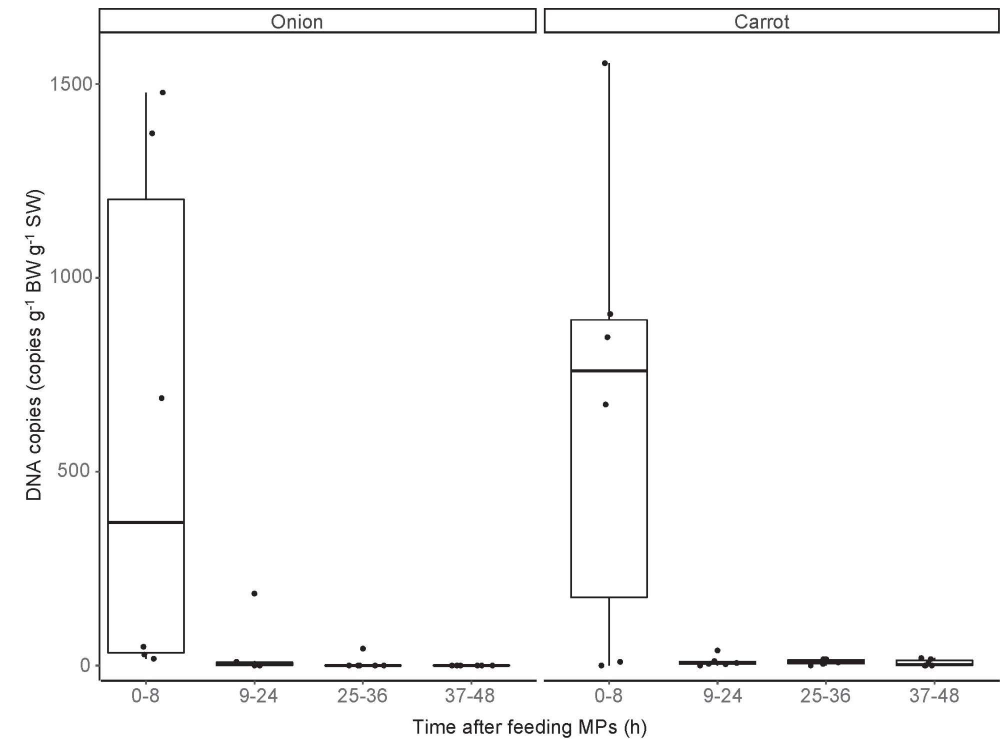

Figure 2.1 | Gut passage time analysis. Total number of onion (left) and carrot (right) DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples excreted by six adult wood mice fed matrix pellets with five onion and five carrot seeds plotted through time after feeding (y-axis). Primer sets used were AC1 and Dcsat1.

Subadult mice fed with one carrot seed excreted droppings that contained 3.4 times more carrot DNA copies compared to adult mice fed one carrot seed (mean subadults $2.2*10^3$, CI: $0.9*10^3$ – $6.8*10^3$ carrot DNA copies g^{-1} BW g^{-1} SW; mean adults $0.7*10^3$, CI: $0.2*10^3$ – $2.0*10^3$ carrot DNA copies g^{-1} BW g^{-1} SW; $W = 28$, $p < 0.05$) (Figure 2.3). This effect was not apparent for subadult and adult mice fed with one and five onion seeds and with five carrot seeds, respectively. Overall, there was no general effect of age on carrot DNA quantification (χ^2 (1, N = 47) = 1.4, $p = 0.2$).

There was no effect of sex on the number of onion and carrot DNA copies in feces for mice fed one seed or five seeds (Figure S2.2). However, the number of onion DNA copies found in fecal samples of mice that had been fed with MPs consisting of one onion seed varied with MP composition ($\chi^2 = 12.5$, df = 3, $p < 0.05$) (Figure 2.4, A). Onion DNA content of fecal samples from mice fed with MPs of a ‘0%

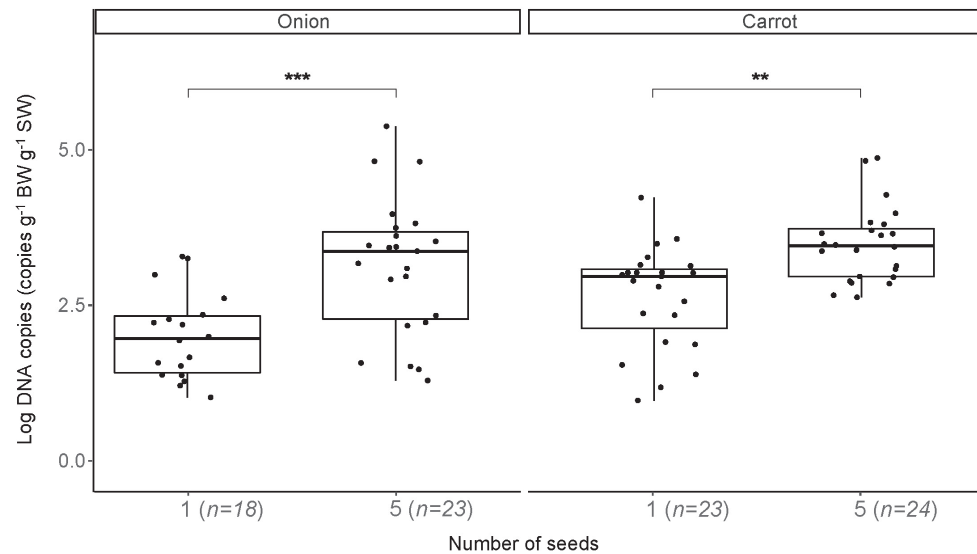

Figure 2.2 | Boxplot showing the difference in the number of log DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples of wood mice fed with one onion and one carrot seed (MP1.2) or five onion and five carrot seeds (MP1.1). Mice of different sex and age were included. Primer sets used were AC1 and Dcsat1. Zeros were removed from this analysis (n = 8 out of 96). Statistically significant differences are depicted (p < 0.0005 '***', 0.005 '**').

non-target seeds / 50% invertebrates / 50% foliage' composition and MPs with a '50/50/0' composition were 17.0 and 12.5 times higher, respectively, compared to onion DNA content of fecal samples from mice fed with MPs with a '50/25/25' diet composition (Dunn test, 0/50/50 – 50/25/25, Z = 2.8, $p < 0.05$; 50/25/25 – 50/50/0, Z = -3.1, $p < 0.005$). This effect was not apparent for adult mice fed with five onion seeds and with one and five carrot seeds, respectively. Overall, a general effect of diet composition on DNA quantification was neither found for onion (χ^2 (3, N = 62) = 3.1, p = 0.4) nor for carrot seeds (Figure 2.4, B).

DNA content in fecal samples of mice fed with one or five onion seeds did not show significant differences when quantified with markers AC1 compared to AC3 (Figure 2.5, A). DNA content in fecal samples of mice fed with one or five carrot seeds also showed no significant differences between markers Dcsat1 and Dcsat3 (Figure 2.5, B). However, when combining all onion data and removing the variance explained by increasing seed number, the overall effect of marker on onion DNA quantification was statistically significant (W = 180, $p < 0.05$).

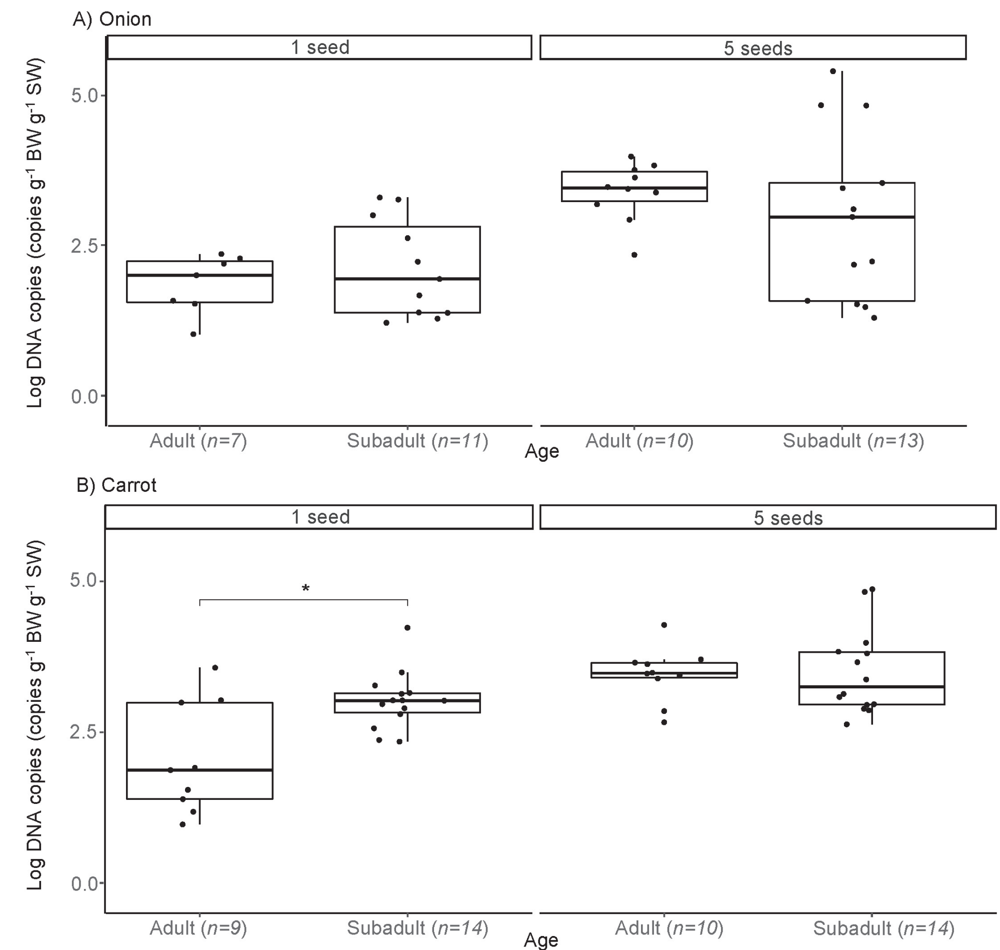

Figure 2.3 | The number of log-transformed onion **(A)** and carrot **(B)** DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples of adult and subadult wood mice fed one seed (MP1.2) or five seeds (MP1.1). Primer sets used were AC1 and Dcsat1. Zeros were removed from this analysis (n = 8 out of 96). Statistically significant differences are depicted (p < 0.05 '*').

Combining all carrot data and removing the variance explained by increasing seed number, the overall effect of the carrot marker used for quantification did not significantly influence the number of carrot DNA copies found (W = 202 , p = 1.0).

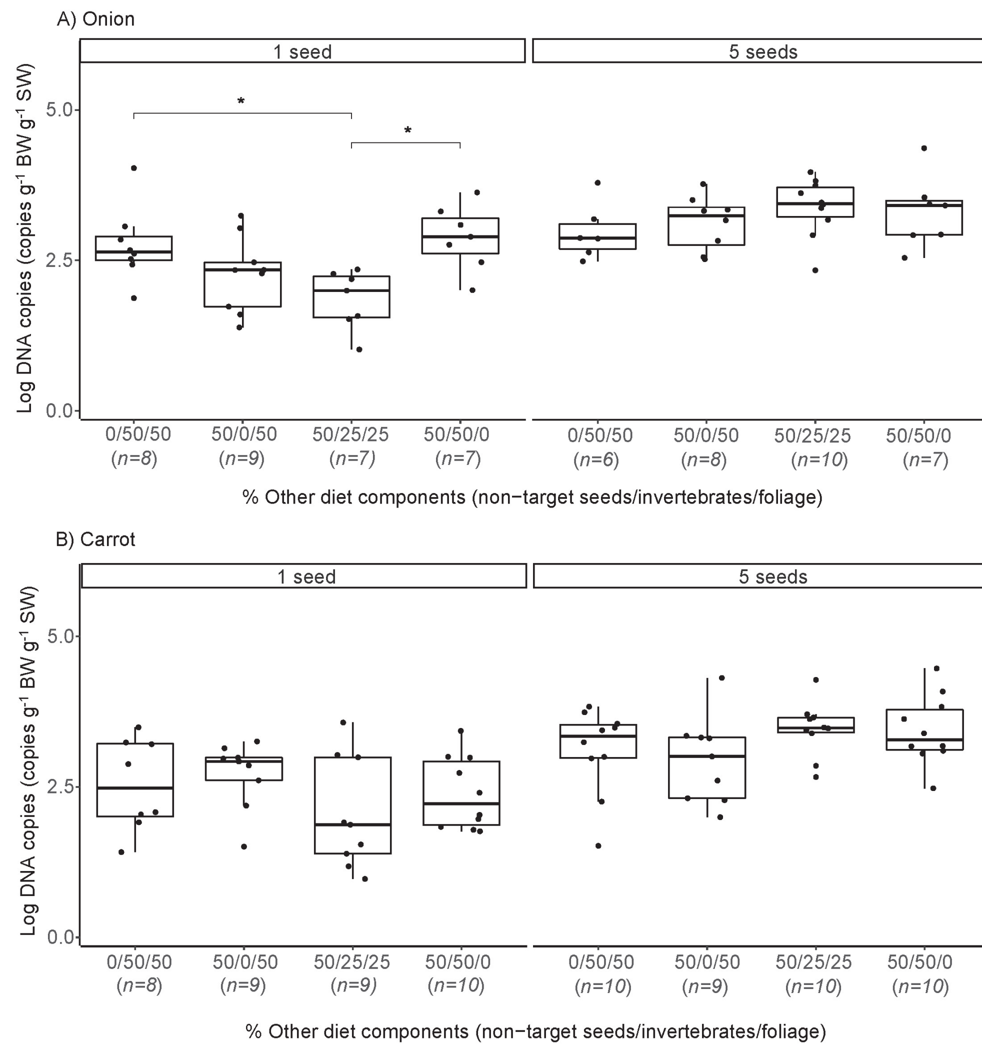

Figure 2.4 | Effect of diet composition (in percentages non-target seeds/invertebrates/foliage) on the number of log-transformed onion **(A)** and carrot **(B)** DNA copies per gram body weight (BW) and per gram sample weight (SW) in fecal samples of adult wood mice fed one seed or five seeds. MP 1.1 -1.2 and 2.1 – 2.6 were used. Primer sets used were AC1 and Dcsat1. Zeros were removed from this analysis (n=23 out of 160). Statistically significant differences are depicted ($p < 0.05$ '*').

The above analyses were also performed including measurements that did not yield any onion or carrot DNA copies (see Appendix S2.2 for all the results including figures and statistics). Aside from the differences within treatment groups (last paragraph of Appendix S2.2), the overall effects of the treatments found in this analysis were the same as the overall effects found in the analyses where the zeros were excluded.

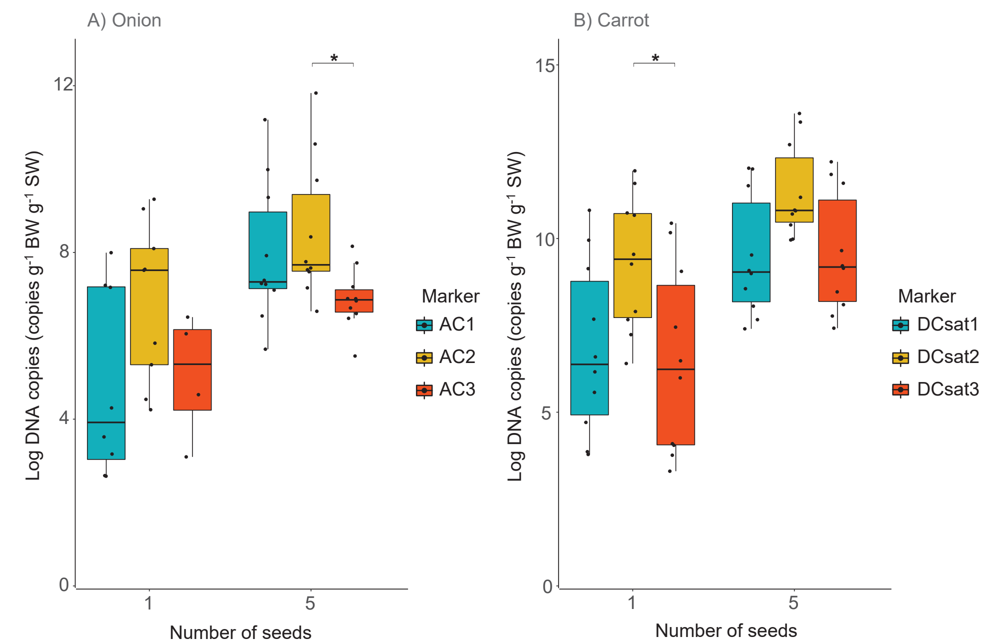

Figure 2.5 | Log-transformed onion **(A)** and carrot **(B)** DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples of wood mice fed different numbers of seeds (x-axis). MP 3.1 – 3.2 were used. N = 10 for each number of onion and carrot seeds fed for each marker. Except for mice fed with one onion seed and marker AC1 where n = 8 and marker AC3 where n = 4. Zeros were removed from this analysis (n = 8 out of 80).

2.5 | Discussion

Quantification of DNA content in wood mice feces was experimentally validated and the method was able to discern between one and five seeds fed. Furthermore, factors such as age, sex and other diet constituents did not alter the digestion patterns. The results here can enhance seed intake estimates, which is valuable information in multiple scenarios. These include risk assessments for small mammals that eat seeds on which plant protection products are applied as coating, studying seed predation, food preference and food competition.

Marker choice did significantly influence onion DNA quantification, although all markers were designed to produce only one amplicon and the same samples were measured with each marker. This result could be due to the presence of NUPTs (nuclear cpDNA) (Richly & Leister, 2004). NUPTs arise when organellar DNA infiltrates nuclear DNA (Richly & Leister, 2004). Ayliffe, Scott and Timmis (1998) stated that NUPTs vary interspecifically in size and copy number as well as intraspecifically in species, within individual plants or even within different tissues of the same individual. Each marker targeted a different part of the chloroplast locus and could therefore potentially result in targeting different or additional NUPTs. However, NUPTS and cpDNA are both amplified and can be used as indicators of target DNA. It is important to note that NUPTS also may cause differences in detectability of target DNA. AC3 did not detect any DNA in 6 out of 10 one onion seed samples. When these zero measurements were included, marker AC3 scored consistently lower in onion DNA copies compared to AC1 for all seed numbers, although non-significant. This does suggest that reliable quantification of marginal quantities can only be done by first investigating how different markers influence detectability (see Di Bernardi et al., 2021). In this study, we therefore based our results on AC1 and Dcsat1 only.

We found that seed DNA passed the wood mice gut within 24 hours without variation, which is in the range of passage time in mammals (Lambert, 1998). Tsuji, Miura, Kotoge, Shiraishi and Murai (2015) proved that gut passage time in herbaceous/omnivorous mammals accelerates with increased food intake. This suggests that increasing the number of seeds consumed further, may require additional calibration. In terms of detectability, this study has shown that as low as one ingested seed was detectable in wood mice feces. Even trace amounts of DNA of up to 2 copies per measured PCR sample (22 µL) were detectable, demonstrating extremely high sensitivity using this fecal DNA quantification technique.

Although sex and age differences in food intake and digestion patterns are known (Karasov & Douglas, 2013), effect sizes are usually very small and highly variable (Asarian & Geary, 2013). This was in concordance with our findings of large intragroup variation and a lack of general effects of sex and age. Although samples sizes are small for each group (n = 7-14), even if sample size would increase, an overall effect of sex and age on quantification is not expected due to the variable nature of these effects (Asarian & Geary, 2013). The absence of sex or age effects on quantification imply that there is no need to differentiate between sex and age when wood mice are not pre-selected, for instance when wood mice were to be caught in the field.

Furthermore, there was no congruent and overall no significant effect of diet composition on target seed DNA content in fecal samples. Theoretically, Karasov and Douglas (2013) suggest that the expression of digestive enzymes and nutrient transporters approximately matches the dietary load of their respective substrates. As such, changing diet composition in wood mice to a more invertebrate rich or more cellulose-rich diet would suggest an adaptation in digestibility of their GIT. Additionally, Hetland and Svihus (2001) found that there is a great ability of organisms to adjust the gut passage time and increased gut volume to increasing feed consumption. These adaptations together may change the digestibility of diet constituents and this could explain that there was no effect of diet composition on DNA quantification as these adaptations could have potentially leveled the differences in respective substrates. This result implies that quantification of seed intake in e.g. field conditions is possibly independent of the diet eaten in the field. This suggests that when wild wood mice would be caught in a realistic field scenario, there is no need to account for what a wood mouse might have eaten during, after, or before intake of onion and/or carrot seeds, if even possible.

Advances in DNA methodologies have led to improvements in the ability to detect species and communities in less-favorable (due to rapid DNA degradation) sampling environments such as aquatic environments, GITs and feces (Andres, Sethi, Lodge, & Andrés, 2020). Mitochondrial or plastid DNA is mostly favored for quantification research since it is present in higher copy numbers, but the ability to detect trace amounts of DNA also opens up the possibility to target nuclear DNA. Andres et al. (2020) have documented the application of multiple amplicon-

based methods to obtain intraspecific nuclear genetic information from eDNA samples and to estimate the absolute abundance of a species in eDNA samples. Quantification using nuclear DNA approaches has the capacity to estimate the number of (exact) genetic contributors in an DNA sample and thereby might overcome the correlative relationships between DNA concentrations and relative species abundance which is frequently challenged by large variation (Andres et al., 2020; Iversen, Kielgast, & Sand-Jensen, 2015) and heavily impacted by taxon-specific amplification biases (Andres et al., 2020; Kelly, Shelton, & Gallego, 2019).

In this study, we used newly designed species-specific primers to quantify cryptic diet constituents. In theory, our approach can also be used with universal primers that would target a whole taxon. However, in practice this would make absolute quantification problematic. The use of a universal primer would be beneficial in terms of utility, costs and effort but quantification would almost always be hampered by taxon-specific amplification biases (Piñol, Senar, & Symondson, 2018). Furthermore, a direct comparison with metabarcoding approaches is not valid because metabarcoding practices have to date never been shown to be fully quantitative, but semi-quantitative at best, and is more suited to produce whole dietary breadth taxonomic lists and relative read abundances (Pringle & Hutchinson, 2020).

Apart from taxon-specific amplification biases, copy number variation biases are also illustrated within this study as shown by the differences in target seed DNA ratios present in feces of mice fed one vs. five target seeds (for onion the ratio between DNA copies was 1: 52.5 and for carrot DNA copies 1:5.8). This entails that for every new target, a calibration study like this study would be needed and the DNA sequence of the target needs to be known upfront. This study may act as a guideline for setting up these target adjustments to be able to quantify other cryptic diet constituents.

We have shown reliable quantification of one and five onion and carrot seeds, with no detectable bias by sex, age and diet composition, which suggests that further quantification of increased seed numbers should be possible with this approach. Moreover, the same trend for all onion and carrot markers in terms of seed fed versus DNA content was found (i.e. higher seed numbers led to higher DNA content). This proof of concept allows future studies to examine the possibility of building calibration curves with ingested seeds versus DNA content. This opens up the possibility to use these calibration curves to estimate intake of seeds (or other small diet constituents) from wild wood mice and potentially other small mammals in a natural setting. Enhanced insights into seed intake (e.g. minimum number of seeds eaten) can for example be beneficial to, risk assessments studies where seed coating procedures are applied or exposure to environmental contaminants need to be measured, and to studies of animal ecology (resource use, seed predation, diet choice, trophic interaction, competition).

2.6 | Supporting information and data accessibility

Additional supporting information can be found in the online version of the article at the publisher's website (scan the QR code below). Raw onion and carrot DNA copy counts as measured by ddPCR per mouse fed different MPs were uploaded to Dryad. These counts include mouse morphological data (body weight, sex and age). Furthermore, a data file was uploaded to estimate gut passage time of target seed DNA. Both files are available at Dryad: https://doi.org/10.5061/dryad.pnvx0k6nz.

3|

DNA-based seed intake quantification for enhanced ecological risk assessment of small mammals

Kevin Groen | Jens Jacob | Susanne Hein | Emilie A. Didaskalou |
Peter M. van Bodegom | Joerg Hahne | Krijn B. Trimbos |

Published in **Ecotoxicol. Environ. Safety**, 2023, *259*, 115036.
DOI: 10.1016/j.ecoenv.2023.115036

3.1 | Abstract

To prevent the non-acceptable effects of agrochemicals on arable fields, Environmental Risk Assessment (ERA) aims to assess and protect against a wide range of risks due to stressors to non-target species. While exposure to stress is a key factor in ERA models, exposure values are difficult to obtain and rely on laboratory studies with often debatable relevance to field situations. To improve intake estimates, data from realistic field-based scenarios are needed. We developed calibration curves relating known seed numbers of up to 20 onion and carrot seeds consumed by wild-caught wood mice (*Apodemus sylvaticus*) to the seed DNA content in the feces. Based on these inferred quantitative relationships, a field trial was run to determine seed intake in a natural setting using realistic levels of seed spillage. Onion DNA was detected in the fecal samples of the wood mice caught in the field, which resembled a seed intake of up to 1 onion seed. No intake of carrot seeds was detected. This is the first-ever study to quantify seed intake in a realistic field scenario using a DNA-based analysis, showing that accurate seed intake estimates can be obtained. Our approach can help to improve risk assessment models through its minimally-invasive and accurate assessment of seed intake by ERA representative and non-target species, which would otherwise be undetectable with traditional methods. Our novel approach and its results are highly relevant to studies of food intake and diet composition for basic and applied research alike.

3.2 | Introduction

Increased agricultural intensification is considered to be a great threat to terrestrial biodiversity (Stoate et al., 2001). A major part of this threat originates from a loss of appropriate habitats, but has also been linked to the application of agrochemicals (herbicides, insecticides, fungicides, growth enhancers and chemical fertilizer) on arable fields (Tscharntke et al., 2005; Emmerson et al., 2016; Geiger et al., 2020). To prevent non-acceptable effects of agrochemicals, Environmental Risk Assessment (ERA) aims to assess and protect against a wide range of risks to non-target species and ensure a high level of protection of human, animal and environmental health (Storck et al., 2017). Especially when it comes to agrochemical authorization and its usage patterns, a strictly regulated ERA process takes place according to guidance developed by the European Food Safety Authority (EFSA, 2009). An ERA consists of a tiered procedure via a set of toxicity studies, hereby using predicted exposure values to calculate potential risk on multiple endpoints (Storck et al., 2017; Brühl & Zaller, 2019). Such assessments also include safety factors that increase the conservativeness of a potential risk approval (EPRS, 2019). If the risk is deemed acceptable, agrochemicals are allowed on the market and considered safe for a defined period until the next ERA is due for re-registration (Storck et al., 2017; Brühl & Zaller, 2019).

In the models that are used for ERA, exposure is a key factor (EFSA, 2009). However, exposure values are often difficult to obtain and therefore usually based on data and predictions (e.g. extrapolations or interpolations) from laboratory studies with often debatable relevance to field situations (Chapmen, 1995; Hart et al., 2003). As an example, estimates on the intake of contaminated food sources by representative focal species in nature are mostly based on invasive and tedious stomach content analyses through visual estimation under a microscope (Hyslop, 1980). Rindorf & Lewy (2004) showed that these traditional methods are prone to bias, with biased intake estimates of up to 150%. Moreover, visually obtained intake estimates may also be hampered by the fact that diet remains are notoriously difficult to identify especially with partially digested material, such as soft plant and animal tissue. Additionally, to our knowledge, very little is known about the consumption of soft plant and animal tissue by small mammals. As a result, these biases may lead to over- or underestimation of exposure which in turn hamper accurate ERA. In addition, ecotoxicologists continue to question the

validity and relevance of laboratory studies to field situations, since the artificial conditions in laboratory settings often cannot be simply transferred to field responses (Chapmen, 1995; Vijver et al., 2017; Hilbers et al., 2018). To improve intake estimates, data from realistic field-based scenarios are needed.

DNA-based approaches used in dietary studies (e.g., using feces as a source of DNA of diet constituents) are currently receiving attention (see Creer et al., 2016) and these approaches are now extensively used by ecologists for the qualitative and semi-quantitative assessment of the diet of herbivores (Dell'Agnello et al., 2019; Hibert et al., 2013; Soininen et al., 2015), carnivores (Alberdi et al., 2020; Deagle et al., 2009; Shehzad et al., 2012) and omnivores (De Barba et al., 2014; Robeson et al., 2017). DNA-based diet analysis has the potential to overcome traditional biases in assessing diets and is already able, to a certain extent, to quantify diet constituents in a laboratory setting (see Chapter 2). Therefore, we aimed to use DNA-based diet analysis to estimate seed intake for wood mice (*Apodemus sylvaticus*) in both laboratory and field settings to create realistic field-based scenarios of exposure to contaminated seeds. In Chapter 2, we established that seed intake by wood mice can be quantified with DNA markers and showed that sex and age did not affect the detectability of seed intake. However, they only quantified seed intake for a maximum of up to 5 (small) vegetable seeds in the wood mouse diet. Here, we developed calibration curves relating known seed numbers of up to 20 seeds consumed by wild-caught wood mice to the seed DNA content in the feces. On basis of these new findings, a field trial (first-ever) was run to determine small seed intake in a natural setting using realistic levels of seed spillage. This study can help to improve risk assessment models through a less invasive and accurate assessment of seed intake by non-target species including ERA representative focal species. In addition, the results are highly relevant to studies of food intake and diet composition for basic and applied research alike.

3.3 | Material and methods

3.3.1 | Focal species

The wood mouse (*Apodemus sylvaticus*) is used as the representative focal species for the ERA of small omnivorous and granivorous mammals (EFSA, 2009). The wood mouse feeds on (small) seeds and is therefore potentially exposed to seed treatments. Moreover, wood mice inhabit field margins when shelter is available and occur in agricultural fields and can therefore have access to freshly drilled seeds and other parts of plants (Pelz, 1986).

3.3.2 | Live trapping

Wood mice were live-trapped with Ugglan traps in the surroundings of Muenster, Germany (51.97° N, 7.55° E). Traps were set in a forest habitat or along hedgerows and/or tree rows. The trapping regime was identical to previous studies (Chiron et al., 2018; Hein & Jacob, 2018; and see Chapter 2). In brief, traps were pre-baited with rolled oats for 3 nights before restocking with apple chunks, peanut curls, rodent pellets and rolled oats as bait and wood wool for nesting material. Traps were activated for 3 to 5 nights and checked about every 12 hours. After capture, the individuals were individually marked with a passive integrated transponder (PIT) tag (LUX-Ident, Lanškroun, Czech Republic), sexed and weighed with a spring scale (Pesola, Schindellegi, Switzerland) to the nearest gram. Mice were housed in standard rodent cages with wood shavings, a turned-over clay flowerpot for nesting, standard pellets (Altromin 1324; Altromin, Lage, Germany) as food source and tap water *ad libitum* at standard holding conditions at reversed day-night cycle. All procedures involving animals were covered by permission of the German federal state of North Rhine-Westphalia, permit 84-02.04.2016.A540.

3.3.3 | Pellet preparation and feeding trial

We produced custom-made food pellets (FPs) to deliver known proportions of known diet components to wood mice (see Chapter 2 for details). In short, components of FPs were wheat kernels (non-target seeds), mealworms (invertebrates) and wheat leaves (foliage). These ingredients were selected to represent the main food categories at relevant proportions – seeds 50%, invertebrates 25%, foliage 25% - for wood mice reported in natural habitats (Abt & Bock, 1998). Matrix pellets (MPs) were produced; these were FPs spiked with a given amount of onion (*Allium cepa*) and carrot (*Daucus carota var. sativus*) seeds (mean seed weight for onion 3.8 mg ± 0.3 mg (SD), and for carrot 1.8 mg ± 0.3 mg (SD)) at varying proportions of components of MPs (Table 3.1). In this study, we focused on onion and carrot seeds because these plant species are frequently grown in European horticulture, with 182,210 ha used for onion and 119,010 ha for carrot production in the EU in 2018 (Eurostat, 2020).

The feeding trial routine was conducted according to the method described in Chapter 2. In short, each feeding trial started with placing randomly selected mice in fresh cages. After a 12-hour fasting phase, 10 individual adult mice (5♂ and 5♀) were fed one MP (t = 0, see Table 3.1 for the different MPs fed and the replication). All fecal samples 24 hours after MP consumption were collected. Droppings per individual were collected in a collection tube, labeled and dried in a drying cabinet (UF 110, Memmert GmbH, Büchenbach, Germany) for 2-4 hours at 35 °C to ensure homogenization of the droppings in a bead mill. After the trial, individuals were returned to the cages with wood shavings and a flowerpot. All individuals were weighed before each trial. Captured individuals were re-used in trials after a resting period of at least 3 days. Wood mice, not used anymore for ongoing feeding trials, or after all feeding trials were finished, were released at the place of capture.

Potential differences in digestive patterns between wood mice held in the laboratory and those living outdoors were mitigated by 1) minimizing the period mice spend in the lab before being used in trials and 2) offering diet components in the lab that resemble their natural food, 3) deriving mice from the wild for laboratory trials and 4) using the target species in both settings instead of the classical caged laboratory house mouse. Furthermore, even if the passage time in the wood mice gut was slightly different between lab and field conditions we would assume similar results. As we showed in Chapter 2 that 95% of DNA already passes the GI tract after 8 hours and we covered a sampling period of 24 hours in the feeding trial to encompass any variation in the passing of target DNA through the GI tract.

Table 3.1 | Composition of matrix pellets (MP) and the number of wood mice tested per feeding trial (note that individuals were reused after a break of at least 3 days). MPs main constituents were wheat kernels (as non-target seeds), mealworms (as invertebrates) and wheat leaves (as foliage). O = onion; C = carrot.

MP	Non-target seeds	Inverte-brates	Foliage	Target seeds	♂ adult	♀ adult
4.1	50%	25%	25%	0O + 0C	5	5
4.2	50%	25%	25%	1O + 1C	5	5
4.3	50%	25%	25%	3O + 3C	5	5
4.4	50%	25%	25%	5O + 5C	5	5
4.5	50%	25%	25%	10O + 10C	5	5
4.6	50%	25%	25%	15O + 15C	5	5
4.7	50%	25%	25%	20O + 20C	5	5

3.3.4 | Field trial

Two field trials were conducted in October 2019 on an agricultural field neighboring a wood strip at the premises of JKI in Muenster, Germany (51.97° N, 7.55° E). Two thousand m^2 of the agricultural field were prepared for sowing by standard agricultural techniques. Forty plots of $50m^2$ (5 x 10 m) were established along a hedge with trees and understory. In the first trial, 7 onion seeds/m^2 and 15 carrot seeds/m^2 (considered as medium density of exposed seeds) were distributed on the surface. In the second trial, 14 onion seeds/m^2 and 30 carrot seeds/m^2 (considered as high density of exposed seeds) were distributed on the surface. Onion and carrot seeds were not sown but distributed on the surface to resemble medium and high densities found in the end rows during field studies under good agricultural practice and are estimates of how much a drilling machine loses at headlands during a U-turn (Roy et al., 2019). The choice of seed density in the field trial was based on the occurrence of seeds on the surface to resemble realistic conditions and not on potential consumption by wood mice. To ensure an equal seed distribution over a plot, an aliquot of seeds corresponding to the required seed density per plot was mixed with sand. The seed-sand mixture was

distributed by hand per plot. Directly afterwards, 96 Ugglan multiple capture live traps were set (ca. 2 traps per plot) and equipped with a sensor that immediately indicated a capture of an animal by sending a signal to a pager (Notz et al., 2017). Traps were activated at midnight to allow wood mice (as nocturnal species) time to feed before capture. Caught animals were weighed, sexed and reproductive activity recorded, individually marked with a PIT tag (LUX-Ident, Lanškroun, Czech Republic), released at the point of capture and fecal pellets were sampled. Trapping was continued for up to 5 days per trial run. Care was taken to remove all droppings before setting traps again.

3.3.5 | DNA extraction and quantification

Mouse droppings per individual were weighed, and each sample was divided into subsamples to not exceed the maximum starting amount (< 200 mg dry weight) as stated in the protocol of the extraction kit. DNA extraction was performed using the Dneasy Plant Maxi kit (Qiagen) with an introduction of a stool inhibitor removal step (using the INHIBITEX (Qiagen) tablets from the QIAamp DNA Stool kit). For in-depth information regarding the extraction optimization and choice made see Appendix S3.1. Before sample lysis, the mouse droppings were homogenized in a bead mill using 5 mm stainless steel beads. After DNA extraction, each extract was quantified with four species-specific primer sets. Two sets for each plant species as described in Chapter 2 to accurately account for DNA fragmentation by digestion. This means that for each plant species quantification results are doubled (e.g. one fecal sample results in two measurements for each plant species; one measurement per primer set). DNA quantification was done (***in duplo***) using droplet digital PCR (ddPCR) according to the manufacturer's protocol (ddPCR Supermix for Probes, Bio-Rad). In short, 1 or 5 µL DNA (depending on the MP i.e. depending on whether 1 or 5 target seeds had been used in the feeding trial, to prevent overloading; 5 µL for MPs 4.1 – 4.3 and 1 µL for MPs 4.4 – 4.7), 11 µL ddPCR supermix for probes (Bio-Rad), 1 µL target primers (10 µM) and 1 µL Taqman probes (5 µM) supplemented to 22 µL total volume with RNAse/DNAse free water were mixed and loaded on to a QX200 droplet generator. After droplet generation, the droplets were transferred to a thermal cycler machine. After PCR, the droplets were read on a QX200 droplet reader (Bio-Rad). Threshold values for determining positive droplets were determined using the Quantasoft software (v1.7, Bio-Rad). Positive droplets of duplicate measurements were merged using

the same software to strengthen quantification statistics. The threshold for a positive signal was set based on a positive control sample (***A. cepa*** and ***D. carota sat.*** DNA only). Droplets above the threshold were counted as positive events. No-template controls were used as negative controls for the test samples. Count estimates for each sample were compared to the maximum confidence interval (95%) of the negative controls to determine if DNA concentrations were statistically different from zero. Raw DNA concentrations of droplet digital PCR were given in DNA copies/µL. These were recalculated to total DNA copies in the sample (DNA content) and used for further statistical analyses.

3.3.6 | Data analysis

The total number of DNA copies per sample was normalized for varying body weight (BW; in grams) of mice and varying sample weight (SW; in grams) as this may otherwise affect quantification (e.g. see Stunkard (1983) for effects of BW on diet metabolism). Distributions of DNA content were non-normal and of a relatively small sample size. Means and confidence intervals (95%) were therefore determined by bootstrapping (R=10000, Bca method) (Carpenter & Bithell, 2000) using the ***rcompanion*** package (Mangiafico, 2021). Multiple comparisons between seed numbers (0 to 20 seeds) and seed density (medium vs. high seed density) were tested using pairwise Wilcoxon tests adjusted with Benjamini-Hochberg correction (Wilcoxon, 1945; Benjamini & Hochberg, 1995). Linear regressions of log-transformed data (log(DNA copies)+1 ~ number of seeds fed) were performed to get estimates and a linear equation for both the calibration curve of onion and carrot seeds. We assessed the compliance to model assumptions by means of graphical validation of the normality of the residuals. Due to large variation of the DNA content within seed number groups and the non-normal distribution of the residuals, we log-transformed the data to acquire normality. Subsequently, seed intake estimates of wild-caught wood mice in the field trial were predicted by using the average value of DNA content found in the fecal samples of all mice from the field trial against the linear equation from the calibration curve of onion and carrot seeds using the ***chemCal*** package (***inverse.predict*** function, Massart et al., 1997; Ranke, 2022). All figures were produced using the ***ggpubr*** package (Kassambara, 2020) and, if needed for appropriate visualization purposes, data were presented log-transformed. All statistics were performed in R 3.6.3 (R Core team, 2014).

All steps taken in the laboratory study and the data analyses were performed to be able to quantify seed intake in wood mice in a realistic field scenario. Zero copies of target DNA measured in the field might reflect three scenarios: 1) the wood mouse in question did not eat the target seed and therefore the DNA is not present in its feces; 2) the wood mouse did eat the target seed but too long ago for target DNA to be present in feces (due to digestion); 3) an experimental error. In the field, it was impossible to distinguish between the scenarios above. We therefore, argue that these zero values are not of any added value towards an application of this study in a realistic field scenario, where wood mice are caught in the wild. However, the zeros measured are true values and should not be neglected. Therefore, we have run the complete analysis twice, first with the zeros omitted (Results), and second with the zeros included (Appendix S3.2). The samples that contained zeros copies of target DNA, because mice were indeed fed zero seeds (MP4.1), were used in both analyses. The outcomes of both analyses were compared for any substantial differences between the two approaches (see section 3.4).

3.4 | Results

We quantified and plotted DNA content (DNA copies/BW/SW) in the fecal samples of wood mice fed from 0 up to 20 seeds (Figure 3.1, see Table S3.1 for booted means). Multiple comparisons between DNA content per seed number showed that onion and carrot DNA content differed from zero seeds for each seed number >0 consumed. There was an increase in DNA content from 0 to 10 seeds but a plateau with no statistically discernible differences for higher seed numbers (up to 20) for onion and carrot DNA ($p > 0.2$). DNA content did not differ between 1 and 3 and 3 and 5 seeds for onion and carrot DNA. Hence, calibration was possible for up to 10 seeds in increments of 5 for both onion and carrot DNA. Therefore, linear regression models were developed, for 0 to 10 seeds only, to obtain calibration curves per seed species (Figure 3.2). The calibration curve for log-transformed onion DNA content showed a strong positive trend with a regression coefficient of 0.31 onion DNA copies g^{-1} BW g^{-1} SW per seed number (Adj. R^2 = 0.58, F (82 df) = 116.4, $p < 0.0001$). The calibration curve for log-transformed carrot DNA content also showed a strong positive trend with a regression coefficient of 0.26 carrot DNA copies g^{-1} BW g^{1} SW per seed number (Adj. R^2 = 0.57, F (98 df) = 131.6, $p < 0.0001$).

In the field trials, 17 fecal samples were collected (from 11 different wood mouse individuals) and target DNA was quantified. DNA content in the wood mice feces was similar for trials done with medium and high seed density for both onion ($W = 3$, $p = 0.33$) and carrot seeds ($W = 1$, $p = 1.00$) and both densities were pooled (Figure 3.3). Figure 3.2 also presents the results of the DNA content found in the field trial samples in comparison to the measured onion and carrot DNA content during the laboratory feeding trials. We found that 9 out of the 17 fecal samples (52.9%) contained onion DNA. Applied to the number of mouse individuals caught, this accounted for 7 out of the 11 individuals (63.6%) that had consumed onion seeds. We found that only 3 out of the 17 fecal samples (17.6%) contained carrot DNA. This accounted for 3 out of the 11 (27.3%) mouse individuals caught that had consumed carrot seeds. The total number of onion DNA copies g^{-1} BW g^{-1} SW quantified in fecal samples of wild-caught wood mice during the field trial was on average 31.5 (CI: 19.5-39.2, N = 9) and resembled the number of copies found when wood mice were fed 1 onion seed during the feeding trial. The total number of carrot DNA copies g^{-1} BW g^{-1} SW quantified in fecal samples of wild-caught wood mice during the field trial was on average 30.1 (CI: 24.8 – 35.9, N = 3) and

was even lower than the number of copies found for wood mice that were given no carrot seeds (0 carrot seed number, Figure 3.1B) during the feeding trial. Using the calibration curves of Fig. 3.2 and filling in the log-transformed onion and carrot DNA copies g^{-1} BW g^{-1} SW of the field trial samples; the model predicted a seed intake of 1.08 (SE: 1.15) onion seeds and 0.00 (SE: 1.93) carrot seeds.

The above analyses were also performed including measurements that did not yield any onion or carrot DNA copies (see Appendix S3.2 for all the results including figures and statistics). A difference between the two analyses was found within a comparison between seed numbers 3 and 5 (Figure S3.4) for onion DNA content only (which was not apparent in the analyses with zeros excluded). Another difference between the two analyses was found in the total number of onion DNA copies g^{-1} BW g^{-1} SW found in fecal samples of wild-caught wood mice during the field trial. With zeros included, onion DNA copy number resembled the number of copies found when wood mice were fed 0 onion seeds during the feeding trial (instead of 1 in the analyses with zeros excluded). In this case, the calibration curve models predicted a seed intake of 0.00 (SE: 0.76) onion seeds and 0.00 (SE: 1.29) carrot seeds. This thus reflects no intake of onion and carrot seeds in the field, whereas the data with zeros excluded did.

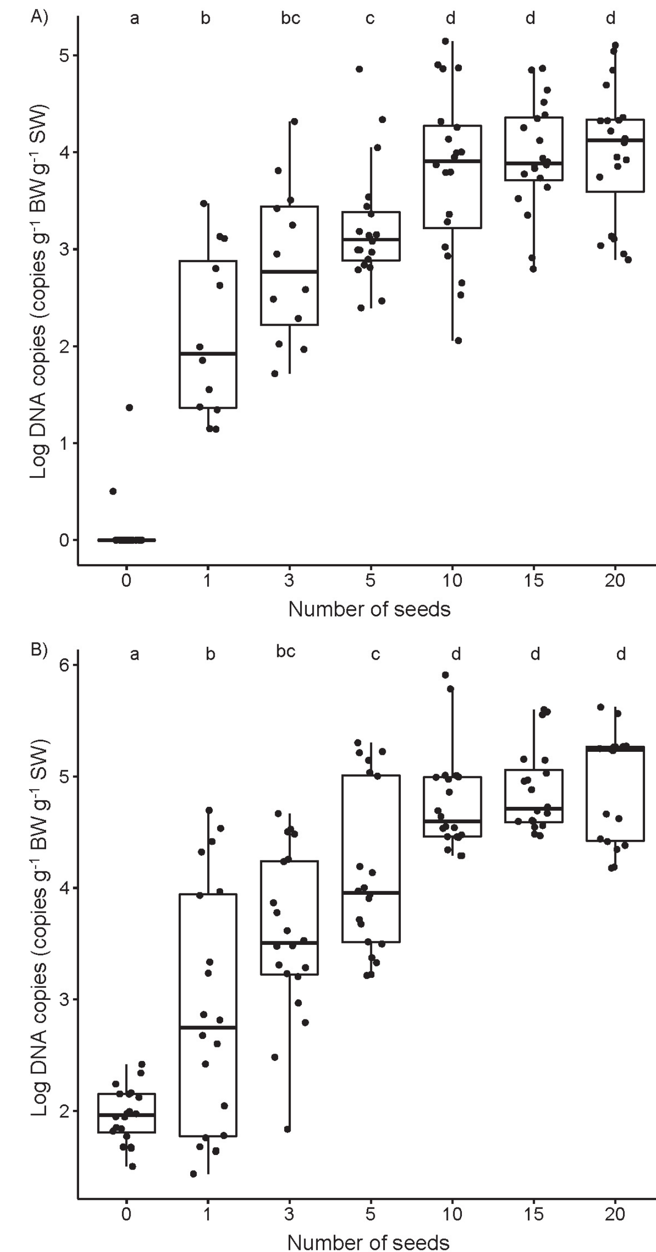

Figure 3.1 | Log-transformed onion **(A)** and carrot **(B)** DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples of wood mice fed different numbers of seeds (x-axis). Different letters a – d indicate a statistically significant difference between seed numbers in multiple comparisons ($p < 0.05$). N = 20 for each seed number except for graph A for 1 seed and 3 seeds N = 12 and for graph B for 20 seeds N = 18. Negative measurements (zeros) were removed from this analysis, except for seed number 0.

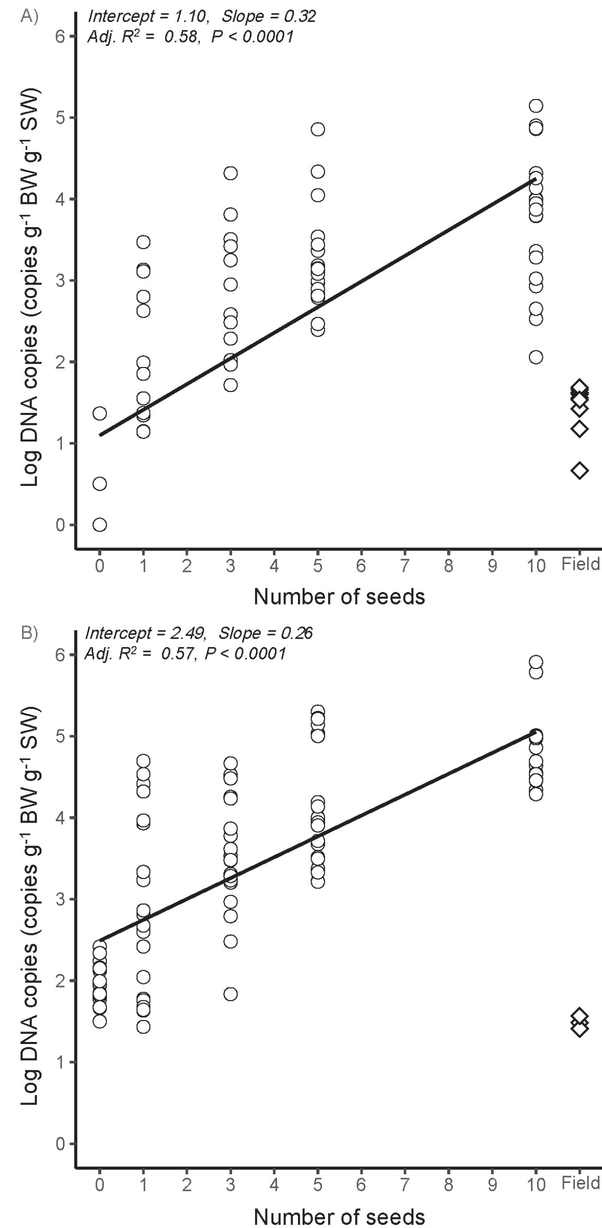

Figure 3.2 | Linear regression of log-transformed onion **(A)** and carrot **(B)** DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples of wood mice and seed numbers fed to wood mice in the laboratory feeding trial (0 to 10 seeds, circles). Black lines resemble linear trend lines. Statistic test results are given in the graphs for each seed species. Additionally, the DNA content of the fecal samples collected in the field trials (tilted squares) are also given to show the comparison between the field and laboratory feeding trial data. For laboratory feeding trial samples, N = 20 for each seed number for onion and carrot DNA except for graph A for 1 seed and 3 seeds N = 12. For field trial samples, N = 9 for onion DNA and N= 3 for carrot DNA. Negative measurements (zeros) were removed from this analysis, except for seed number 0.

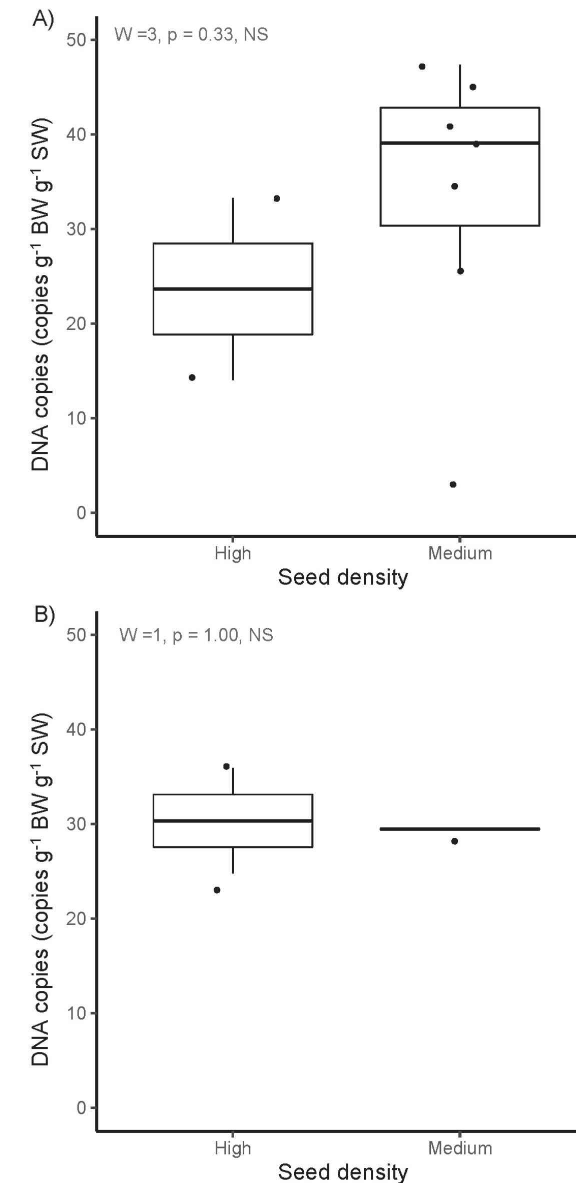

Figure 3.3 | Onion **(A)** and carrot **(B)** DNA content (copies per gram body weight (BW) and per gram sample weight (SW)) of fecal samples collected in the field trial with different seed densities (high and medium seed density) distributed on the field. Statistic test results are given in the graphs for each seed species. No significant (NS) differences were detected. In graph A, N = 2 for high density and N = 7 for medium density measurements. In graph B, N = 2 for high density and N = 1 for medium density measurements. Negative measurements (zeros) were removed from this analysis.

3.5 | Discussion

To our knowledge this is the first-ever study to quantify seed intake in a realistic field scenario using a DNA-based analysis. Calibration curves from 0 to up to 10 onion and carrot seeds were developed for the relation of seed numbers eaten by wood mice and DNA content of these seeds in feces, experimentally validated in a laboratory setting and applied in a field trial to quantify seed intake in a wild wood mouse population. Onion DNA was detected in the fecal samples of the wood mice caught in the field, which resembled a seed intake of up to 1 onion seed. Carrot DNA was also detected, although minimally, in the fecal samples of the mice caught in the field. However, unlike onion seed intake, the amount of carrot DNA copies was not sufficiently high to suggest any intake of carrot seeds by wood mice in the field.

This result can be explained by the fact that fecal samples of wood mice fed 0 carrot seeds in the laboratory trials did also contain (minimal) traces of carrot DNA (~100 DNA copies g^{1} BW g^{-1} SW). We allowed a 3-day resting period between each feeding trial run plus an extra fasting phase of 1 day to avoid detecting remnant target seed DNA in later trials. Therefore, it seems unlikely that such remnants biased results. Additionally, in Chapter 2 we showed that >97% of the DNA already passed the gut 24 hours after feeding carrot seeds. However, minimal traces of DNA (1.5%) did pass 37-48 hours after feeding wood mice 5 carrot seeds (see Chapter 2). We chose to add these 0 seed quantification values to the calibration curve and prevented the curve to intercept at the origin to correct for any of such 'late' gut passage occurrences. Importantly, the number of carrot DNA copies found in the field samples was even significantly lower than the number of copies found for mice fed 0 seeds which clearly indicates 0 carrot seed intake in the field.

To our knowledge seed intake has never been quantified in a realistic field scenario using DNA-based methods. This study is thus a proof of concept that DNA-based methods are applicable and useful to estimate seed intake (although with some experimental error/variation). Based on only the fecal samples of mice that did contain onion or carrot DNA, quantifiable seed intake was detected for onion seeds only. The inclusion of zeros (measurements where no target DNA was present) resulted in substantially lower average seed intake estimates of (near) zero seeds. This difference was expected with fecal samples from the field trial where almost 50 percent of samples did not contain onion DNA and more than 80 percent did not contain carrot DNA.

This result does imply that very few target seeds are consumed and if so, onion seeds are preferred over carrot seeds. This might be explained by the bigger size of onion seeds (more than 2 times heavier in weight) as this might potentially correspond to higher nutritional values and thus a more favorable tradeoff between effort and gain when foraging (Hernández et al., 2019). Furthermore, no effect of seed density was found on the number of DNA copies found in wood mice fecal samples for both onion and carrot seeds. This suggests that a higher number of these seeds available does not necessarily lead to higher consumption.

The calibration curves in this study were obtained by running linear regressions for known seed numbers and their corresponding DNA copies. Regular calibration curves tend to use single-point estimates (Hart et al., 2003). Here we chose to use probabilistic approaches as distributions and regression to get the best fit for our data. Our measure for goodness of fit obtained in this way provides a more complete and balanced description for the accuracy of risk assessment models (Van Loco et al., 2002; Hart et al., 2003). Although large variation within seed number DNA copies is apparent and inherent to digestive processes, which mostly favor random processes under the influence of the gut microbiome (Cresci & Bawden, 2015), our models explain a substantial part of the variation in our data (R^2> 0.55). Nonetheless, larger sample sizes per seed number would increase the power of these regressions and would improve calibration power (making the now large confidence intervals smaller). Additionally, less variation would theoretically allow for an extension of the calibration curve up to more than 10 seeds. However, in light of our field trial results demonstrating seed numbers below 5, improving the calibration curve for more than 10 seeds was considered irrelevant for this study.

Estimates of seed intake of the whole wood mouse population that were only based on (and averaged over) the positive samples led to higher estimates of seed intake. In the laboratory trials, only < 1% of the measurements for carrot DNA and < 5% of the measurements for onion DNA did not result in the detection of DNA content although mice had consumed 1, 3, or 20 (20 seeds only applicable to carrot DNA) target seeds (see Table S3.2, Appendix S3.2).

Therefore, considering positive samples only may be preferred in an ERA, even if this potentially leads to an overestimation. With the aim of ERA, an overestimation

might be preferred over an underestimation, since this could contribute to an improved protection of wild animals from the risk of exposure.

The quantification approach used in this study may be extended to other diet constituents that are consumed similar to the foraging of seeds by wood mice. A foraging behavior where delayed consumption occurs, e.g. some rodents collect large seeds that they find in their environment and deposit it in a hoard, rather than directly consuming them (Jones et al., 1990; Quy et al., 2005), would not favor accurate quantification in a real field scenario. It is unlikely that hoarding also occurs for small seeds, although this should be further investigated. Furthermore, note that to create an applicable DNA-based protocol for a new model organism or diet constituent new calibration curves are required for each that govern time and costs. Also, note that sampling feces should be conducted in the same period, or only shortly after, sowing occurs. If feces were to be sampled too long after sowing (when sprouting has already started), non-seed material of the same plant species (e.g. sprouts) is present and intake of this non-seed material will increase DNA copies in the feces causing an overestimation of the number of ingested seeds.

This study has proven that a less invasive assessment of seed intake by a relevant focal species can be obtained through DNA-based analysis of fecal samples. While we did not test this explicitly, when seed intake is combined with treatments by agrochemicals, our approach can be used to assess the exposure through feed intake by those agrochemicals in the European ERA process. This method can also be suitable for other exposure scenarios in other species and could be used as a less invasive tool in risk assessment and for a multitude of purposes in basic and applied research. Ultimately, this approach could optimize exposure models ensuring an improved level of protection for wild animals.

3.6 | Supporting information and data accessibility

Additional supporting information can be found in the online version of the article at the publisher's website (scan the QR code below). Raw onion and carrot DNA copy counts as measured by ddPCR in the fecal samples per mouse fed different MPs and the field trial samples are available at Mendeley Data: https://doi.org/10.17632/r7pfjmxxcd.1.

4|

DNA metabarcoding illuminates the contribution of small and very small prey taxa to the diet of lions

Kevin Groen | Sophie Beekenkamp | Hans H. de Iongh | Francis Lesilau | Mumbi Chege | Luka Narisha | Michiel Veldhuis | Laura D. Bertola | Peter M. van Bodegom | Krijn B. Trimbos |

Published in **Environmental DNA**, 2023, *00*, 1-11.
DOI: 10.1002/edn3.457

4.1 | Abstract

Knowledge of food web interactions is essential for understanding the role of carnivores in an ecosystem and designing appropriate conservation and management strategies to preserve them. These interactions can only be understood by studying carnivores' diets and obtaining comprehensive and unbiased diet data. For large carnivores - which typically rely on large herbivores as prey - the role of smaller prey species has not received attention. This study aims to quantify the contribution of small (5-50 kg) and very small (<5 kg) prey taxa in the diet of lions (*Panthera leo melanochaita*) in four Kenyan National Parks (NPs). We use DNA metabarcoding to achieve higher-resolution insights into prey composition, which is less biased toward large prey species compared to traditional methods, such as carcass counts. Our study identified 24 prey taxa in a total of 171 lion fecal samples. Small and very small prey taxa together contributed 18.7% out of 278 prey occurrences in all fecal samples. With comparable small prey presence (ranging from 8% to 15%) in the diet for each NP studied. This approach proved to be useful in detecting small and very small prey species in the diet of lions and can therefore be used in future research to uncover the diverse diet composition of these large carnivores. The consistent presence of smaller prey species in the diet indicates that lions generally supplement their large prey diet with smaller prey.

4.2 | Introduction

Predator-prey interactions are the basis for understanding a species' role in an ecosystem. Detailed knowledge about a predator's diet provides useful information on which prey species are affected by the predator, the level of competition with other predators, their dietary flexibility and dependence on the presence of specific prey. Given their generally wretched conservation status (Ripple et al., 2014) and perceived strong influence on ecosystems through top-down control (Vogel, Somers & Venter, 2018; Atkins et al., 2019), a comprehensive insight into apex predator diets is of utmost importance to designing appropriate conservation and management strategies to preserve them (Xiong et al., 2017; van der Heyde et al., 2021).

Lion (*Panthera leo*), an apex predator, prefers prey species within the weight range of 96-632 kg – e.g. gemsbok (*Oryx gazella*), African buffalo (*Syncerus caffer*), blue wildebeest (*Connochaetes taurinus*), giraffe (*Giraffa camelopardalis*), plains zebra (*Equus quagga*) – with an optimum prey weight around 350 kg (Hayward and Kerley 2005; Clements et al., 2014). Smaller prey species (<50 kg) are believed to be avoided by lions since they are too low in energetic content for lions to obtain enough energy from hunting to be sustainable (Carbone et al., 1999; Hayward & Kerley, 2005; Lesilau, 2019; Barnardo et al., 2020). Nonetheless, non-preferred prey species can contribute up to 36% of the lion diet in Hluhluwe–iMfolozi Park, South Africa, even when preferred prey species were also high in abundance (Barnardo et al., 2020). Furthermore, it has been observed that small prey species (5-50 kg) such as small antelopes (Bauer et al., 2008; Davidson et al., 2013; Lesilau, 2019) and very small prey species (<5 kg) such as small birds and rodents (even mice, see Davidson et al., 2013; Sogbohossou et al., 2011; Lesilau, 2019) are part of a lion's diet as well. Lion encounters with small prey species (e.g. Steenbok (*Raphicerus campestris*), Hare (*Lepus sp.*) and Porcupine (*Hystrix africaeaustralis*)), regularly (68.7%) resulted in a hunt (Hayward and Kerley 2005). Encounter rates increase when small prey occur in high densities, reducing the costs of actively pursuing small prey (Clement et al., 2014).

It remains unclear however, whether the selection of smaller prey species by lions is an exception (e.g. due to incapability of the lion to hunt larger prey or lack of larger prey in the ecosystem) or whether these smaller prey species have been overlooked in the past due to a bias in the research methods used

(Power, 2002; Hayward & Kerley, 2005). Diet composition estimates based on carcass counts include a bias towards large prey species, because the detection chance and the fast and often complete consumption of smaller prey lead to a potential underestimation of small prey species in lion diets (Davidson et al., 2013; Clements et al., 2014; Barnardo et al., 2020). Additionally, sometimes lions cover the kill and make carcass counts unsuitable (Schaller, 1972). Moreover, the detection of smaller prey species with microscopic hair morphology analysis of prey hair in lion scats is more difficult than detecting larger prey species because of the low body mass or the absence of hairs in the scat (i.e. reptiles) (Lesilau, 2019). To adequately manage prey populations to sustain lions, the role of small (5-50 kg) and very small (<5 kg) prey needs thus to be addressed. However, apart from the study of Lesilau (2019) in Nairobi National Park, the role of smaller prey species in the diet of lions has not been studied yet.

DNA metabarcoding is a powerful method to detect the presence of species in environmental DNA samples, such as fecal samples (Rubbmark et al., 2018). DNA metabarcoding has been used as an approach to overcome the biases of traditional diet analysis studies since DNA metabarcoding of fecal samples has shown to be successful in studying a more complete foraging niche of predator species (Symondson, 2002; Shehzad et al., 2012; Xiong et al., 2017; Forin-Wiart et al., 2018; Lesilau, 2019; Thuo et al., 2019; Beng & Corlett, 2020). For example, the study by Forin-Wiart et al. (2018) identified 29 prey taxa in the diet of domestic cats (***Felis silvestris catus***), including rodents, birds, and reptiles. Metabarcoding approaches may thus allow evaluation of the contribution of small and very small prey species in lion diets.

In this study, we aim to fill the knowledge gap which results from the bias towards large prey species in conventional methods, by specifically focusing on the contribution of small (5-50 kg) and very small (<5 kg) prey taxa in the diet of lions (*Panthera leo melanochaita*) using DNA-metabarcoding. We chose four ecologically different Kenyan National Parks (NPs) to obtain a broad spectrum of the lion's diet and therefore food-web interactions. Our study is the first-ever study, to our knowledge, that focuses on the proportions of smaller prey taxa in the diet of lions in multiple Kenyan NPs using a DNA-based approach.

4.3 | Materials and Methods

4.3.1 | Study sites

Four National Parks in Kenya with different ecosystems were selected for data collection to obtain a broad spectrum of lion prey and predator-prey dynamics (Figure 4.1, see Appendix S4.1 for all the species considered for the diet analysis). Meru National Park (MNP, 884 km^2), a partly fenced savannah bushland, was established as a National Park in 1967 and is located in the Eastern Province of Kenya (0°20'- 0°10' S; 38°0'- 38°25' E) (Sitienei, Jiwen & Ngene, 2014). Vegetation and ecosystem types within MNP predominantly consist of thorny ***Acacia-Commiphora*** bushland and ***Acacia*** wooded grassland (Bundotich et al., 2016). Established in 1974, located Southwest of MNP, close to the Tanzanian border and in the Rift Valley province, lies Amboseli National Park (ANP, 392 km^2, 02°37 S; 37°15 E), a non-fenced savannah grassland with seasonal flooding (Okello et al., 2008). Lions here have the opportunity to disperse to other reserves and to the surrounding environment (Huqa, 2019). Approximately 7 km south of the city Nairobi, situated in the Nairobi Province, lies Nairobi National Park (NNP, 117 km^2, 01°20'- 01°26' S; 36°50'- 36°58' E). This park, established in 1946, consists predominantly of savannah grassland and was semi-fenced in 1955, with about 56% of the park's northern border being fenced to create a separation between the city and the National Park (Lesilau, 2019). Northwest of NNP and south of the city of Nakuru lies Lake Nakuru National Park (LNNP, 188 km^2, 0°18'- 0°30' S; 36°2'- 36°9' E) which is located in the Rift Valley Province, like ANP (Kassilly et al., 2008). The park was established in 1961 and completely enclosed with an electric fence in 1986. The landscape, enclosing a saline lake, consists of grasslands, swamps and marsh, with rocky cliffs and outcrops. There are areas of woodland and rocky hillsides covered with bushland and forest (Kassily et al., 2008). All NPs have two rainy seasons: long rains from March to May and short rains from October to December with dry spells in between. Lion densities in the four NPs were not exceeding carrying capacity of the respective parks at the time of data collection and all four parks contain prey populations that show stable or increasing trends (Lindsey et al., 2017; Elliot et al., 2020).

4.3.2 | Sample collection and DNA extraction

Lion scat samples were collected between the 4th of February and the 16th of April 2019 (Figure 4.1). Scat samples were collected opportunistically along transects during patrols thrice a week. Stuart & Stuart (2013) pocket guide was used to identify the scats and additional information was noted down (date and time of collection, GPS coordinates, habitat type, weather conditions, and scat freshness). The categories for freshness of scats were specified from 1 to 4 (1 = inside and outside of the scat were soft; 2 = inside soft, outside hard; 3 = inside and outside hard; 4 = only hairs left) with 1 being the freshest. Five pinches were taken from inside the scat with sterilized forceps, preserved in vials with 1 mL 99% ethanol and stored in a fridge at the end of each fieldwork day. After all fieldwork was completed, the samples were transferred to a -20°C freezer. In total, 313 scats were collected (ANP: 66, LNNP: 58, MNP: 70, NNP: 119). To extract DNA from the scat samples, the protocol for 'Isolation of DNA from Stool for Pathogen Detection' of the QIAamp DNA Stool Mini Kit handbook was followed. DNA extraction was performed in a room dedicated to processing low-quantity DNA samples with regular decontamination cycles of the laboratory materials inside a UV cabinet to prevent contamination. Samples of each NP were extracted separately to ensure no contamination between NPs.

4.3.3 | Scat identification and DNA metabarcoding

After extraction, each sample was checked with a lion-specific PCR for the presence of lion DNA to make sure the correct scats were collected and subsequently used in the diet analysis. We used lion (*Panthera leo*) specific primers (specificity checks were done *in-silico*) that targeted the cytochrome b gene (5'-> 3': forward primer; TCCACCTCCTGTTCCTCCAT and reverse primer; AGGATTGGCGGGGGTATAGT). The PCR reaction (25 µL) consisted of 1 µL DNA extract and 16.75 µL milliQ water, 2.5 µL KlearTaq buffer, 2.5 µL dNTP, 1 µL forward primer, 1 µL reverse primer and 0.25 µL KlearTaq polymerase. The PCR program started with an initial denaturation step of 15 min at 95 °C, followed by 40 cycles of 40 s at 94 °C, 30 s at 48.5 °C and 60 s at 72°C with a final extension step of 7 min at 72°C. Only samples that tested positively for lion DNA were used in subsequent DNA metabarcoding analysis. Additionally, location and freshness of

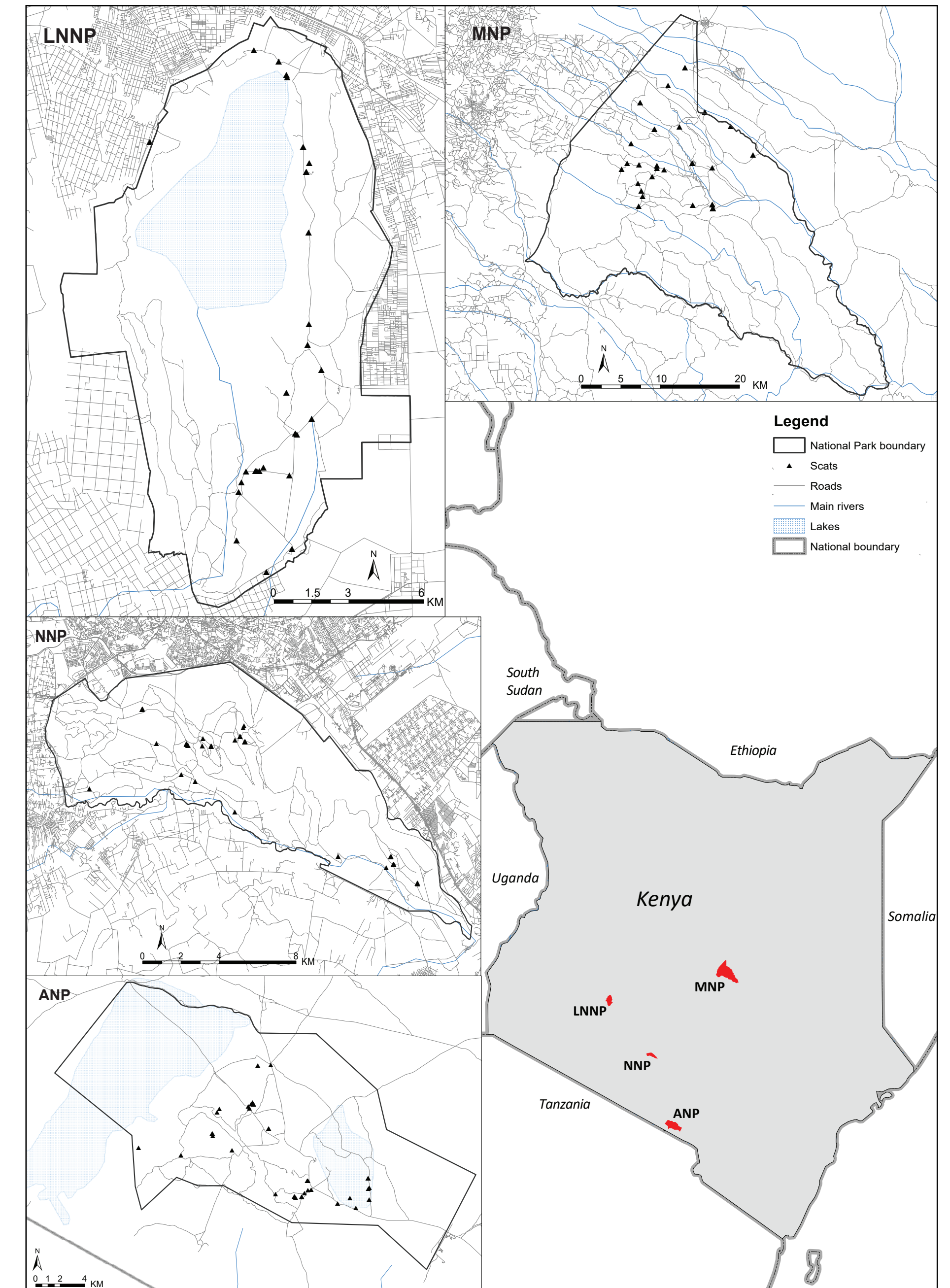

Figure 4.1 | Maps of the study sites where scat samples were collected: Amboseli National Park (ANP), Lake Nakuru National Park (LNNP), Meru National Park (MNP), and Nairobi National Park (NNP). Locations for the scat collection sites are indicated with black triangles. The map on the bottom right corner shows Kenya with the four study sites depicted in red. The maps were generated with data from UNEP-WCMC and IUCN (2022) in ArcGIS v10.6 (ESRI, Redlands, CA, USA).

scats were considered when selecting samples for sequencing to make sure that multiple prides were taken into account (when present) and that DNA extraction from the freshest scats (categories 1, 2 and 3) was used to prevent degradation bias as much as possible. After this selection procedure, a total of 171 DNA extracts (44 DNA extracts from ANP, 31 from LNNP, 50 from MNP and 46 DNA extracts from NNP) were analyzed using DNA metabarcoding.

A second PCR with vertebrate-specific primers, Mod_RepCOI_F and VertCOI_7216_R, to amplify a 244-bp fragment of the cytochrome c oxidase subunit I (COI) gene was used, which has been demonstrated to have high-resolution power for identifying the genera across most vertebrate taxa (Reeves et al., 2018). *In-silico* analysis of primer mismatches against East African vertebrate taxa can be found in Appendix S4.1. Since lions tend to eat only once a day, and digestion, and therefore defecation, is quick (Schaller, 1972) we expect only a few prey species per scat and high prey copy numbers. We, therefore, did not include a blocking primer, as with few prey species per scat, possibly reduced sequencing depth due to swamping of host DNA is less of an issue in this particular dietary analysis (see also Piñol et al., 2014). Amplification was carried out in a final volume of 20 µL, using 3 µL DNA extract and 15 µL TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, Waltham, United States), 0.1 µM Mod_RepCOI_F with Nextera forward tag attached and 0.1 µM VertCOI_7216_R with Nextera reverse tag attached. The PCR program started with an initial denaturation step of 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C, 30 s at 48.5 °C and 40 s at 72°C with a final extension step of 5 min at 72°C. During the third amplification, Nextera i7/i5 index adapters were added to the amplicons for the purpose of pooling at sample level. Positive PCR controls (POS), DNA extracts of Chilean flamingo (*Phoenicopterus chilensis*, geographically restricted to South America; BirdLife International, 2018) and wood mouse (*Apodemus sylvaticus*), and no template PCR controls (NTCs) were included in the metabarcoding amplifications to check for contamination. Additionally, for every park, negative field control(s) (NEG) (by dipping sterilized forceps used for sampling in 99% ethanol) were included in the diet analysis. Final PCR products were pooled in equimolar concentrations and sequencing was carried out on the Illumina MiSeq (v3 Kit, PE300) platform (Illumina Inc., San Diego, CA, USA) at the NGS facility of BaseClear, Leiden, The Netherlands.

4.3.4 | Reference database

By using a custom reference database, a more robust result can be achieved since no species are under or overrepresented in the database and mismatches with species not occurring at the study site can be prevented (Nakahara et al., 2015). A custom reference database was created by selecting species for each genus of terrestrial mammals and reptiles and each family of birds, occurring in Kenya, based on field guides (Stevenson & Fanshawe, 2002; Stuart & Stuart, 2013; Branch, 2014). Fish were not taken into account as, to our knowledge, there is not any scientific literature suggesting active hunting behavior of lions towards fish. Bird sequences were selected at family-level instead of genus-level since taxation by family can be a higher taxonomic surrogacy for taxation by genera (Kallimanis et al., 2012). In total, 194 sequences (Appendix S4.1) were added to a custom database using Geneious (Geneious Prime® 2021.0.3), based on sequences from the Barcode of Life data system (Ratnasingham & Hebert, 2007) and GenBank (Sayers et al., 2022), including 21 large mammal genera (27 sequences), 66 small mammal genera (69 sequences), 61 bird families (61 sequences), and 37 reptile genera (37 sequences).

4.3.5 | Bioinformatics and taxonomic identification

The custom reference database sequences were uploaded to the Galaxy web platform (galaxy.naturalis.nl) and a bioinformatics pipeline was created in Galaxy (Afgan et al., 2018) to analyze the output of the MiSeq sequencing data. Data preparation consisted of merging demultiplexed reads based on forward and reverse primers by using FLASH (Magoč & Salzberg, 2011) with a minimum overlap of 10 bp and a maximum overlap of 300 bp and a mismatch ratio of 0.25. The forward and reverse primers were trimmed by using the Cutadapt sequence trimmer (Martin, 2013). Subsequently, only sequences with a length of 240-260 bp and with a minimum quality of 25 were kept using the PRINSEQ tool (Schmieder & Edwards, 2011). Next, OTUs were created by clustering reads using >98% similarity, with a minimum accepted abundance of two before clustering (i.e. removing all singletons), and the presence of chimera sequences was checked by using VSEARCH (Rognes et al., 2016). The created OTUs were BLASTed to the custom reference database using BLAST+ (Camacho et al., 2009). A least

common ancestor (LCA) analysis (Hoogeveen, 2019) was performed to account for missing identifications (identity threshold >98%) and a table including all OTUs with their number of reads per sample was created. Since low amount of contamination is inevitable with NGS technologies, especially when using universal primers, despite strict conformance to good laboratory practices for minimizing contamination risks (Pompanon et al., 2012; De Barba et al., 2014) we used the reads in the control samples (POS, NTC, NEG) to filter out any contamination by subtracting the number of sequence reads found in the controls per taxon from the number of reads found in the samples. Lastly, a taxonomy worksheet was created, displaying all occurrences for every taxonomic unit for every sample. If samples had missing identifications at genus-level and composed ≥1% of the total number of reads in a sample a higher taxonomic level was used.

4.3.6 | Data analysis

We calculated the sequence hits for each species, when possible, or otherwise genus in each sample. Since this study aimed to detect the occurrence of small (5-50 kg) and very small (<5 kg) prey taxa in the diet of lions, prey taxa were placed into one of three categories, adapted from Lesilau (2019), based on their minimum weight (see Appendix S4.1): medium to large prey taxa (>50 kg), small prey taxa (5-50 kg), and very small prey taxa (<5 kg). No distinction was made between large (>200 kg) and medium-sized (50-200 kg) prey taxa (Bauer et al., 2008). Next to these three prey categories based on weight, we added a prey category called 'mesopredator', consisting of sequence hits of ***Canis sp.*** (black-backed jackal, African wolf, or domestic dog), ***Leptailurus sp.*** (serval) and ***Ichneumia sp.*** (white-tailed mongoose). Mesopredators were placed in a separate category because we cannot rule out that this signal is the result from field contamination. These species are known to over-mark scats from other carnivores for designation of their own territory or other communication signals (e.g. see Wikenros et al., 2017; Lesilau, 2019; Di Bernardi et al., 2021). These mesopredator genera are all known to use their olfactory glands or urine and scat for scent marking (Estes et al., 2012) and may thus give a positive signal in our results because of their behavior and not as actual prey. As lions do tend to sparsely kill mesopredators (Mills & Funston, 2003), we chose not to exclude these taxa from the lion diet results.

Spotted hyenas (*Crocuta crocuta*) are the only known predators to actively scavenge on lion scats, thereby introducing their DNA into lion scats (Owen-Smith & Mills, 2008; Yirga et al., 2012). Therefore, these sequences were removed from the analysis. All ***Panthera sp.*** sequences (40% of total sequences) were also removed from the analysis as these were attributed to the host species (see Appendix S4.2 for more data on percentages of host DNA compared to the total reads per sample for each NP). Human DNA found by DNA metabarcoding is likely a result of sample processing in the lab, as no attacks on humans were observed or heard of during the time of sampling, and thus these sequences were also omitted from the results.

Each presence of a prey taxa (regardless of the number of sequences of that prey taxon) within a fecal sample was counted as an occurrence, where presence of multiple taxa means multiple occurrences within one fecal sample. The diet of lions was quantified as a proportion of occurrence (%TX) of an individual prey taxon over all occurrences found in all fecal samples (Xiong et al., 2017). To obtain proportions of occurrences per park, only the respective fecal samples of that particular park were used.

Differences in the number of taxa per sample per park were tested using Kruskal–Wallis rank sum tests and a subsequent post hoc Holm-adjusted Dunn test (Dunn, 1964), due to the non-normality of the data. Figures were made using the ***moonBook*** and ***webr*** packages (Moon, 2015; Moon, 2020) and statistical calculations were done using the ***FSA*** package (Ogle et al., 2021) in R (R Core Team, 2021).

4.4 | Results

The sequencing run of the 171 scat samples from four Kenyan NPs generated 10.7 million reads with a mean quality (Q-score) of 34.9 (SD 0.7). The mean number of reads found per sample was 100,330, 95% CI [0 - 210,325] reads, for samples collected in ANP; 42,400, CI [38,749 - 46,051] reads for samples collected in LNNP; 45,790, CI [40,264 - 51,314] reads for samples collected in MNP; and 47,346, CI [45,326 - 49,365] reads for samples collected in NNP. After merging reads, trimming primers, and quality control, mean reads per sample was 26,102, 95% CI [0 - 53,325] reads for ANP; 11,275, CI [9,815 - 12,734] for LNNP; 11,488, CI [10,477 – 12,498] for MNP; and 14,469, CI [13,559 – 15,378] reads for NNP (see Appendix S4.2 for the number of reads per taxa per sample for each NP). After exclusion of the taxa we consider not relevant for this study (see Methods), these reads were assigned to 2 families, 22 genera and 1 species (Figure S4.1). Controls (POS, NTC and NEG) contained little contamination (Table S4.1). After correction for these contaminations, 24 taxa, of which 2 families, 21 genera, and 1 species remained. The number of prey taxa per sample varied from 1 to 5 with a mean of 1.6 (SD 1.1). Combining the lion diets of the four parks resulted in a total of 278 prey occurrences, of which 74% of the diet was composed of medium to large prey taxa, with Bovidae (43%) and Equidae (25%) as largest components in this prey category (Figure 4.2). The other 26% was composed of small (11%), very small (8%) prey taxa and mesopredator (8%) taxa. *Lemniscomys* (striped grass mouse), *Aepyceros* (impala) and *Madoqua* (dik-dik) composed the top-three small and very small prey genera by contributing 5%, 4% and 3% to the diet, respectively. Additionally, small prey genera included *Papio* (baboon), *Nanger* (gazelle) and *Hystrix* (porcupine), and very small prey taxa included *Phoenicopterus roseus* (greater flamingo), *Gallus* (chicken) and *Charadrius* (plover). Mesopredator DNA found in the scat samples of lion were attributed to *Leptailurus* (serval), *Canis* (jackal or domestic dog) and *Ichneumia* (white-tailed mongoose) by 4%, 2%, 1% of total occurrences, respectively.

Differences in the lion diet composition between ANP, LNNP, MNP, and NNP were found (Figure 4.3). Lowest medium to large prey proportions were found in LNNP (53%), and highest proportions in MNP (83%). Small prey proportions were relatively comparable among parks, although slightly larger in MNP (15%) and lowest in NNP (8%). The proportion of very small prey varied strongly, with especially low proportions in MNP (2%) compared to the highest proportions in LNNP (19%). The same trend was found for mesopredator occurrences, where highest proportions were present in LNNP (16%) and no occurrence in MNP.

The maximum number of taxa found per sample in the respective parks was four for ANP, MNP and LNNP with a mean of 1.8 (SD 1.0), 1.0 (SD 0.9) and 1.2 (SD 0.8), respectively. Maximum in NNP was five with a mean of 2.3 (SD 1.2) taxa per sample. The number of taxa found per sample (including all prey categories) differed significantly between the NPs (Figure S4.2, $H = 37.1$, [$N = 171$], $p < 0.0005$). Only ANP and NNP, and LNNP and MNP did not significantly differ from each other in number of taxa per sample found.

The diet of lions in ANP was composed of 70% medium to large prey taxa and 21% small (10%) and very small (11%) prey taxa, including three mammal genera (*Nanger*, *Aepyceros* and *Papio*) as part of the small prey category, one mammal genus (*Lemniscomys*) belonging to the very small prey, and two bird taxa (*Phoenicopterus roseus* and *Charadrius*). The other 9% of the lion diet in ANP was contributed to three mesopredator genera, with the highest occurrence of *Leptailurus*. In LNNP, 53% of the diet was composed of medium to large mammal prey.

Furthermore, small (13%) and very small (19%) prey taxa together contributed 32% of the diet. These prey categories were represented by two mammal genera (*Madoqua* and *Papio*) belonging to the small prey category, and one mammal genus (*Lemniscomys*) as part of the very small prey. Three genera of mesopredators (16% of the total diet in LNNP) were found, with *Leptailurus* ranking as the most frequently occurring. Medium to large prey taxa, small prey taxa, very small prey taxa and mesopredator taxa contributed 83%, 15%, 2% and 0% to the diet of lions in MNP, respectively. The total small and very small prey (17%) consisted of two mammal genera (*Madoqua* and *Papio*), one rodent genus (*Hystrix*) and one bird genus (*Gallus*). In NNP, the contribution of medium to large prey taxa to the diet was 77% and the rest was composed of small (8%) and very small (6%) prey taxa and 8% belonged to mesopredator taxa. Small and very small prey taxa (14%) were composed of two mammal genera (*Aepyceros* and *Madoqua*) which belong to the small prey category, one mammal genus (*Lemniscomys*) as part of the very small prey, and one bird species (*Phoenicopterus roseus*). Three mesopredator genera were present in the results of NNP, with the highest occurrence of *Leptailurus*, as also observed in ANP and LNNP.

4

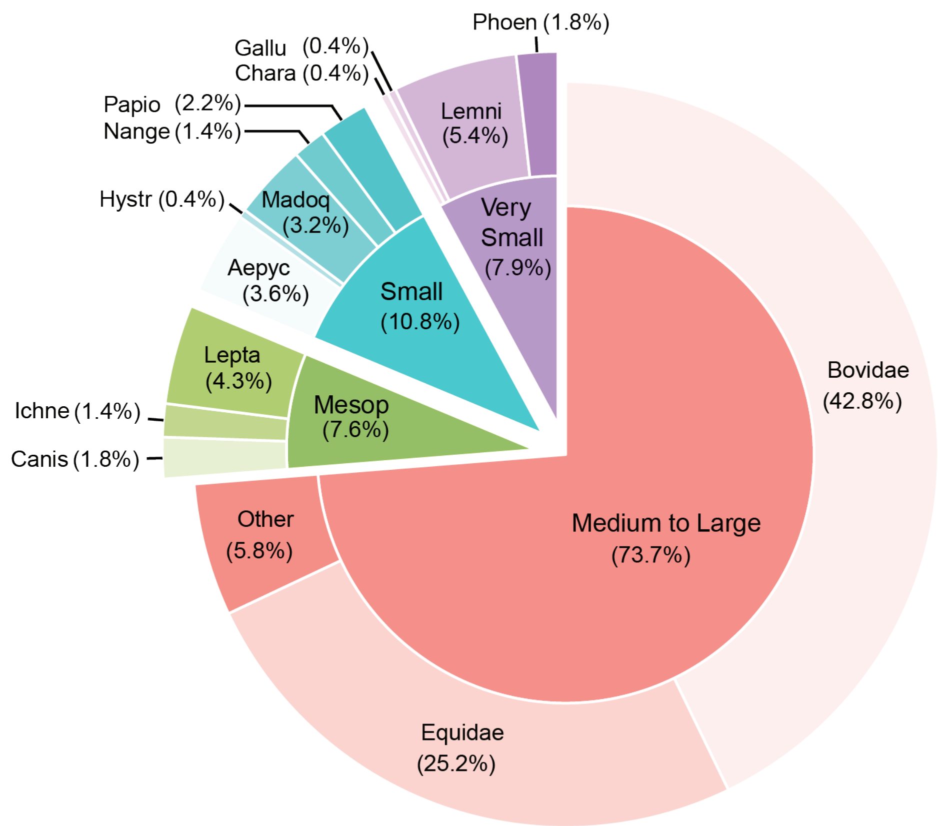

Figure 4.2 | Proportions of occurrences (%TX) of the four prey categories in the diet of the lion in four National Parks (NPs) in Kenya. Prey taxa identified and their contribution in percentages are given, and the diet contribution of medium to large, small, very small taxa are depicted, as well as presence of mesopredator (Mesop) taxa. Diet composition by prey taxon and prey category is presented as a proportion of occurrence (%TX). Total prey occurrences found were 278 in a total of 171 scat samples. The 'Other' in the 'Medium to Large' prey category consists of *Giraffa* (3%), Suidae (2%), *Hippopotamus* (0.4%) and *Crocodylus* (0.4%). In the 'Medium to Large' category the Equidae consisted of only one genus; *Equus*, whereas the Bovidae consisted of 7 taxa; *Syncerus* (20%), *Connochaetes* (14%), *Alcelaphus* (6%), *Bos* (1%), Bovidae sp. (0.7%), *Kobus* (0.4%), and *Tragelaphus* (0.4%). Taxa abbreviations used: *Aepyceros* (Aepyc), *Charadrius* (Chara), *Gallus* (Gallu), *Hystrix* (Hystr), *Ichneumia* (Ichne), *Lemniscomys* (Lemni), *Leptailurus* (Lepta), *Madoqua* (Madoq), *Nanger* (Nange) and *Phoenicopterus roseus* (Phoen).

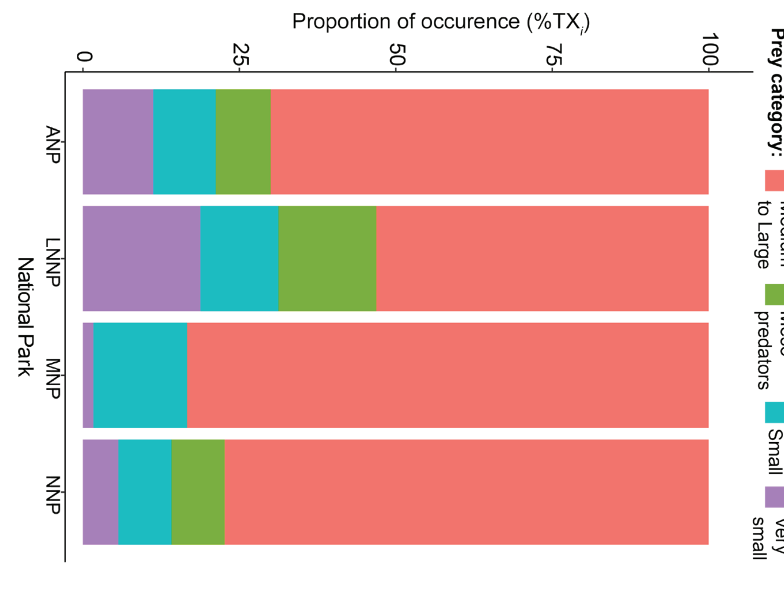

Figure 4.3 | Proportions of occurrences (%TX) of the four prey categories in the diet of the lion given for four National Parks (NPs) in Kenya on the left. On the right, small, very small and mesopredator proportions of occurrences (%TX) are further specified per taxon for each NP. Total occurrences in Amboseli National Park (ANP) were 80, in Lake Nakuru National Park (LNNP) 32, in Meru National Park (MNP) 60 and in Nairobi National Park (NNP) 106. Taxa abbreviations used: *Aepyceros* (Aepyc), *Hystrix* (Hystr), *Madoqua* (Madoq), *Nanger* (Nange), *Lemniscomys* (Lemni), *Phoenicopterus roseus* (Phoen), *Charadrius* (Chara), *Gallus* (Gallu), *Leptailurus* (Lepta) and *Ichneumia* (Ichne).

4.5 | Discussion

This study specifically addressed the role of small (5 - 50 kg) and very small (<5 kg) prey in the lion diet across multiple Kenyan NPs without a bias towards larger prey species. We found 24 prey taxa and 278 prey occurrences in 171 fecal samples. Out of these 278 prey occurrences, 205 (74%) were attributed to medium to large prey (>50 kg). Of these 205 medium to large prey occurrences more than half (58%, 109 occurrences) were attributed to the Bovidae family. Small and very small prey taxa in the diet of lions were also successfully determined using our DNA-based approach and contributed 19% (out of all prey occurrences) to the diet of lions in Kenya. A total of 7.6% of all occurrences were contributed to mesopredator taxa.

4.5.1 | The composition of small and very small prey in a lion's diet

In this study, the small prey taxa *Aepyceros* (impala) and *Madoqua* (dik-dik) were detected most frequently in the diet of lions. This is consistent with findings of Davidson et al. (2013) who showed that small antelopes could explain 17% of the lions' diet in Hwange National Park, Zimbabwe and that lions feed on small antelopes opportunistically when they are encountered in the environment. Occurrences of very small prey taxa originated mostly from *Lemniscomys* (striped grass mouse). Surprisingly, bird taxa composed the other occurrences of very small prey taxa found in the diet of lions. Lesilau (2019) showed that DNA analysis compared to hair analysis increased the contribution of very small prey species to the lion diet from about 2% to 9%. However, the primers (12SV5F/12SV5R) used in the research of Lesilau (2019) are known to be biased towards amplifying only mammal DNA which explains the lack of birds in his dataset (Riaz et al., 2011; Shezad et al., 2012). Our results are likely more robust compared to Lesilau's (2019) study as we used a vertebrate-specific primer and used 171 samples in our analysis, instead of only 10 samples used in Lesilau's study. Previous studies, using traditional methods, have also shown contribution of small prey species to the diet of lions (Lehmann et al., 2008; Davidson et al., 2013; Barnardo et al., 2020) meaning that lion predation is not size-partitioned (Hopcraft et al., 2010). However, the presence of very small prey species (<5 kg) in the diet of lions was only anecdotally observed (Davidson et al., 2013; Lesilau, 2019).

4.5.2 | Differences in diet between national parks

Differences were found between lion diets in the four National Parks. ANP and NNP showed a higher number of prey taxa per sample compared to LNNP and MNP which suggests that lions in these parks have a more diverse diet. In LNNP, we found the largest contribution of smaller prey compared to the other NPs. Probably this is due to the low number of individual lions in LNNP (only 11 individuals >1 year old, based on Elliot et al., 2020) which results in small pride sizes and thus small hunting groups. As group hunting allows lions to take larger prey (Hayward & Kerley, 2005), the small pride size in LNNP might explain why lions in LNNP hunt less hazardous, smaller prey.

MNP lions, on the other hand, had the highest contribution of medium to large prey species in the diet. The study by Loarie, Tambling & Asner (2013) found a strong link between hunting behavior and dense vegetation. As MNP predominantly consists of wooded grassland and bushland (Bundotich et al., 2016), the higher contribution of larger prey species in the diet might be explained by denser vegetation compared to the three other NPs (Hopcraft et al., 2005). Hay et al. (2008) also showed that larger prey, are more vulnerable to predation in dense bush due to ambush hunting by lions.

4.5.3 | Considerations for DNA-based diet analyses

The sensitivity of DNA-based diet analysis aids detection of small taxa, but this sensitivity also means that it will detect species that may have not been eaten by the animal of focus. In our study, we found substantial presence of mesopredator DNA (especially serval) in the fecal samples of lions, which we interpret as over-marking by mesopredators (Wikenros et al., 2017). Yet, it is known that lions tend to aggressively exclude and kill other carnivores to display dominance and prevent competition (Mills & Funston, 2003) as well as compete for, or scavenge on, mesopredator kills.

Therefore, the origin of mesopredator DNA cannot be fully attributed to over-marking alone. The role of over-marking and competition in interspecific interactions between intraguild species remains largely unknown (Allen et al., 2016). Mesopredator visits to apex predator scats may be seen as a trade-off between obtaining information on a potential food source (e.g. prey killed by lions) and the potential risk of predation by an apex predator (Wikenros et al., 2017). Another pathway of contamination is through secondary predation (Tercel et al., 2021). However, we expect this to occur minimally in the diet of lions, as lions tend not to eat the inside of the digestive tract of prey (Schaller, 1972). Additionally, passing the digestive tract twice (that of mesopredator and lion) may severely degrade DNA beyond amplification quality. Moreover, only in 45% of the fecal samples that contained very small prey DNA we also found DNA of mesopredators. This illustrates that secondary predation does not explain finding very small prey in the diet of lions. A final pathway of contamination is via ecological transfer: scats attract coprophagous taxa (mostly insects) which in turn attract small predators, such as insectivores, that feast on these prey species additionally scats may attract osteophagous animals. Via saliva or feeding remains or directly by touching the fecal samples, these small predators can transmit their DNA into the scat of lions. Although we have not witnessed Arthropoda in the fecal samples collected, these ecological transfers may be excluded by complementary analysis of morphological remains of the complete scat. For example, presence of bone remains from very small species, such as *Lemniscomys sp.*, in the fecal sample would suggest that they were actually a prey species while the absence could point in the direction of ecological transfer. The same caution also applies to bird taxa found in the diet. A combination of DNA and morphologically based methods might be complementary in such cases.

In light of all this, inferring the role of smaller prey in the lion's diet based on occurrences might lead to an overestimation of the importance of small prey species, but not for making comparisons among study areas (Ferreras & Fernandez-de-Simon, 2019). Instead of using occurrences to count diet, which might overestimate small prey, relative read abundances can be used. However, Deagle et al. (2018) states that both approaches are similarly accurate when the mean number of food taxa in samples is small, as in our case where we observed an average of only 1 to 3 species per sample (Figure S4.2). Smaller prey probably constitute less in terms of biomass and energy intake than larger prey towards a lion's diet. However, conversion factors that can transfer DNA content in feces to actual biomass intake are greatly lacking (but do exist for the studies of food remains in feces and gut contents, e.g. see Wachter et al., 2012 and Chapter 2). Hence, as DNA-based approaches are increasingly being used for diet studies, feeding studies relating biomass to DNA content are needed but currently remain challenging (Deagle et al., 2018).

Eventually, DNA-based estimates of biomass intake could be used to infer energetic gain of lions complementing their diet with smaller prey species, shedding light on the question of why lions eat smaller prey. Could it be energetically viable? Although Carbone et al. (1999) and Owen-Smith & Mills (2008) suggest that species under 20 kg in mass contribute little to the prey base for lions, due to too low energetic gain. Or do lions complement their diet by adding specific nutritional value? Alternatively, it could be just a form of opportunism or survival strategy (in case larger prey is not accessible), that includes scavenging and consuming road kills, as Lesilau (2019) suggested. Our method proved to be useful in detecting small and very small prey species in the diet of lions and can therefore be used in future research to continue to uncover the diverse diet composition of these large felines. The consistent presence of smaller prey species in the diet indicates that these taxa form an additional food source for lions and that, independent of location and rationale, lions will generally supplement their diet with some smaller prey.

4.6 | Supporting information and data accessibility

Additional supporting information can be found in the online version of the article at the publisher's website (scan the QR code below). The raw output of the NGS run is available at Dryad: https://doi.org/10.5061/dryad.zs7h44jfm.

5|

Exploring the effect of fencing on the diet composition and prey preference of lions using DNA-based diet analysis: a case-study in four Kenyan National Parks

Kevin Groen | Hans H. de Iongh | Francis Lesilau | Mumbi Chege |
Luka Narisha | Megan Verhagen | Kennedy Kariuki | Amy Montanje |
Sam Boerlijst | Iris Noordermeer | Josine Meijer | Dionne Jacobs |
Gert-Jan Goeminne | Mateo Bal | Michiel Veldhuis |
Laura D. Bertola | Peter M. van Bodegom | Krijn B. Trimbos |

In prep

5.1 | Abstract

Fences, omnipresent and often man-made structures worldwide, play a crucial role in biodiversity conservation. While their short-term effects, such as protecting large carnivores from poaching and livestock predation, are well-known, the long-term consequences, including behavioral shifts in lions and their prey, remain incompletely understood. Notably, there has never been a direct, simultaneous comparison of lion diet and preferences in both fenced and unfenced natural areas using identical methodologies and timeframes. To address this gap, we harnessed the power of being able to analyze large sample sizes using DNA metabarcoding to enable a direct comparison of lion diet composition and prey preference between four different National Parks (NPs) as a means to study the effect of fencing on lion diet. Our objective was to investigate whether differences existed in the diet preferences and compositions of lions between fenced and unfenced NPs. The findings revealed distinct changes in lion diet composition and prey preferences along a gradient from fully fenced to completely unfenced NPs. Remarkably, the fenced park exhibited an almost opposing pattern in terms of prey preferences, particularly concerning specific prey body weights, compared to the unfenced NP. Lions in the small, fenced reserve displayed a pronounced preference for smaller prey, contrasting with the preferences observed in other NPs. Partly fenced NPs yielded varied diet results, generally aligning within the preference boundaries of both fenced and unfenced NPs and lacking outliers for specific prey species. Despite the limited statistical power arising from the study of only four NPs, our research provides evidence that fencing influences lion diets. Exploratory studies like these contribute to unraveling the impacts of fences on ecosystems across trophic levels, ultimately benefiting the conservation of large carnivores.

5.2 | Introduction

Fences are ubiquitous, widespread and often manmade infrastructures existing all over the globe and frequently used to conserve and protect biodiversity (Woodroffe et al., 2014; Løvschal et al., 2017; Jakes et al., 2018). The intended aims of fencing include preventing the introduction of invasive species and reducing human-wildlife conflicts and thus persecution, if a fence is managed and maintained correctly (Ewen et al., 2011; Kesch et al., 2015). Additionally, fences are a powerful tool for conserving and restoring wildlife in landscapes that are highly modified by human activity and these areas are increasingly viewed as nuclei for more extensive restoration efforts entailing progressive removal of fencing (Woodroffe et al., 2014). On the other hand, fences can obstruct large-scale animal movements and constrain herbivores' abilities to escape predators. Additionally, it can cause ecosystem fragmentation, genetic isolation, reduced conservation value of buffer zones, and fence materials can be utilized for wire snare poaching (Creel et al., 2013; Davies-Mostert et al., 2013; Woodroffe et al., 2014). In addition, fences can cause an altered landscape of fear (i.e. predation pressures), by which prey species may alter their feeding behavior, and create a negative ecological cascade causing destabilization of the ecosystem inside the fenced area (Cassidy et al., 2013). This dichotomy and plethora of potential effects on the ecosystem of using fences as management practice evidently illustrates the currently limited comprehension regarding fencing impacts on ecosystem functioning (Trinkel et al., 2016).

Predators, especially apex carnivores, form a key component of an ecosystem, with their top-down effects, and are suited to study ecosystem functioning alterations due to fencing (Ford & Goheen, 2015; Ford et al., 2014; Feit et al., 2019). A high-profile debate concerning the use of fences to protect African lions highlights a more general need to evaluate the role of fencing in predator conservation (Packer et al., 2013a; Creel et al., 2013; Packer et al., 2013b). Packer et al. (2013a) state that lion populations in fenced reserves are significantly closer to their estimated carrying capacities and have reduced costs of management than unfenced populations. Whereas Creel et al., (2013) prioritize and argue for large and intact ecosystems to conserve lions, instead of fencing. In that context, the high lion densities found by Packer et al. (2013a) might actually indicate that food webs are profoundly altered by fencing (Woodroffe et al., 2014). For example, Louw et al. (2012) demonstrate that lions seem capable of locating

the preferred prey species with more ease in reserves where the movement of the prey species is constrained by fences and also Davies-Mostert et al. (2013) shows that boundary subjected (fenced) areas are likely to affect carnivores' hunting strategies and thereby their diet composition. Moreover, both Power (2002) and Lehmann et al. (2008) conducted a study on prey selection in a single small, fenced reserve and also showed deviations from the general findings for lion diet composition. Thus, predator-prey dynamics and diet composition within fenced reserves can change, and might even lead to dietary range shifts, in response to being held in a constrained habitat (Dupuis-Desormeaux et al., 2016). Furthermore, fencing confines predator populations into smaller areas, and might thereby induce density-dependent effects, especially when the carrying capacity of a fenced area is reaching its maximum for a predator species (Lindsey et al., 2017). In summary, short term effects of fencing are generally known (Packer et al., 2013a; Woodroffe et al., 2014; Kesch et al., 2015) and aid in the protection of large carnivores (e.g. reduced poaching and livestock predation), however long term effects (e.g. behavioral changes in lions and their prey) are not fully understood (Jakes et al., 2018; McInturff et al., 2020). Moreover, while these studies do indicate or propose that fencing influences a carnivores diet, a direct comparison of lion diet and preferences in fenced and unfenced nature areas using the exact same methodology and timeframe has never been made.

Therefore, we set up to study the diet of lions in four differently fenced or unfenced Kenyan National Parks (NPs), as an explorative case study. Specifically, we aim to address whether differences are found in diet preferences and diet composition of lions between fenced and non-fenced NPs. To accomplish this we established a method where we could analyze feeding behavior simultaneously in these NPs to prevent confounding effects of temporal dynamics and observer bias (e.g. biased census data, see Louw et al., 2012). Therefore, we adopted the method of Chapter 4. They performed DNA-based diet analysis as it favors lower costs, reduced effort and time and increased dietary resolutions, hence a better assessment of the large carnivore dietary ranges and components as compared to traditional scat analysis and carcass counts, which is needed to accurately compare spatial differences in lion diets. Ultimately, this understanding on the effect of fencing on lion feeding behavior could benefit in improving large predator conservation practices.

5.3 | Materials and Methods

5.3.1 | Study sites

To study the effect of fencing on the diet of lions, four NPs in Kenya were selected for their varying degree of fencing and their presence of lions (Figure 5.1). Northwest of Nairobi and south of the city of Nakuru lies Lake Nakuru National Park (LNNP, 188 km^2, 0°18'- 0°30' S; 36°2'- 36°9' E) (Kassilly et al., 2008). The park was established in 1961 and is completely enclosed with an electric fence since 1986. The landscape, enclosing a saline lake, consists of grasslands, swamps and marsh, with rocky cliffs and outcrops. There are areas of woodland and rocky hillsides covered with bushland and forest (Kassily et al., 2008). Meru National Park (MNP, 884 km^2), a partly fenced (70km, 24% of the total perimeter) savannah bushland, was established as a National Park in 1967 and is located in the Eastern Province of Kenya (0°20'- 0°10' S; 38°0'- 38°25' E) (Sitienei et al., 2014). Vegetation and ecosystem types within MNP predominantly consist of thorny Acacia and Commiphora bushland and Acacia wooded grassland (Bundotich et al., 2016). Approximately 7 km south of the city Nairobi, situated in the Nairobi Province, lies Nairobi National Park (NNP, 117 km^2, 01°20'- 01°26' S; 36°50'- 36°58' E). This park, established in 1946, consists predominantly of savannah grassland and was semi-fenced in 1955, with about 56% (65 km) of the park's border being fenced to create a separation between the city and the National Park (Lesilau, 2019). Established in 1974, located Southeast of NNP, close to the Tanzanian, border lies Amboseli National Park (ANP, 392 km^2, 02°37 S; 37°15 E), a non-fenced savannah grassland with seasonal flooding (Okello et al., 2008). Lions here have the opportunity to disperse to other reserves and to the surrounding environment (Huqa, 2019).

We have chosen to use the number of kilometers fence to order the parks from fully fenced to not fenced (LNNP; fully fenced → MNP; partly fenced for 70 km → NNP; partly fenced for 65 km → ANP; not fenced). In general, road accessibility within the NPs was satisfactory. However, in the case of MNP, the southern part of the park posed challenges due to poor or non-existent roads and dense vegetation. This resulted in disproportionate sampling across the surface of the National Park. Nevertheless, prey transects and scat sampling sites did overlap, and availability and diet occurrences could thus be related. Moreover, as we

ordered the NPs by the degree of fencing, our transect and sampling sites in MNP did concur with the location of the fenced park boundaries and the potential effect of fencing is thus most likely to occur in the part surveyed. So due to the partial sampling, the apparent fencing proportion is likely substantially higher than 24%. All NPs have two rainy seasons: long rains from March to May and short rains from October to December with dry spells in between.

5.3.2 | Lion surveys

Lion population numbers and social structure in the NPs can influence feeding ecology (Hayward & Kerley, 2005). Therefore, we performed lion surveys to estimate population numbers and social structure in the four different NPs. Fieldwork took place from 4th February, 2019 until 17th April, 2019. During this period, we performed direct counts from opportunistic observations and call-up surveys where different vocalizations were broadcasted to attract lions to a survey point to obtain data for population and social structure. Lions were distinguished from each other by their unique whisker spot pattern (using photos of vibrissae patterns) and by investigating typical characteristics such as unique marks, scars, ear notches and presence of scrotum (Pennycuick & Rudnai, 1970). We kept track of the land surface area surveyed (in km^2) for the presence of lions per NP using a GPS unit (Garmin eTrex). Furthermore, lions were grouped into two age categories namely cubs (0-1 year) and adults (> 1 year) as it was not possible to assign gender to cubs by sighting. Additional notes were made on date, time, sex, and if the lion was in a group or pride and the composition of that group or pride.

5.3.3 | Prey transects

Prey availability was measured for calculating prey preferences. Therefore, we conducted prey transects to count available prey species for lions in each NP simultaneously during the study period. All vertebrate prey species (above 5 kg body weight) within 500 meters (measured using a Mileseey Golflaser rangefinder) on each side of the transect (Figure 5.1) were counted and identified. For birds, this includes ostrich and kori bustard, and for reptiles, amongst other the Nile crocodile, since they all have a bodyweight > 5kg. Species were identified using the field guide from Stuart & Stuart (2013) and prey weights were taken from the same guide. Prey transects were conducted two days a week. Every transect day, transect counts were conducted while driving around the park in clockwise direction (at 06:30 in the morning) and in the opposite direction (at 15:00 in the afternoon). This order was alternated every week. Transects were 2 km long following a park track and chosen to cover the majority of habitats in the park.

5.3.4 | DNA-based diet analysis

A DNA-based diet analysis was carried out according to the method described in Chapter 4. In short, lion scat samples were collected between the 4th of February and the 16th of April 2019 (Figure 5.1). Scat samples were collected opportunistically along transects during patrols thrice a week. Stuart & Stuart (2013) pocket guide was used to identify the scats and additional information was reported (date and time of collection, GPS coordinates, habitat type, weather conditions, and scat freshness). The categories for freshness of scats were specified from 1 to 4 (1 = inside and outside of the scat were soft; 2 = inside soft, outside hard; 3 = inside and outside hard; 4 = only hairs left) with 1 being the freshest. Five picks were taken from inside the scat with sterilized forceps, preserved in vials with 1 mL 99% ethanol and stored in a fridge at the end of each fieldwork day. After all fieldwork was completed, the samples were transferred to a -20°C freezer. To extract DNA from the scat samples, the protocol for ‘Isolation of DNA from Stool for Pathogen Detection’ of the QIAamp DNA Stool Mini Kit handbook was followed. After extraction, samples were tested for lion DNA to ensure correct scat collection.

Next, samples were selected for freshness (≤ 3) to prevent degradation bias as much as possible and location to make sure that multiple prides were taken into account (when present). Subsequently, a PCR with vertebrate-specific primers, Mod_RepCOI_F and VertCOI_7216_R, was performed to amplify a 244-bp fragment of the cytochrome c oxidase subunit I (COI) gene which has been demonstrated to have high-resolution power for identifying the genera across most vertebrate taxa (Reeves et al., 2018). Amplification was carried out in a final volume of 20 µL, using 3 µL DNA extract, 15 µL TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, Waltham, United States) and 0.1 µM of forward and reverse primer. A single cycle of 10 min at 95 °C, followed by 45 cycles of

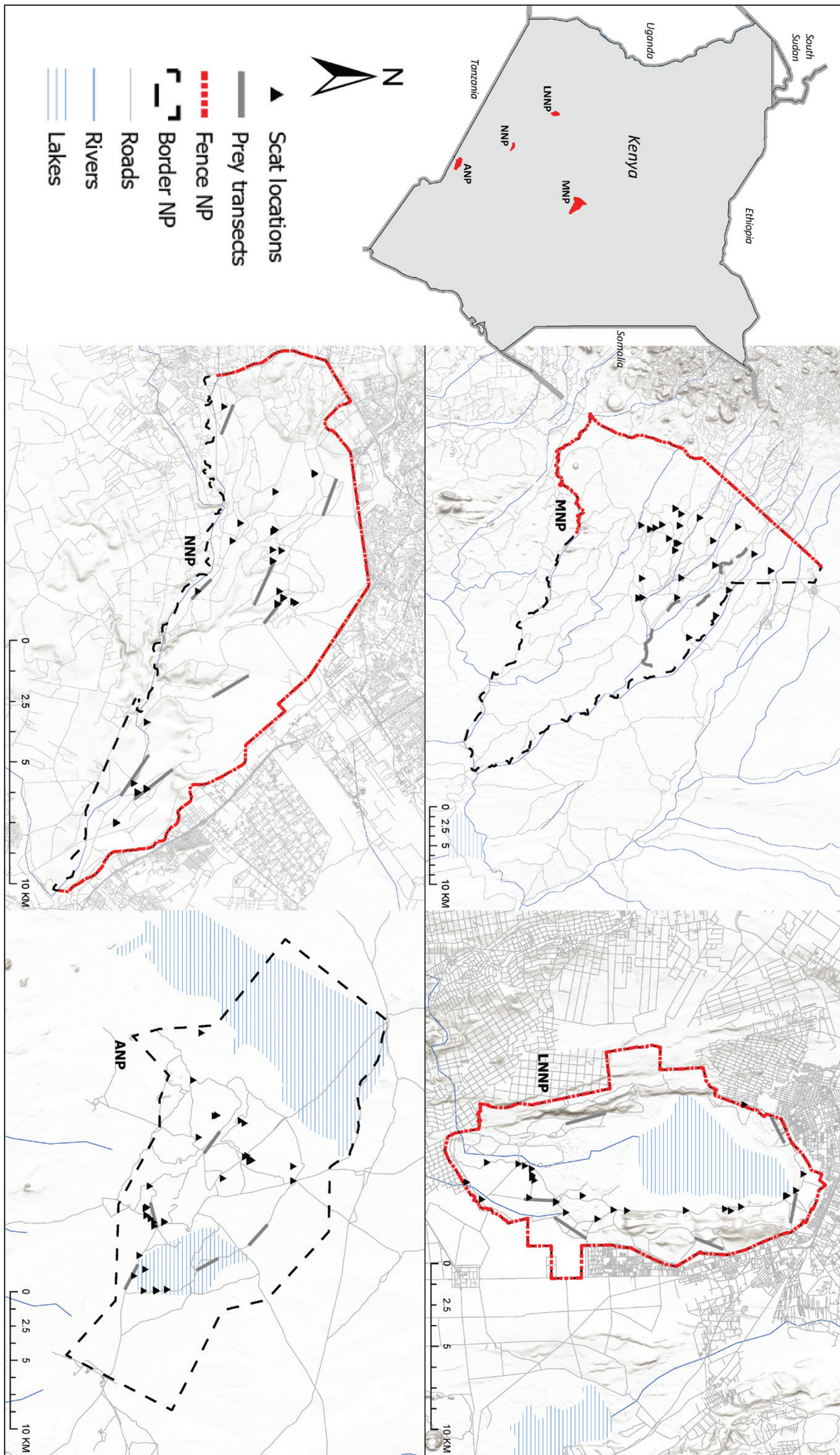

Figure 5.1 | Map of the study sites with fences depicted (red borders). Grey lines represent prey transects conducted and black triangles show where scat samples were collected in (following anticlockwise direction) fully fenced Lake Nakuru National Park (LNNP), partly fenced Meru National Park (MNP) and Nairobi National Park (NNP) and non-fenced Amboseli National Park (ANP). The map on the top left corner shows Kenya with the four study sites depicted in red. The southern part of MNP was inaccessible due to bad or non-existing roads and dense vegetation. The map was adapted from Chapter 4.

15 s at 95 °C, 30 s at 48.5 °C and 40 s at 72°C with a final extension step of 5 min at 72°C was used as PCR programme. Finally, Nextera i7/i5 index adapters were added to the amplicons for the purpose of pooling at sample level. Positive (POS) controls; DNA extracts of Chilean flamingo (*Phoenicopterus chilensis*, geographically restricted to South America; BirdLife International, 2018) and wood mouse (*Apodemus sylvaticus*), and no-template controls (NTC) were included in the metabarcoding amplifications to check for contamination. Additionally, for every park, negative field control(s) (NEG), by dipping sterilized forceps used for sampling in 99% ethanol, were included in the diet analysis. Final PCR products were pooled in equimolar concentrations and sequenced on the Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA).

Data output of the NGS run was prepared by merging the demultiplexed reads, primer trimming and sequence trimming as detailed in Chapter 4. Next, OTUs were created and the presence of chimera sequences was checked. The created OTUs were BLASTed to the custom reference database used in Chapter 4. Subsequently, a least common ancestor analysis was performed to account for missing identifications and a table including all OTUs with their number of reads per sample was created. The reads in the control samples (POS, NTC, NEG) were used to filter out any contamination by subtracting the number of sequence reads found in the controls per taxon from the number of reads found in the samples. Lastly, a taxonomy worksheet was created, displaying all occurrences for every taxonomic unit for every sample. If samples had missing identifications at species-level and composed ≥1% of the total number of reads in a sample a higher taxonomic level was used. For details on sequencing results, e.g. generated reads, quality scores and mean number of reads, see Chapter 4 and NGS raw data output can be found at: doi:10.5061/dryad.zs7h44jfm.

5.3.5 | Data analysis

We calculated the number of sequence hits for each species in each sample. Next, we omitted all hits from species below 5 kg as these were not counted during the prey transects and no abundance data was thus available (see Chapter 4 for results on prey < 5kg in the lion diet). Furthermore, we removed reads from mesopredators (*Canis sp.*, *Leptailurus sp.*, *Crocuta sp.* and *Ichneumia sp.*), humans (*Homo sapiens*) and host DNA (*Panthera sp.*) following the reasoning and analysis performed in Chapter 4.

The presence of a prey taxa (regardless of the number of sequences of that prey taxon) within a fecal sample was counted as an occurrence, where presence of multiple taxa means multiple prey within one fecal sample. The diet of lions was quantified as a proportion of occurrence of an individual prey taxon over all occurrences found in all fecal samples (i.e. frequency of occurrence per food item, expressed as a percentage of the number of occurrences of one food item of the total number of occurrences of all food items). To obtain proportions of occurrences per park, only the respective fecal samples of that particular park were used. Lions, as carnivores, feed sporadically and in discrete foraging events therefore prey occurrences may provide a meaningful indication of how often each taxon is predated thereby justifying the use of occurrence data to interpret the lion diet (Deagle et al., 2018). However, as the latter study recommends, we also calculated the relative read abundance per prey species and NP, using the formula in Deagle et al. (2018), for completeness.

The resulting diet information based on occurrences does not take into account the difference in prey availability per NP. Therefore, to compare the diet between parks and account for differences in prey abundance, we used the Jacobs' index (D) which calculates a prey preference using the formula: $D = (r - p) / (r + p - 2rp)$. Where r represents the number of times a species is killed as a proportion of the total number of kills and p represents the proportional availability of that species (Jacobs, 1974). In this study, the proportion of occurrence (%TX) (as described above) of a species in the diet is interpreted as r and the relative species abundance of that species is used as availability (p). The relative species abundance was calculated by adding the number of individuals counted for a species and dividing that number by the total number of animals counted per NP. The Jacobs' index produces values from -1 to 1. A positive index ($D > 0.1$) indicates a preference while a negative index ($D < -0.1$) means a species is avoided and consumed less frequently than expected based on the abundance of that species (Hayward & Kerley, 2005). All figures were made using the *ggplot2* package (Wickham, 2009) in R (R Core Team, 2022). We added trend lines to scatter plots using locally weighted smoothing (LOESS, formula = y ~ x). Species accumulation curves were produced using the *specaccum* function (default settings) in the *vegan* package (Oksanen et al., 2022).

5.4 | Results

5.4.1 | Lion surveys

Totaled for all NPs, we observed 122 lions during the lion surveys (Table 5.1). With highest number of individuals (45), of which 15 cubs, was found in Amboseli NP, but highest density (31.6 ind./100 km^2) was found in Nairobi NP. Lowest abundance (9 ind.) and density (6.7 ind./100 km^2) were found in Lake Nakuru NP. Number of lion prides were highest in Amboseli with 5 prides found, and an average group size of (45 ind. / 5 prides) 9 individuals. Group sizes in Nairobi , Meru and Lake Nakuru NP were 12, 8 and 5 lions per pride, respectively.

Table 5.1 | Lion population numbers and social structure, including land surface area surveyed, for each National Park (NP) based on our lion surveys.

National Park (NP)	Land surface area surveyed (km^2)	Abundance (ind.)*	Density (ind./100 km^2)	Cubs (lions < 1-year-old)	Sex ratio (♀:♂)**	Number of prides
Amboseli NP	392	45	11.5	15	1.7 : 1.0	5
Nairobi NP	117	37	31.6	12	1.6 : 1.0	3
Meru NP	381	31	8.1	10	1.1 : 1.0	4
Lake Nakuru NP	135	9	6.7	3	1.0 : 1.0	2

* All individuals (adults + cubs)
** Lions >1-year-old only

5.4.2 | Prey transects

We recorded a total of 94 490 individuals (ANP: 33 505, NNP: 24 939, MNP: 26 374, LNNP: 9 672) spread over 30 prey species in 24 transects (ANP: 5, NNP: 8, MNP: 5, LNNP: 5) for all NPs combined. The total transect length was 1 920 km, combined with a coverage of 500 meter on each side of the transect, we thus surveyed an area of 1 920 km^2. Highest number of individuals within prey transects were observed for plains zebra (*Equus burchellii*) (18 792 total individuals; highest number in NNP with 7 382 individuals), shoat (*Ovis aries*/*Capra aegagrus hircus*) (15 931 total individuals; highest number in MNP with 13 400 individuals) and blue wildebeest (*Connochaetes taurinus*) (118 470 total individuals; highest number in ANP with 10 350 individuals). Highest number of individuals within prey transects

in fully fenced Lake Nakuru NP are found for African buffalo (*Syncerus caffer*) with 5 114 individuals and second highest for plains zebra with 1 728 individuals. Prey availability (proportions of counted individuals per species versus total individuals of all species per NP) for all prey species observed are given in Table S5.1. Sixteen out of the 30 (53%) prey species counted were not found in the diet of lions. All species found in the diet of lions were observed in the prey transects (14), except for two, more cryptic, species which were not observed inside the prey transects, although observed outside transects, namely crested porcupine (*Hystrix cristata*) and the Nile crocodile (*Crocodylus niloticus*).

5.4.3 | DNA-based diet analysis

5.4.3.1 | Comparison of lion diet composition

The DNA-based diet analysis resulted in 16 assigned prey species, with a body weight greater than 5 kg, in 171 scat samples (Figure 5.2). The number of species found in the diet of lions in ANP is 10, in NNP 7, in MNP 9 and in LNNP 7. Sampling effort per NP was compared using species accumulation curves (Figure S5.1). Relative read abundance per prey item and per NP can be found in Table S5.1. Proportions of occurrences did not significantly differ from percentages of relative read abundances (Wilcoxon signed-rank test; V = 348 (N =33), p = 0.23).

Lion diet composition based on proportions of occurrences of prey items in, non-fenced, Amboseli NP is similar to the diet composition found in, partly fenced, Nairobi NP, while diet composition of lions in Meru NP (partly fenced) and Lake Nakuru NP (fully fenced), strongly diverge (Figure 5.2A). In both, the two least fenced, NPs (ANP and NNP) the highest proportions of occurrences were found for plains zebra (ANP 46.88 %, NNP 38.20 %) and blue wildebeest (ANP 31.25 %, NNP 22.47 %). Plains zebra is, however, less observed in the diet of lions in the most fenced NPs (MNP and LNNP, 8.47 % and 4.76 %, respectively). Blue wildebeest does not occur in MNP and LNNP and is thus not present in the diet. In contrast, the proportion of occurrences for the diet of lions in most fenced NPs (MNP and LNNP) was strongly dominated by African buffalo (54.24 % and 47.62 %) but was lacking for least fenced NPs (ANP, 3.13 % and NNP, 13.48 %). Although livestock was regularly observed during prey transects in each NP (average availability in transects 9.4 %), except LNNP, occurrences of livestock in the diet of lions were few and found only in MNP where 5.08 % of the lion diet was contributed to cow (*Bos taurus*). Proportion of occurrences plotted against minimum prey weight demonstrate that lions in unfenced ANP and partly fenced NNP, generally prey more frequently on prey species lighter than 500 kg whereas in the most fenced NPs, MNP and LNNP (Figure 5.2B) most occurring prey weight lies within 500-600 kg body weight.

5.4.3.2 | Comparison of lion prey preference

To fully assess diet differences between NPs, we took prey availability within NPs into account. Lion prey preference in each NP, as calculated by the Jacobs' index (JI), is shown for different functional groups (see Table S5.1 for group assignment) (Fig. 5.3A) and prey body weight (Fig. 3B). Figure 5.3 represents prey species found in the DNA-based diet analysis only. Index values for prey species found during prey transects but not in the diet are treated as avoided (D = -1) and are added to Table S5.1 (but are thus not included in Figure 5.3).

Rodents and reptiles are only found in the diet of lions in Meru NP, both were not frequently counted during transects and therefore receive a high index value (D = 1) and are therefore considered preferred. Similarly, Dik-dik's in NNP and LNNP also got a D = 1 value for preference, as they were not observed but found in the diet using DNA analysis. As these results might have an (substantial) impact on the trendline of MNP, NNP and LNNP in Fig 3B, and to evaluate robustness, we added the same figure without the results of rodents and reptiles in MNP and Dik-dik's in NNP and LNNP to the supplementary material (Figure S5.2). Removing these datapoints did not alter overall trends which illustrates the robustness of the results.

The sole livestock species found in the diet of lions is cow and is marginally favored (D = 0.18). Suids are equally preferred in all NPs (D = 0.3 – 0.5), except MNP, where no suids were found in the lion diet. Primates are highly preferred in all NPs (D > 0.8), except NNP where no primates were found in the diet of lions. Most apparent differences in prey preference between NPs were found within the equids, large ungulates and antelopes. In fully fenced LNNP, lions tend to avoid equids (D = -0.6), where these are generally preferred in the other NPs (D > 0.2). Additionally, lions in LNNP show less preference, although still preferred (D < 0.5), for large ungulates (African buffalo and giraffe) compared to the partly and non-

fenced NPs which show high preference (D > 0.5). Antelopes are generally favored by lions among all NPs, with highest mean preference for antelopes in LNNP (D > 0.5) and lowest mean preference in partly fenced NNP (D =0.2) due to highly species-specific prey preferences for this functional group.

Differences in prey preferences in terms of prey body weight were especially found in the only enclosed NP, Lake Nakuru, where lions clearly prefer lower prey weights (~100 – 200 kg) and avoid prey of higher body weight (200 to 500 kg). Prey preference for prey heavier than 700 kg is high in all NPs (D > 0.5). Meru NP follows the same strong preference as LNNP for lighter prey. However, lions here do not at all avoid heavier prey and actually favor them. Contrastingly, lions in Nairobi NP generally avoid light prey and prefers medium sized prey (~300 kg) and prey above 500 kg. Lions in non-enclosed Amboseli NP do not avoid any prey but generally tend to have a larger preference for heavier prey (> 400 kg) compared to lighter body weights (around 250 kg).

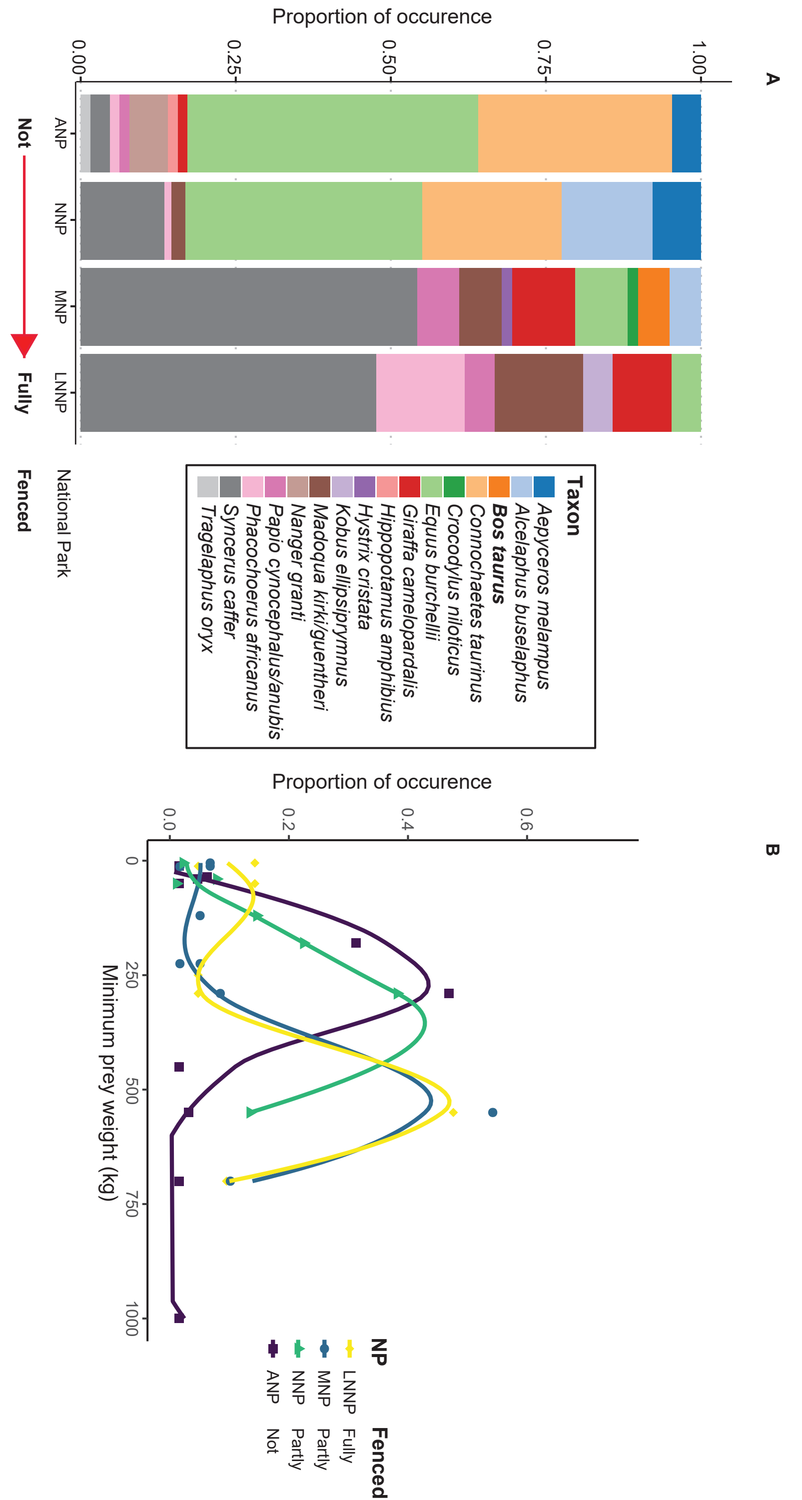

Figure 5.2 | Lion diet composition based on prey occurrences (prey body weight > 5 kg) in the diet of lions in four different fenced National Parks (NPs) in Kenia. ANP = Amboseli NP where N = 44 scat samples, NNP = Nairobi NP where N =46, MNP = Meru NP where N = 50, and LNNP = Lake Nakuru NP where N = 31 scat samples. The extent of fencing is added to the legend of the figure, ordered by km fence. **(A)** Proportions of occurrence found in the diet of lions ordered from non-fenced ANP to fully fenced LNNP. Livestock species are given in bold. **(B)** Proportions of occurrence plotted against minimum prey body weight. A LOESS trendline is added for each NP with smoothing parameter α = 0.8 where each point represents a prey species.

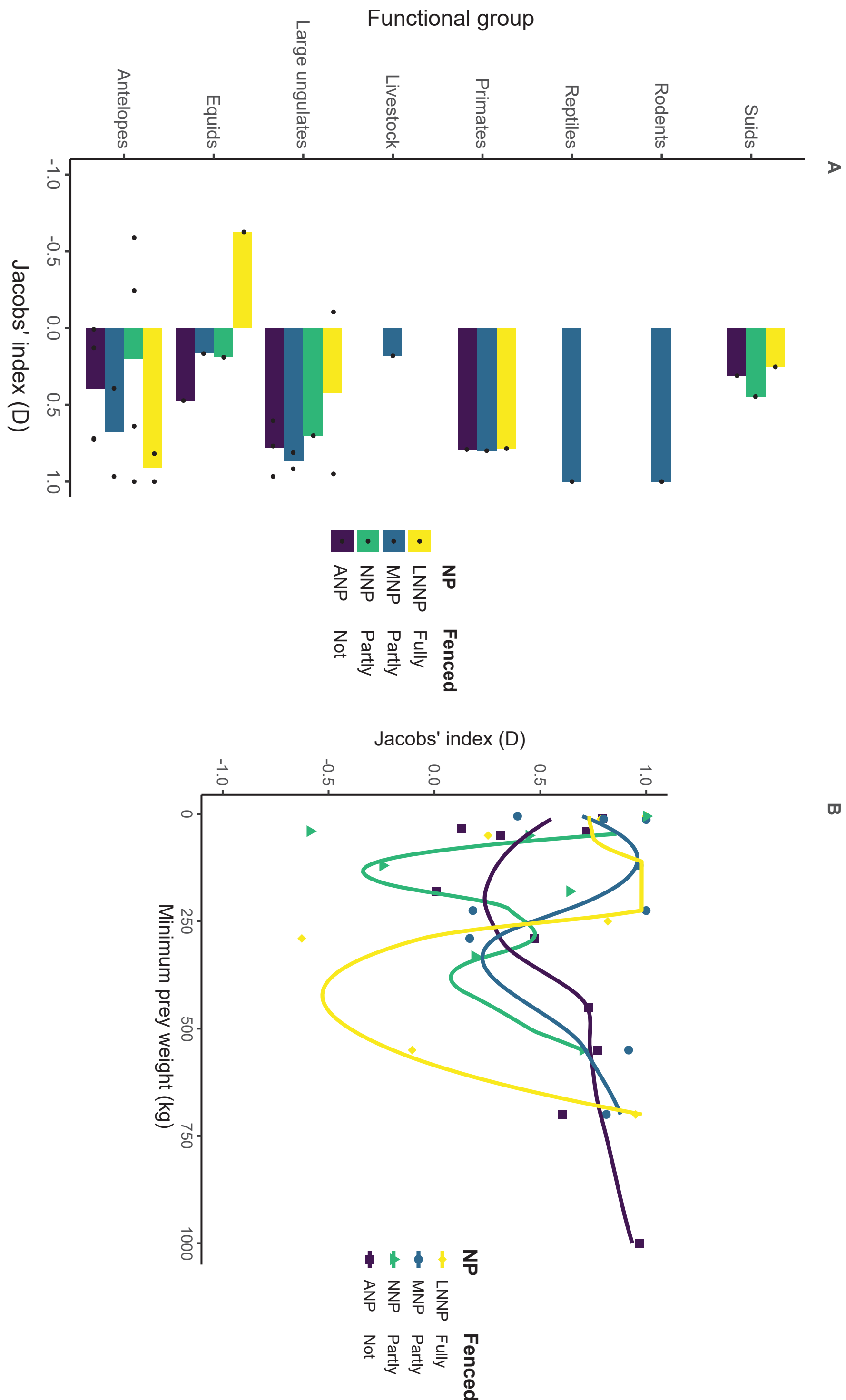

Figure 5.3 | Lion prey preference (prey body weight > 5 kg) based on the Jacobs' index, in which prey availability is included, in four different fenced National Parks (NPs) in Kenia. ANP (N = 44 scat samples) = Amboseli NP, NNP (N = 46) = Nairobi NP, MNP (N = 50) = Meru NP, and LNNP (N = 31) = Lake Nakuru NP. Prey preference is given per functional group **(A)** and per species plotted against prey body weight **(B)** for each NP. Black dots represent individuals species belonging to that functional group in figure A. Functional groups are assigned according to the information in Table S5.1. Please note that for this figure, only species found in the DNA-based diet analysis are used. Preferences of species not found in the diet but observed during prey transects are given in Table S5.1. For Figure B, a LOESS trendline was added for each NP with smoothing parameter α =0.98.

5.5 | Discussion

We found a contrasting lion diet composition and prey preference between the fully enclosed NP (LNNP) and the completely unfenced NP (ANP). Strikingly, LNNP also shows almost an opposite pattern in terms of prey preferences for specific prey body weights compared to ANP. The lions in this small and fenced reserve show high preference for smaller prey (< 250 kg), especially antelopes (highest mean preference of all NPs), and avoidance for prey weights that are generally preferred in other NPs and according to the scientific literature (most preferred prey weight of lions is considered to be 350 kg according to Hayward & Kerley, 2005). Partly fenced NPs (NNP and MNP) generally show varying diet results, but are nearly always situated within the preference boundaries set by the prey preferences of LNNP and ANP and show no particular outliers for certain prey species.

The lions in the least fenced NPs preyed most dominantly upon plains zebra, followed by blue wildebeest, whereas African buffalo prevailed as dominant prey in the diet of lions in the most fenced NPs. Both plain zebra and African buffalo do occur in all NPs, while prey availability of buffalo is extremely high in LNNP (52.9 %) compared to the other NPs (< 5.0%). Although highest proportions of occurrences were found in LNNP, lions in LNNP showed no preference for African buffalo. In all other NPs lions showed high preferences for buffalo. Additionally, plains zebras were also non-preferred by lions in the enclosed LNNP, whereas the lions in other NPs preferably hunt on this prey species. Availability of plain zebra was generally similar for each NP (~20 %), with proportionally slightly less zebras in MNP (6.2%).

Overall, lions in LNNP demonstrate remarkable differences in diet composition and preference in comparison to the other NPs, which clearly shows that feeding behavior in the only fully fenced NP in this study is substantially different to the unfenced and partly fenced NPs. Completely attributing this difference to fencing is difficult and caution should be taken, as diet can be influenced, aside from prey density, by other factors (e.g. climatic events such as droughts and lion demography, see Hayward & Kerley (2005) and Huqa (2019)). Although, by sampling all NPs during the same period we have tried to minimize the effect of seasonal climatic events. Furthermore, large regional differences in weather between the NPs were not observed. However, in regards to lion demography

within NPs, we need to stress that lion population numbers in LNNP are the lowest among all four NPs and the obtained diet might thus be the result of a smaller lion population, including lowest number of lion individuals, lowest density, fewest prides and lowest groups size. However, this argument of lion demography does not fully explain the resemblance of Meru NPs' trend to that found in LNNP (trend of MNP is similar to LNNPs' trend except with less extreme preferences) as lion numbers in MNP are higher, with larger group size. So, we suggest that also fencing - in addition to or in combination with population structure – is an important explanation for the observed patterns.

Our results corroborate that lions tend to hunt smaller prey when lion numbers are low (as collective hunting by lions allows lions to take larger-bodied prey) and avoid larger prey such as buffalo which agrees with our results (Hayward & Kerley, 2005). Furthermore, we noted high lion offspring mortality due to conflicts with African buffalo in LNNP due to the extremely high presence of these prey species in the park. Notwithstanding, a small lion population might also be an indirect effect or characteristic of an enclosed, unbalanced system with less genetic diversity and no (im)migration possibilities (Creel et al., 2013; Creel et al., 2019; Naha et al., 2023). As Mesa-Cruz et al. (2016) also demonstrated that outside of protected areas (i.e. unnatural systems), large cats shifted their diet to smaller prey which concurs with our results.

Lions in ANP, the only unfenced NP in this study, showed a preference for all prey species considered in this study, except 2 antelopes, which were not preferred nor avoided. Highest preferences were seen for species > 250 kg, although proportions of occurrences also showed high proportions of smaller prey in their diet. Combined, these results suggest that in open unfenced areas lions can be regarded as opportunistic hunters, that hunt everything they can get, but with preference for heavier prey. This concurs with other lion prey preference studies in natural systems (i.e. unfenced) where prey might be more difficult to get, since it is not confined to a closed area, and prey selection is not always possible when hungry (Creel et al., 2013; Davies-Mostert et al., 2013; Woodroffe et al., 2014).

As discussed before prey body weight preferences of the lions in partly fenced MNP follow the same trend as the lions in LNNP however with less extremes. When we consider that the area surveyed within MNP is mostly within bounds of the fenced part of the NP, and is thus likely most affected by it, we could infer that the prey preference trend that is observed (MNP behaving like LNNP), might thus likely be directly attributed to the effect of fencing. Combined with the fact that NNP, also partly fenced but surveyed across the whole park area, does not follow the same trend as MNP or LNNP (but rather shows more agreement with the trend of ANP) supports our argument that fencing was a driver of the patterns found. We do note that both ANP and LNNP could benefit from increased sampling effort as accumulation curves show that the asymptote has not been reached, which is reached for NNP and MNP, and the curve is still climbing. Although total number of species found in the diet did not differ much between NPs (7 species for LNNP, 7 for NNP, 9 for MNP and 10 species for ANP). Additionally, Trites and Joy (2005) calculated that for 8 or 11 species in a diet, between 46 and 31 scats need to be collected to infer differences between diets among locations with enough statistical power (based on a diet with linearly declining proportions of occurrences for prey species). Scat samples collected within NPs differed between 31 and 50, we therefore, although barely, abide by these set numbers and therefore we do not expect a difference in observed patterns with increased sampling effort.

Multiple studies show deviating lion ecology in small enclosed nature areas (Lehmann et al., 2008; Packer et al., 2013a; Creel et al., 2013), which forms an increasing body of evidence of fence-induced influences on ecosystem functioning. This study adds to this evidence by directly comparing diet composition and preferences between NPs with varying degree of fencing. However, as only 4 NPs have been studied here and only two of four NPs were either completely fenced or unfenced stating statistically based differences with regards to the effect of fencing was not possible. Nonetheless, despite statistical power issues and alternative explanations, we provide strong evidence that fencing influences the diets of lions. We therefore do stress the need for further research into the impact of fences on wildlife ecology. Diet studies would for instance benefit from scenarios where fences are being erected, and a before and after situation can both be studied, combined with for example remote sensing techniques to evaluate changes in vegetation over time or monitor herbivore activity (Kröschel et al., 2017). Such studies could help disentangling the impact of fences on ecosystems along trophic levels and ultimately benefit the conservation of large carnivores.

5.6 | Supporting information and data accessibility

No non-public available data is used for this publication.

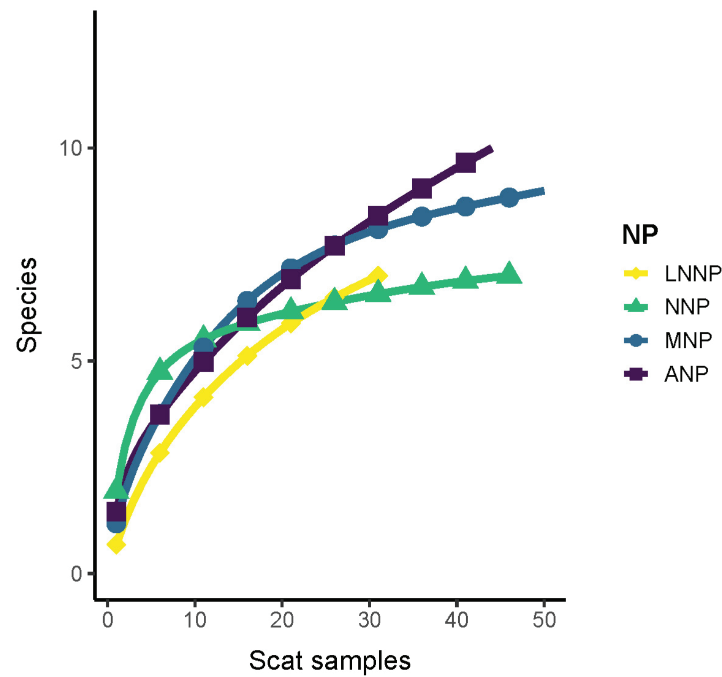

Figure S5.1 | Species accumulation curves for scat samples collected in four National Parks in Kenia. ANP (N = 44) = Amboseli NP, NNP (N = 46) = Nairobi NP, MNP (N = 50) = Meru NP, and LNNP (N = 31) = Lake Nakuru NP.

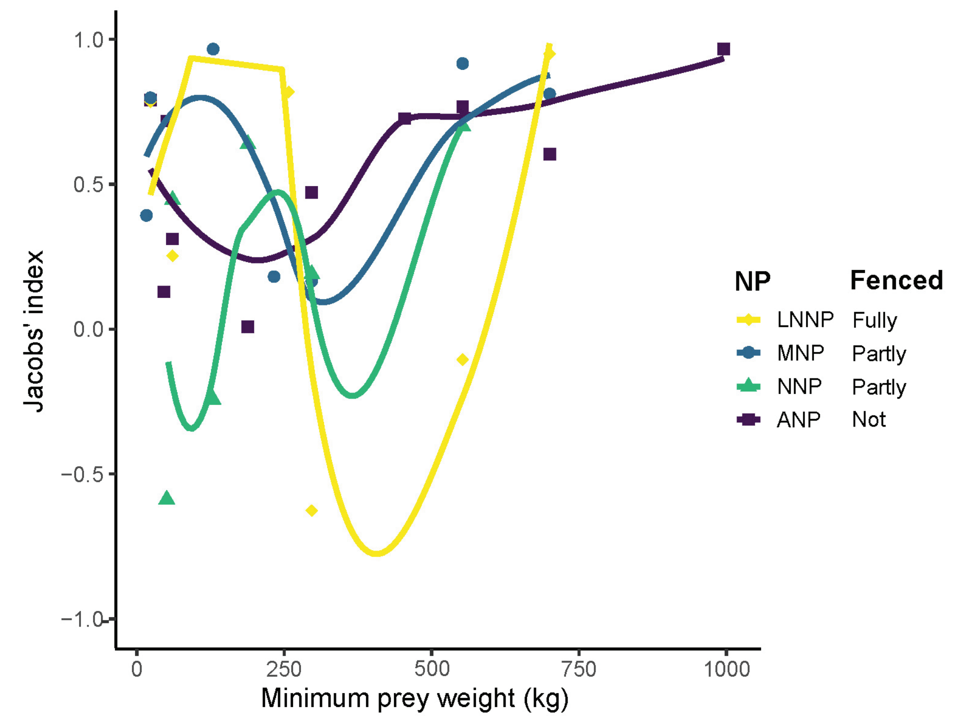

Figure S5.2 | Lion prey preference (prey body weight > 5 kg) based on the Jacobs' index, in which prey availability is included, in four different fenced National Parks (NPs) in Kenia. ANP (N = 44 scat samples) = Amboseli NP, NNP (N = 46) = Nairobi NP, MNP (N = 50) = Meru NP, and LNNP (N = 31) = Lake Nakuru NP. Prey preference is given per species plotted against prey body weight for each NP. Please note that for this figure, only species found in the DNA-based diet analysis are used. However, data points with a Jacobs' index value of 1 (i.e. species found in diet but not observed during transects) are excluded (but shown in Table S5.1). Preferences of species not found in the diet but observed during prey transects are also given in Table S5.1. A LOESS trendline was added for each NP with smoothing parameter α =0.98.

Table S5.1 | Overview of prey species analyzed during this study with corresponding prey availability based on results of prey transects and proportions of occurrences in lion diets found based on the results from the DNA-based diet analysis. Prey preferences are calculated using the Jacobs' index (D). Species that receive index values of D > 0.5 are considered highly preferred prey (++) and values of D < -0.5 are highly avoided prey (--). Species with D > 0.1 and ≤ 0.5 are preferred prey (+) and D < -0.1 and ≤ -0.5 are avoided prey (-). Index values for prey species found during prey transects but not in the diet are treated as avoided (D = -1.0). A blank space means that a particular prey species does not occur in that particular NP. An x denotes that the calculation of the Jacobs' index was not possible due to no availability and no occurrence in the diet of that prey species, but the prey species is known to occur in the area. Species' taxonomic families are given as well as assigned functional groups (l. u. = large ungulate). Functional groups 'elephant' and 'bird' are not presented in Figure 5.3A, as no occurrences were found in the diet of lions for these groups in any NP, but are included here for completeness.

Species[a]	Scientific name	Family (functional group)	Body mass (min - max, kg)	Preference [D] (Jacobs' index)								Availabilty [p] (%)				Proportion of occurrence [r] (%)				Relative read abundance (%)[b]			
				ANP		*NNP*		*MNP*		*LNNP*		*ANP*	*NNP*	*MNP*	*LNNP*	*ANP*	*NNP*	*MNP*	*LNNP*	*ANP*	*NNP*	*MNP*	*LNNP*
African buffalo	*Syncerus caffer*	Bovidae (l. u.)	550 - 700	++	0.77	++	0.70	++	0.92	~	-0.1	0.42	2.67	4.89	52.87	3.13	13.48	54.24	47.62	2.44	<u>21.18</u>	<u>73.06</u>	49.97
Baboon	*Papio cynocephalus/ anubis*	Cercopithecidae (primate)	12 - 45	++	0.80	--	-1.0	++	0.80	++	0.78	0.19	0.45	0.81	0.60	1.56	0	6.78	4.76	1.22	0	4.73	5.56
Black rhinoceros	*Diceros bicornis*	Rhinocerotidae (l. u.)	800 - 1100			--	-1.0		x	--	-1.0		0.10	0	0.01		0	0	0		0	0	0
Blue wildebeest	*Connochaetes taurinus*	Bovidae (antelope)	180 - 250	~	0.008	++	0.64					30.89	6.00			31.25	22.47			<u>39.37</u>	20.29		
Bohor reedbuck	*Redunca redunca*	Bovidae (antelope)	35 - 65	--	-1.0	--	-1.0				x	0.12	0.17		0	0	0		0	0	0		0
Bushbuck	*Tragelaphus sylvaticus*	Bovidae (antelope)	45 - 80		x	--	-1.0	--	-1.0			0	0.03	0.004		0	0	0		0	0	0	
Common duiker	*Sylvicapra grimmia*	Bovidae (antelope)	18 - 21		x	--	-1.0		x		x	0	0.01	0	0	0	0	0	0	0	0	0	0
Common eland	*Tragelaphus oryx*	Bovidae (antelope)	450 - 900	++	0.73	--	-1.0	--	-1.0	--	-1.0	0.25	1.83	0.20	1.12	1.56	0	0	0	0.01	0	0	0
Common ostrich	*Struthio camelus*	Struthionidae (bird)	63 - 150	--	-1.0	--	-1.0	--	-1.0	--	-1.0	0.79	2.23	0.43	0.08	0	0	0	0	0	0	0	0
Common warthog	*Phacochoerus africanus*	Suidae (suid)	50 - 105	+	0.31	+	0.45	--	-1.0	+	0.25	0.83	0.43	0.61	9.04	1.56	1.12	0	14.29	0.47	1.43	0	16.67
Cow	***Bos taurus***	**Bovidae (livestock)**	**225 - 395**	**--**	**-1.0**	**--**	**-1.0**	**+**	**0.18**		**x**	**11.31**	**0.12**	**3.58**	**0**	**0**	**0**	**5.08**	**0**	**0**	**0**	**1.18**	**0**
*Crested porcupine**	*Hystrix cristata*	*Hystricidae (rodent)*	*13 - 27*		*x*		*x*	*++*	*1.0*		*x*	*0*	*0*	*0*	*0*	*0*	*0*	*1.69*	*0*	*0*	*0*	*0.02*	*0*
Dik-dik's	*Madoqua kirki/ guentheri*	Bovidae (antelope)	5 - 6		x	++	1.0	+	0.39	++	1.0	0	0	3.07	0	0	2.25	6.78	14.29	0	0.24	4.88	<u>5.59</u>
Donkey	***Equus africanus asinus***	**Equidae (livestock)**	**80 - 480**	**--**	**-1.0**		**x**	**--**	**-1.0**		**x**	**0.05**	**0**	**4.03**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Dromedary	***Camelus dromedarius***	**Camelidae (livestock)**	**300 - 600**	**--**	**-1.0**		**x**	**--**	**-1.0**			**0.16**	**0**	**16.23**		**0**	**0**	**0**		**0**	**0**	**0**	
East African oryx	*Oryx beisa*	Bovidae (antelope)	175 - 175		x			--	-1.0			0		0.06		0		0		0		0	
Elephant	*Loxodonta africana*	Elephantidae (elephant)	2800 - 6500	--	-1.0			--	-1.0			7.51		0.17		0		0		0		0	
Gerenuk	*Litocranius walleri*	Bovidae (antelope)	30 - 50		x			--	-1.0			0		0.25		0		0		0		0	
Giraffe	*Giraffa camelopardalis*	Giraffidae (l. u.)	700 - 1400	++	0.60	--	-1.0	++	0.81	++	0.95	0.39	1.50	1.16	0.27	1.56	0	10.17	9.52	0.02	0	<u>3.64</u>	11.11
Grant's gazelle	*Nanger granti*	Bovidae (antelope)	35 - 80	+	0.13	--	-1.0	--	-1.0	--	-1.0	4.89	2.84	4.15	3.49	6.25	0	0	0	2.89	0	0	0
Hartebeest	*Alcelaphus buselaphus*	Bovidae (antelope)	120 - 150		x	-	-0.24	++	0.97			0	21.96	0.09		0	14.61	5.08		0	11.48	2.60	
Hippopotamus	*Hippopotamus amphibius*	Hippopotamidae (l. u.)	1000 - 2000	++	0.97	--	-1.0		x		x	0.03	0.43	0	0	1.56	0	0	0	0.37	0	0	0
Impala	*Aepyceros melampus*	Bovidae (antelope)	40 - 50	++	0.72	--	-0.59	--	-1.0	--	-1.0	0.80	24.72	0.94	11.94	4.69	7.87	0	0	2.45	8.46	0	0
Kori bustard	*Ardeotis kori*	Otididae (bird)	5.5 - 19	--	-1.0		x	--	-1.0		x	0.23	0	0.05	0	0	0	0	0	0	0	0	0
Lesser kudu	*Tragelaphus imberbis*	Bovidae (antelope)	62 - 100		x		x	--	-1.0			0	0	0.55		0	0	0		0	0	0	
Mountain reedbuck	*Redunca fulvorufula*	Bovidae (antelope)	30 - 30			--	-1.0				x		0.08		0		0		0		0		0
Nile crocodile**	*Crocodylus niloticus*	Crocodylidae (reptile)	*225 - 750*		x		x	++	1.0		x	0	0	0	0	0	0	1.69	0	0	0	0.04	0
Plains zebra	*Equus burchellii*	Equidae (equid)	290 - 340	+	0.47	+	0.19	+	0.17	--	-0.63	24.01	29.60	6.21	17.87	46.88	38.20	8.47	4.76	50.75	36.91	9.84	5.56
Shoat	***Ovis aries/ Capra hircus***	**Bovidae (livestock)**	**45 - 100**	**--**	**-1.0**	**--**	**-1.0**		**-1.0**		**x**	**7.02**	**0.72**	**50.81**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Thomson gazelle	*Gazella thomsoni*	Bovidae (antelope)	15 - 25	--	-1.0	--	-1.0			--	-1.0	10.11	4.01		2.14	0	0		0	0	0		0
Waterbuck	*Kobus ellipsiprymnus*	Bovidae (antelope)	250 - 270	--	-1.0	--	-1.0		-1.0	++	0.82	0.003	0.02	1.71	0.50	0	0	0	4.76	0	0	0	5.56
White rhinoceros	*Ceratotherium simum*	Rhinocerotidae (l. u.)	1400 - 2300			--	-1.0		x	--	-1.0		0.07	0	0.07		0	0	0		0	0	0

* *Not a representative result as transects likely miss this nocturnal animal and results in no availability,* ** Common but not in transects

[a] **Livestock species in bold**, [b] <u>Differences between prop. of occurrence vs. rel. read abundance >5% are underlined</u>

6|

General Discussion

Interpreting diet data obtained by genetic approaches might seem straightforward as genetic codes are assigned to species which subsequently results in a taxonomic list reflecting the consumed prey/food items. However, inferring a consumer's true diet does not only rely on assigning the right taxa (or the right presence/absence measurements) to the (present) genetic sequences in your samples, unfortunately. A consumer's true diet represents the actual consumption of all potential diet items available in the ecosystem, while the estimated diet represents the diet identified by DNA-based diet analysis (Figure 6.1a). A mismatch between true and estimated diet can't be prevented (as you cannot sample indefinitely), and thus the aim of diet estimation is to maximize the overlap of the true and estimated diet. However, errors arise from, either, true diet not detected by the selected method (methodological bias) or potential diet items in the environment not ingested by the consumer but detected by the method (biological and environmental biases) (Figure 6.1b).

In this thesis, I dove into the realm of DNA-based diet analyses by investigating these biasing factors (in Chapters 2 to 5), that can influence the fate of DNA in dietary samples quantitatively and qualitatively. Through the use of droplet digital PCR, we optimized DNA extraction, mitigated PCR inhibition, and examined the influence of DNA markers on detectability, despite the inherent challenge of DNA degradation due to digestive processes (Chapter 2). Our newly developed approach enabled robust and minimally invasive quantification of small diet constituents in feces. In a realistic field scenario, we tested this method by analyzing the effect of biological factors, meal size, and digestibility on DNA quantification in wood mice feces, establishing calibration curves that allowed for accurate dietary intake estimates (Chapter 3). Additionally, we utilized DNA metabarcoding to investigate the contribution of small prey species to the diet of lions, revealing biases in non-genetic methods and highlighting the prevalence of field contamination (Chapter 4). Finally, applying these insights to an ecological context, we compared lion diet composition and prey preferences across different National Parks, finding distinct patterns related to the presence of fencing (Chapter 5). Our study underscores the potential of DNA-based analysis for ecological research, while also identifying its limitations and the need for complementary methods. Investigating and elucidating these biases further and the implications thereof hopefully aids the research field in moving forward toward estimating a customer's true diet as closely as possible which I discuss in this final chapter. I will start by discussing the wealth of opportunities, but also the limitations,

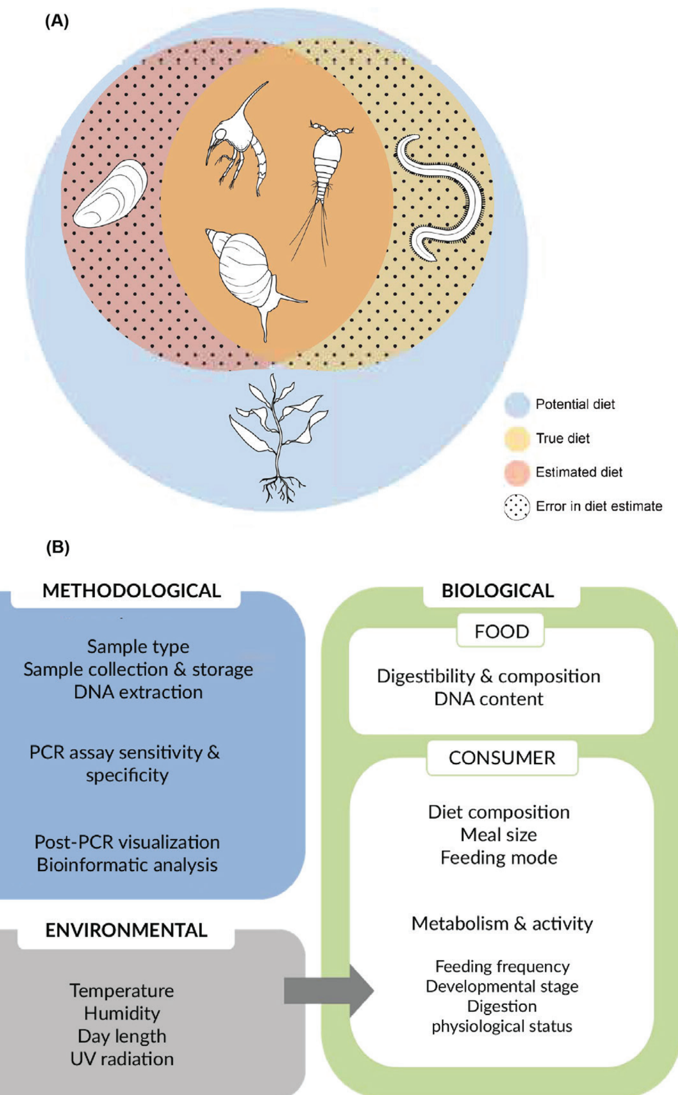

Figure 6.1 | (A) A consumer's true diet (yellow shaded area) represents some fraction of all potential diet items (blue shaded area) available in the ecosystem, while the estimated diet (red shaded area) represents diet identified by DNA-based diet analysis. Mismatches between yellow and red shaded areas represents error in the diet estimation (black dotted area), thus the aim of diet estimation is to maximize the overlap of the true and estimated diet (orange shaded color) qualitatively as well as quantitatively. Errors can arise from the true diet not being detected by the selected method (right black dotted area) and potential diet items in the environment not ingested by the consumer but detected by the method (left black dotted area). Figure adapted from Nielsen et al., 2017. **(B)** A range of factors affect the fate of food DNA in dietary samples and influence the interpretation of molecularly derived trophic data. These factors can be grouped into methodological, biological and environmental aspects. Figure adapted from Traugott et al., 2020.

6

presented by this innovative approach. Next, I will continue by linking qualitative and quantitative diet assessments, and discussing where the research field might be heading in the near future. Next, I will debate the complementarity of genetic methods and non-genetic approaches, as current literature suggests that the animal diet can be best inferred by combining approaches. Lastly, the implications of novel approaches in diet analyses for ecological research are explored.

6.1 | Combining qualitative and quantitative DNA-based analysis

Combining high throughput sequencing (HTS) techniques, such as DNA metabarcoding and diagnostic PCR, such as quantitative PCR (qPCR) and droplet digital PCR (ddPCR), have already been implemented in studies to combine information on both prey and consumer DNA For example, it has been utilized to assess sex-specific dietary choices; Schwarz et al. (2018) examined feces of male and female harbor seals in Canada, identifying prey DNA via metabarcoding and using qPCR to determine the sex of the seals. The novel combination of molecular techniques demonstrated that diet differences between males and females were consistent across sites and years suggesting that seals differ in their foraging strategies depending on their sex. Furthermore, individual identification of carnivores from fecal DNA (genotyping) enables individuals to be linked to their diets, and provides the means to assess intra-population and temporal variation in foraging behavior and prey consumption patterns (Fedriani & Kohn 2001).

Although the above combination of techniques opens up new ecological study possibilities (see section 6.3 below), it still remains largely qualitative. Currently, research combining qualitative research with quantitative techniques linking species identification to species abundance remains challenging and scant mostly due to DNA concentrations not always reflecting abundances as we also demonstrated in Chapter 3 (Alberdi et al., 2018). Murray et al. (2011) did compare HTS approaches with qPCR approaches and found that qPCR shows higher sensitivity in terms of amplifying low quantity DNA compared to HTS (although dependent on marker length). This is an important advantage of species-specific qPCR over HTS, in that it can detect species at very low DNA abundances, whereas the nature of universal primers, such as those used in HTS, renders them less specific and less likely to efficiently amplify low copy number targets in the presence of abundant targets (Thomas et al., 2015). However, the higher qPCR detection success did not drastically affect the overall diet estimates of both methods, due to the low abundance of prey species in the study of Murray et al. (2011). Although even more sensitive PCR techniques, with a higher detection rate, such as ddPCR (compared to qPCR) might have enhanced these results (Verhaegen et al., 2016).

In theory, HTS could be first used to study diet breadth and the importance of species of interest in a diet could be then identified and subsequently quantified using diagnostic PCR (as knowledge *a priori* of diet composition is needed for diagnostic PCR) (as did Wang et al., 2023). However, quantified DNA copy numbers still need to be coupled to actual biomass estimates by feeding studies for each prey item as we did in Chapter 3. More importantly, these feeding studies need to be performed separately for each diet item considered. Moreover, these feeding studies get more complicated when incomplete diet items are consumed such as a pride of lions feeding together on one large herbivore (e.g. zebra) carcass. In these cases DNA copy numbers cannot be easily translated into biomass consumed, as quantification is subject to tissue-specific differences in DNA content (e.g. spleen or liver do not contain the same number of DNA copies) which might result in highly varying DNA copy numbers and faulty biomass estimates if not properly calibrated (discussed in Chapter 2).

The utility of using only DNA metabarcoding for quantifying relative species abundances is currently limited by both biological and technical biases which influence sequence read counts as discussed in section 1.5 of the General Introduction. However, Thomas et al. (2015) found promising results by correcting for multiple sources of potential biases by simultaneously sequencing DNA extracts of field-collected diet samples as well as 50/50 mixtures of target species (i.e. mock samples) and a control species in order to generate relative correction factors (RCFs) which greatly improved relative abundance estimates. While 50/50 RCFs may provide a solution to multiple sources of bias in a single correction, there are other sources of bias that are not accounted for using this approach that require consideration. Most notably are biases introduced by differential degradation of species DNA due to either digestion (in the case of diet studies), or other degenerative (environmentally related) processes responsible for degrading environmental DNA (Alberdi et al., 2018). A metabarcoding diet study

with penguins suggested that differential DNA degradation due to digestion was the most significant cause of bias in the study system (Deagle et al., 2010). In those cases, additional bias correction efforts (e.g. lipid correction; Thomas et al., 2014) may be needed in order to achieve a highly accurate representation of mass proportions from DNA sequence counts of environmental samples (Thomas et al., 2015). Furthermore, it should be emphasized that the species-specific correction factors calculated using this approach are also specific to the experimental conditions of the methodological protocol. Therefore, the control materials used to produce RCFs should be re-sequenced each time a different experimental setup is used or an alteration is made to the methodological protocol, such as a change in PCR conditions or the transfer of protocols between laboratories (Thomas et al., 2015).

In summary, it remains challenging to enable accurate reconstructions of the physical diet as estimates are currently confounded by such a range of factors including differential digestion rates of prey between species, DNA per unit biomass variability between tissues and the developmental stage of the prey species to name but a few issues (Thomas et al., 2015). Furthermore, combining HTS and diagnostic PCR approaches and conducting feeding studies or calculating RCFs for each diet item is very time-consuming and therefore hardly feasible (although not impossible). Taking this into consideration together with the many caveats listed above suggests that other methods and techniques need to be considered to obtain the accurate quantification of prey DNA actually contained in fecal matter (but see Shelton et al., 2022 for a statistic modelling approach on quantification of metabarcoding data). Luckily, genetic techniques advance rapidly, and new approaches to estimate species abundance from diet samples are already emerging, which are primarily targeting and analyzing intraspecific genetic variation to infer the abundance of a species (see Wagemaker et al., 2020 and Andres et al., 2023).

Inferring species abundance from intraspecific genetic variation also relies on the use of HTS techniques. However, the difference to conventional metabarcoding is the focus on using different genetic markers (mostly targeting nuclear genomes or (full) organelle genomes) that contain enough intraspecific genetic variation within individuals to decipher genotypes (instead of using DNA concentrations to estimate abundances) from which true species numbers and biomass can potentially be inferred (Andres et al., 2021).

For example, Sethi et al. (2018) applied a likelihood-based framework to data of 14 microsatellite loci (nuclear markers) from stomach content samples of a predatory fish (largemouth bass) to estimate the number of contributors to these DNA mixtures. This analysis indicated a 5-fold increase in estimated predation rates of yellow perch by largemouth bass relative to conventional visual assessment of diet contents which can miss partially digested prey items (Sethi et al., 2018).

With the current knowledge, quantification approaches in Chapters 2 and 3 could instead have been better focused on detecting and analyzing genetic variation within seeds to quantify their abundance in wood mouse fecal samples. Of course, this is dependent on sufficient DNA quality (which is generally poor in fecal samples due to digestion but needs further study) and sufficient genetic diversity within individual seeds to discriminate between haplotypes of individual seeds. Furthermore, optimizing extraction protocols would not have been as easy to conduct as compared to diagnostic PCRs as HTS approaches generally take more time, resources and bioinformatics before results can be inferred. Nonetheless, estimating species abundance from genetic diversity sounds promising and future research will likely expand on this field of research.

While this field is now limited to obtaining single species counts from environmental samples, it would benefit to move towards multispecies abundance estimates, only then metabarcoding of fecal samples can truly quantify full dietary breadth. Hence, see the method of Wagemaker et al. (2020), genotyping by sequencing, which shows promising results for quantifying relative species abundance of plant roots in multispecies plant communities. However, it is not clear whether this method would work in highly degraded DNA mixtures, like in feces or soil or water eDNA (Piñol, 2021). Identifying haplotypes for entire communities from environmental samples may present additional challenges over single-species approaches. Deiner et al., 2017 show that it is possible to amplify whole mitochondrial genomes (> 16 kb) from environmental samples using HTS sequencing. This opens up the possibility of linking species assignments to genetic diversity indices. However, to make use of this method, there is a need for continued research focused on parsing out sequencing noise from real variation to determine intra-species haplotype diversity collected from environmental samples and high-throughput sequencing (Deiner et al., 2017; Andres et al., 2023). Furthermore, mitochondrial DNA (mtDNA) shows different mutation rates

than nuclear DNA (nuDNA) among phyla of animals and is exclusively inherited through either the paternal or maternal line (Allio et al., 2017). Consequently, it remains challenging to genotype individuals only based on mtDNA. Despite these difficulties, organelle metagenomics (e.g., mitochondria for animals or chloroplasts for plants) is gaining popularity, as it is easier to assemble organelle genomes then whole genomes (Piñol, 2021).

The continued advancement of single molecule and long-read technologies, such as the Oxford Nanopore MinION (Laszlo et al., 2014), might improve this approach and it is expected that long-read sequencing technologies, once they are cost effective and error rates are reduced, will become the method of choice for sequencing long-range PCR products, such as mitogenomes, and will allow population genetic analysis of eDNA samples (Denier et al., 2017). Moreover, Nanopore opens up the possibility of directly sequencing in the field, which prevents further degradation of field-collected samples (Wang et al., 2021). In the coming decade it may be possible to monitor genetic diversity for hundreds of species across the tree of life using eDNA sequence data from diet samples and estimate a consumer's true diet qualitatively and quantitatively (directly in the field) (Deiner et al., 2017; Shum & Palumbi, 2021; Weitemier et al., 2021).

6.2 | Complementarity with non-genetic methods

In recent decades, estimates of dietary habits have predominantly relied on visually inspecting undigested remnants present in feces or stomach contents (Steenweg et al., 2015). This method's popularity stems from its rapid application and the ability to gather both (semi)quantitative and qualitative data about the diet, including insights into food source characteristics such as prey age, size, or developmental stage (Klare et al., 2011). Nevertheless, the current array of available dietary tracing techniques, encompassing morphological examination of remains in feces and stomach contents, DNA barcoding, and the use of biomarkers like stable isotopes and fatty acid ratios (see section 1.2.1 of the General Introduction), complicates the selection of the most suitable method for specific research or management inquiries. The concept of employing integrated approaches has recently gained traction, particularly to address the inherent limitations of these dietary tracing methods when used in isolation (Horswill et al., 2018; Matley et al., 2018; Nielsen et al., 2018; Bonin et al., 2020).

Our study of Chapter 4 would likely have benefited from adding an extra dietary method, as extra information on prey age, size, or developmental stage obtained by morphological approaches would increase qualitative dietary information substantially especially elevating issues with prey size related detection rates between molecular and morphological techniques (Hayward & Kerley, 2005; Pereira et al., 2019). While molecular barcoding facilitates time efficient and mostly unbiased species identification across various life stages, a task often challenging based on morphological traits (Packer et al., 2009; Pereira et al., 2019), it may limit insights into the foraging strategies of predators. Take insectivores, for instance, where a predator's preference for eggs, larvae, or adult stages can offer valuable information about foraging strategies as early life stages of insects tend to be less mobile than adults. Therefore, a combination of molecular analysis and a morphological examination of (the hard part of) prey remains is particularly recommended in such cases (see for examples studies like Thalinger et al., 2018 and Pereira et al., 2019).

Moreover, old fecal samples are generally known to contain lower quality DNA and hamper accurate species identification using genetic approaches (Tollit et al., 2009). However, the age of a sample affects morphological identification to a much lower extent (Bonin et al., 2020). For instance, Bonin et al. (2020) demonstrated a consistent and unexpected finding that some food sources were detected in a lower proportion of feces by molecular analyses compared to a morphological approach in the diet of bears. They suggested that even though DNA within samples might be poorly distributed (see Alberdi et al., 2018 and Mumma et al., 2016), DNA quality could also have been an issue here and could have contributed to failures of DNA amplification or the increased occurrence of false negatives. Tollit et al. (2009) observed a reduced detection rate in molecular analyses compared to visual identification of pinniped remains in feces, particularly in cases involving aged feces. They attributed these false negatives to DNA degradation over time and/or exposure to abiotic conditions. Similarly, McInnes et al. (2016) encountered a comparable limitation in the use of molecular tools for studying seabird food habits in free-ranging conditions, highlighting a logistical challenge. Given the relatively high percentage of feces samples in which molecular analyses could not be completed, and considering that some feces were collected up to 14 days after excretion, it underscores the importance of cautiously considering the impact of environmental factors on DNA degradation rates and sampling time for molecular dietary analyses

(Alberdi et al., 2019). Moreover, this highlights the necessity of obtaining feces close to excretion (as fresh as possible) and applying rapid DNA extraction after collection (to stop degradation as soon as possible) for molecular analysis, and implementing correction metrics and models to address potential false negatives or imperfect detection (Alberdi et al., 2019; Monterroso et al., 2019; Morin et al., 2019). Recognizing that time and access constraints are common challenges, particularly in remote areas with free-ranging populations, the combined use of morphological and molecular tools during specific sampling periods or seasons can help overcome logistical limitations.

Furthermore, false positives are prone to occur in molecular analysis of fecal samples collected in the field due to contamination (Traugott et al., 2020). These false positives might be recognized when morphological analysis would have been used to check true consumption events. For instance, DNA of mesopredators was frequently obtained in fecal samples of lions (Chapter 4). However, if no such hairs or bones (of mesopredators) would have been found in morphological analysis, these occurrences could have assuredly been attributed to overmarking of lion scats by mesopredators to mark their own territories. Likewise, cannibalistic interactions remain challenging to detect, as existing molecular diet methods enable the differentiation of prey DNA among species but not yet within the same species (but see section 6.1). In most cases, dietary studies continue to depend on the morphological identification and counting of prey remnants alongside molecular trophic analyses to evaluate intraspecific feeding interactions (Eigaard et al., 2014).

In a study by Bonin et al. (2020), stable isotopes, morphological, and molecular analyses were compared to investigate the diet of free-ranging consumers with distinct diet types, namely carnivores and omnivores. Their results showed that the different methods led to a consistent diet description for carnivores, which have a relatively simple diet mixture, but their outcomes somewhat differed for omnivores, which have a more complex diet. For instance, fish were missed in the diet of bears using the morphological approach but did get picked up by the molecular analysis. Additionally, Bonin et al. (2020) and Tollit et al. (2009) both reported a lower detection rate for molecular analyses compared to visual identification of diet remains in feces in cases involving old feces. Furthermore, they demonstrated that the taxonomic discriminative power of stable isotope analysis could not go as far as for molecular tools, and for morphological analysis to a lower extent. However, Bonin et al. (2020) still suggested that stable isotope analysis is a powerful tool for dietary studies, especially those addressing the contribution of distinct food sources or food categories over long time scales and for longitudinal monitoring but they also caution because reliable estimates with stable isotope analysis require a minimum of a priori information about the consumer's foraging patterns. In general, the combined use of morphological and molecular analyses enhanced the diversity of food sources detected compared to the use of a single method independent of diet (Bonin et al., 2020). Bonin et al. (2020) emphasized that the choice of an appropriate approach to diet description should be a key step when planning dietary studies of free-ranging populations and recommend using more than one dietary determination method, especially for species with complex diet mixtures.

Another possible complementarity between genetic diet analysis and morphological approaches might lie within the field of calibration studies, like our study in Chapter 3. These kind of studies have also been conducted for several predator species using morphological methods (Klare et al., 2011). And it might be possible to use these morphological-based calibration curves, where they relate kilograms fed to a predator versus the biomass collected in fecal samples and use these curves to be applied to genetic diet studies. It would be interesting to see if these calibration curves which have already been acquired for multiple predator species (e.g. wolf, coyote, side-striped jackal, red fox, badger, American mink, European polecat, grizzly bears, black bears, cougars, cheetahs, bobcats and lynxes to only name a few, see Klare et al., 2011 for references) compare to obtained calibration curves with genetic methods. If these relate, diet quantification (as a new feeding study based on genetic methods might not be needed for each prey item) might benefit from the usage of genetic approaches as these have been demonstrated to be more time and cost-efficient than traditional methods (Ando et al., 2020).

6.3 | Implications for ecological research

Ecologists are increasingly interested in performing community-wide assessments in space and time (Bartley et al., 2019; Daam et al., 2019; Eisenhauer et al., 2019; Meyer et al., 2020). However, many contemporary ecological studies face challenges in considering entire communities or trophic networks due to practical

constraints (Creer et al., 2016). Immediate obstacles include the difficulty in identifying taxonomically complex communities, the extensive diversity that needs sampling, and the lack of resources, such as funding for taxonomists, required for species identifications (Creer et al., 2010). In essence, the task of conducting comprehensive community-wide assessments is substantial and challenging, with a shortage of skilled personnel and time to carry out these ongoing tasks (Creer et al., 2016). By focusing on a range of genetic source material (e.g. environmental DNA), habitats, time and spatial scales, we can now comprehensively characterize entire communities and begin to unpick their biotic/abiotic interactions from dietary samples. Moreover, genetic samples can be analyzed in bulk and eliminate the need for taxonomic specialists, which saves time and allows this (spared) time to be reinvested in collecting more samples generally potentially enhancing knowledge of the system and interactions (Baird & Hajibabaei, 2012).

Referred to as a transformative technology, harnessing the information held in DNA, from dietary samples, potentially has the power to overcome many limitations of classical biodiversity assessment and thereby answer unresolved ecological questions (Baird & Hajibabaei, 2012). Chapter 5 is a great example of such an ecological study which would have been difficult to conduct using only traditional ways of biodiversity assessment (as this would cost at least a year to gather the same amount of data). The power of DNA made it feasible to study the diet of lions in different National Parks in the same period and also made the comparison between parks possible (e.g. no presence of observer bias as genetic samples are analyzed together).

Currently, individual identification from fecal DNA allows researchers to link individuals to their diets, enabling the assessment of intra-population and temporal variations in foraging behavior and prey consumption patterns (Monterosso et al., 2019). Advancements in library preparation and sequencing technologies offer the potential, in theory, for even extended genomic coverage (Chiou & Bergey, 2018). This opens the exciting new possibility of non-invasively studying the genetic basis of specific traits, such as factors that influence dietary choices by individuals (e.g. physical condition, aggressive behavior, boldness) (Carroll et al., 2018). Additionally, metagenomic approaches applied to dietary samples theoretically allow for the identification of infectious agents providing insights into health in response to environmental and management changes (such as fencing) (Bahrndorff et al., 2016). These approaches hold even more promise for addressing questions in evolutionary biology and contributing to the conservation management of natural populations. It isn't hard to imagine that in the near future when metagenomic approaches have been well-developed, DNA extracts from the study in Chapter 5 could be reanalyzed to study not only a lions' diet and the identification of lion (including sex and age) individuals but also a lions' individual health and link that to the effect of fencing (and all that information just from scat samples).

Another compelling aspect that can be explored through DNA-based analysis of dietary samples is the microbiome of animals. Presently, studies on the microbiome of wild terrestrial carnivores are limited, but recent research in both model and non-model systems has revealed the profound impact of the gut microbiome on various aspects, including metabolism, nutrition, immune response, adaptation, tolerance to environmental changes, behavior, anxiety levels, and overall fitness (see Monterosso et al., 2019). Furthermore, examinations of wild populations have highlighted that factors such as phylogeny, physiology, diet, habitat quality, spatial location, sex, social system and status, reproductive status, and age influence the microbiome composition of both species and individuals (Bahrndorff et al., 2016). These studies, along with a growing body of literature emphasize the role of microbiomes in shaping individual fitness, underscore the significance and utility of fecal samples for characterizing and comprehending the gut microbiome in populations of wild animals (see for example Gomes et al., 2020). It is anticipated that fecal samples will play an increasingly prominent role in future quantitative and qualitative DNA-based diet studies (Bahrndorff et al., 2016; Monterosso et al., 2019).

As mentioned previously feces can be used to investigate infectious agents/pathogens, this feces serve as carriers for various pathogens posing potential threats to other wildlife, domestic animals, and ultimately, humans (Monterosso et al., 2019). High-throughput sequencing has marked a significant advancement in epidemiologic surveillance. While a limited number of studies have employed HTS technologies to screen fecal samples of mammalian carnivores for viruses (e.g. Li et al., 2010; Bodewes et al., 2014; Conceição-Neto et al., 2017), integrating these tools into surveillance programs is crucial, given the profound impacts infectious diseases can have on both animal and human populations (Scott, 1988; Pedersen et al., 2007).

Inevitably, future ecological studies will steer towards understanding complete ecosystems (instead of species or single interactions) and Meyer et al., 2020 have already demonstrated that using environmental DNA from feces to identify the plant and mammal diet of species on three trophic levels (two carnivores, two omnivores and two herbivores) they could build a fully functioning ecological network. These authors show that genetic approaches form a solution to transform ecological research and thereby opens up possibilities for humans to study complete ecosystems and thereby manage these systems in a way that serves biodiversity conservation and ultimately enhances human society (Meyer et al., 2020).

6.4 | Final thoughts

While discussing the prospects of DNA-based analysis for qualitative and quantitative assessment of animal diet, it occurred to me what amazing things genetic approaches are likely capable of in the (near) future. Combined, with the recent advent of research into real-time monitoring of ecosystem biodiversity with CRISPR technology, which allows for complex community identification from eDNA samples in less than 1 hour (Phelps, 2019; Williams et al., 2019; Baerwald et al., 2020; Shashank et al., 2023) and research such as genotyping individuals of three large carnivore species from just snow tracks (De Barba et al., 2023) I am in awe at what DNA techniques can already achieve. I have no doubt that in the future quantitative and qualitative DNA-based assessment of diet will become (or already is) the new traditional/conventional way of studying an animal's diet.

References

Abt, K. F., & Bock, W. F. (1998). Seasonal variations of diet composition in farmland field mice Apodemus spp. and bank voles Clethrionomys glareolus. ***Acta Theriologica, 43(4)***, 379-389.

Afgan, E., Baker, D., Batut, B., Van Den Beek, M., Bouvier, D., Ech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B. A., Guerler, A., Hillman-Jackson, J., Hiltemann, S., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A., & Blankenberg, D. (2018). The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. ***Nucleic Acids Research, 46(W1)***, W537–W544. https://doi.org/10.1093/nar/gky379

Alberdi, A., Aizpurua, O., Bohmann, K., Gopalakrishnan, S., Lynggaard, C., Nielsen, M., & Gilbert, M. T. P. (2018). Promises and pitfalls of using high-throughput sequencing for diet analysis. ***Molecular Ecology Resources, 19(2)***, 327–348. https://doi.org/10.1111/1755-0998.12960

Alberdi, A., Razgour, O., Aizpurua, O., Novella-Fernandez, R., Aihartza, J., Budinski, I., Garin, I., Ibáñez, C., Izagirre, E., Rebelo, H., Russo, D., Vlaschenko, A., Zhelyazkova, V., Zrnčić, V., & Gilbert, M. T. P. (2020). DNA metabarcoding and spatial modelling link diet diversification with distribution homogeneity in European bats. ***Nature Communications, 11(1***). https://doi.org/10.1038/s41467-020-14961-2

Allen, M. L., Gunther, M. S., & Wilmers, C. C. (2016). The scent of your enemy is my friend? The acquisition of large carnivore scent by a smaller carnivore. ***Journal of Ethology, 35(1)***, 13–19. https://doi.org/10.1007/s10164-016-0492-6

Allio, R., Donegà, S., Galtier, N., & Nabholz, B. (2017). Large Variation in the Ratio of Mitochondrial to Nuclear Mutation Rate across Animals: Implications for Genetic Diversity and the Use of Mitochondrial DNA as a Molecular Marker. *Molecular Biology and Evolution*, *34*(11), 2762–2772. https://doi.org/10.1093/molbev/msx197

Amundsen, P., & Sánchez-Hernández, J. (2019). Feeding studies take guts – critical review and recommendations of methods for stomach contents analysis in fish. ***Journal of Fish Biology, 95(6),*** 1364–1373. https://doi.org/10.1111/jfb.14151

Ando, H., Mukai, H., Komura, T., Dewi, T., Ando, M., & Isagi, Y. (2020). Methodological trends and perspectives of animal dietary studies by noninvasive fecal DNA metabarcoding. ***Environmental DNA, 2(4)***, 391–406. https://doi.org/10.1002/edn3.117

Andres, K. J., Sethi, S. A., Lodge, D. M., & Andrés, J. Á. (2021). Nuclear eDNA estimates population allele frequencies and abundance in experimental mesocosms and field samples. ***Molecular Ecology, 30(3),*** 685–697. https://doi.org/10.1111/mec.15765

Andres, K. J., Lodge, D. M., Sethi, S. A., & Andrés, J. Á. (2023). Detecting and analysing intraspecific genetic variation with eDNA: From population genetics to species abundance. ***Molecular Ecology, 32(15),*** 4118–4132. https://doi.org/10.1111/mec.17031

Angelakis, E., Yasir, M., Bachar, D., Azhar, E. I., Lagier, J., Bibi, F., Jiman-Fatani, A. A., Alawi, M., Bakarman, M. A., Robert, C., & Raoult, D. (2016). Gut microbiome and dietary patterns in different Saudi populations and monkeys. ***Scientific Reports, 6(1)***. https://doi.org/10.1038/srep32191

Asarian, L., & Geary, N. (2013). Sex differences in the physiology of eating. ***American journal of physiology. Regulatory, integrative and comparative physiology***, ***305***(11), R1215–R1267. https://doi.org/10.1152/ajpregu.00446.2012

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(6436)***, 173–177. https://doi.org/10.1126/science.aau3561

Ayliffe, M. A., Scott, N. S., & Timmis, J. N. (1998). Analysis of plastid DNA-like sequences within the nuclear genomes of higher plants. ***Molecular biology and evolution, 15(6),*** 738–745. https://doi.org/10.1093/oxfordjournals.molbev.a025977

Baerwald, M. R., Goodbla, A., Nagarajan, R. P., Gootenberg, J. S., Abudayyeh, O. O., Zhang, F., & Schreier, A. (2020). Rapid and accurate species identification for ecological studies and monitoring using CRISPR-based SHERLOCK. ***Molecular Ecology Resources, 20(4),*** 961–970. https://doi.org/10.1111/1755-0998.13186

Bahrndorff, S., Alemu, T., Alemneh, T., & Nielsen, J. L. (2016). The Microbiome of Animals: Implications for Conservation Biology. ***International Journal of Genomics,*** 2016, 1–7. https://doi.org/10.1155/2016/5304028

Baird, D. J., & Hajibabaei, M. (2012). Biomonitoring 2.0: a new paradigm in ecosystem assessment made possible by next-generation DNA sequencing. ***Molecular Ecology, 21(8),*** 2039–2044. https://doi.org/10.1111/j.1365-294x.2012.05519.x

Barnardo, T., Tambling, C. J., Davies, A. B., Klein-Snakenborg, S., Asner, G. P., le Roux, E., Cromsigt, J. P. G. M., Druce, D. J., & Kerley, G. I. H. (2020). Opportunistic feeding by lions: non-preferred prey comprise an important part of lion diets in a habitat where preferred prey are abundant. ***Mammal Research, 65(2),*** 235–243. https://doi.org/10.1007/s13364-020-00481-3

Bartley, T., McCann, K. S., Bieg, C., Cazelles, K., Granados, M., Guzzo, M. M., MacDougall, A. S., Tunney, T. D., & McMeans, B. C. (2019). Food web rewiring in a changing world. ***Nature Ecology and Evolution, 3(3),*** 345–354. https://doi.org/10.1038/s41559-018-0772-3

Bauer, H., Vanherle, N., Silvestre, I. Di, & Iongh, H. H. D. (2008). Lion - prey relations in West and Central Africa. ***Mammalian Biology, 73(1),*** 70–73. https://doi.org/10.1016/j.mambio.2006.11.006

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. ***Biodiversity and Conservation, 29(7),*** 2089-2121. http://doi.org/10.1007/s10531-020-01980-0

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. ***Journal of the Royal Statistical Society: Series B (Methodological), 57(1),*** 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

BirdLife International. (2018). ***Phoenicopterus chilensis***. The IUCN Red List of Threatened Species 2018: e.T22697365A132068236. https://dx.doi.org/10.2305/IUCN.UK.2018-2.RLTS.T22697365A132068236.en. Accessed on 15 June 2023.

Bonin, M. A., Dussault, C., Taillon, J., Lecomte, N., & Côté, S. D. (2020). Combining stable isotopes, morphological, and molecular analyses to reconstruct the diet of free-ranging consumers. ***Ecology and Evolution, 10(13),*** 6664–6676. https://doi.org/10.1002/ece3.6397

Boukhdoud, L., Saliba, C., Parker, L. D., McInerney, N. R., Kahale, R., Saliba, I., Maldonado, J. E., & Kharrat, M. B. D. (2021). Using DNA metabarcoding to decipher the diet plant component of mammals from the Eastern Mediterranean region. ***Metabarcoding and Metagenomics, 5***. https://doi.org/10.3897/mbmg.5.70107

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(3),*** 530–540. https://doi.org/10.1111/j.1755-0998.2010.02974.x

Branch, B. (2014). A photographic guide to snakes other reptiles and amphibians of East Africa. Struik Nature.

Brühl, C. A., & Zaller, J. G. (2019). Biodiversity Decline as a Consequence of an Inappropriate Environmental Risk Assessment of Pesticides. ***Frontiers in Environmental Science, 7***. https://doi.org/10.3389/fenvs.2019.00177

Bundotich, G., Jirmo, T., Mukeka, J., Davidson, Z., & Kenana L. (2016). Meru lion and other large carnivores census report 2016. Kenya Wildlife Service

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: architecture and applications. ***BMC bioinformatics, 10***, 421. https://doi.org/10.1186/1471-2105-10-421

Carbone, C., Mace, G. M., Roberts S. C. & Macdonald D. W. (1999). Energetic constraints on the diet of terrestrial carnivores. ***Nature, 402(6759)***, 286–288. https://doi.org/10.1038/46266

Carpenter, J., & Bithell, J. (2000). Bootstrap confidence intervals: when, which, what? A practical guide for medical statisticians. ***Statistics in medicine, 19(9),*** 1141–1164.

Carroll, E. L., Bruford, M. W., DeWoody, J. A., Leroy, G., Strand, A. E., Waits, L. P., & Wang, J. (2018). Genetic and genomic monitoring with minimally invasive sampling methods. ***Evolutionary Applications, 11(7),*** 1094–1119. https://doi.org/10.1111/eva.12600

Casper, R. M., Jarman, S. N., Deagle, B. E., Gales, N. J., & Hindell, M.A. (1997). 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(1–2),*** 144-154.

Cassidy, L., Fynn, R. W., & Sethebe, B. (2013). Effects of restriction of wild herbivore movement on woody and herbaceous vegetation in the Okavango Delta Botswana. ***African Journal of Ecology, 51(4),*** 513–527. https://doi.org/10.1111/aje.12061

Castle, S., Allan, N., Clifford, D. L., Aylward, C. M., Ramsey, J. J., Fascetti, A. J., Pesapane, R., Roy, A., Statham, M. J., Sacks, B. N., & Foley, J. E. (2020). Diet composition analysis provides new management insights for a highly specialized endangered small mammal. ***PLOS ONE, 15(10),*** e0240136. https://doi.org/10.1371/journal.pone.0240136

Chapmen, P. M. (1995). Extrapolating laboratory toxicity results to the field. ***Environmental Toxicology and Chemistry, 14(6)***, 927–930. https://doi.org/10.1002/etc.5620140601

Chiou, K. L., & Bergey, C. M. (2018). Methylation-based enrichment facilitates low-cost, noninvasive genomic scale sequencing of populations from feces. ***Scientific Reports, 8(1)***. https://doi.org/10.1038/s41598-018-20427-9

Chiron, F., Hein, S., Chargé, R., Julliard, R., Martin, L., Roguet, A., & Jacob, J. (2018). Validation of hair tubes for small mammal population studies. ***Journal of Mammalogy, 99(2),*** 478–485. https://doi.org/10.1093/jmammal/gyx178

Clare E. L. (2014). Molecular detection of trophic interactions: emerging trends, distinct advantages, significant considerations and conservation applications. ***Evolutionary applications, 7(9),*** 1144–1157. https://doi.org/10.1111/eva.12225

Clements, H. S., Tambling, C. J., Hayward, M. W., & Kerley, G. I. H. (2014). An Objective Approach to Determining the Weight Ranges of Prey Preferred by and Accessible to the Five Large African Carnivores. ***PLoS ONE, 9(7),*** e101054. https://doi.org/10.1371/journal.pone.0101054

Conceição-Neto, N., Godinho, R., Álvares, F., Yinda, C. K., Deboutte, W., Zeller, M., Laenen, L., Heylen, E., Roque, S., Petrucci-Fonseca, F., Santos, N., Van Ranst, M., Mesquita, J. R., & Matthijnssens, J. (2017). Viral gut metagenomics of sympatric wild and domestic canids, and monitoring of viruses: Insights from an endangered wolf population. ***Ecology and Evolution, 7(12),*** 4135–4146. https://doi.org/10.1002/ece3.2991

Coyle, H. M. (2014). Development of a Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Assay for Plant Species. ***Journal of Forensic Research and Criminal Studies, 1***, 1-5.

Creel, S., Becker, M., Durant, S. M., M'soka, J., Matandiko, W., Dickman, A., Christianson, D., Dröge, E., Mweetwa, T., Pettorelli, N., Rosenblatt, E. I., Schuette, P., Woodroffe, R., Bashir, S., Beudels-Jamar, R., Blake, S., Borner, M., Breitenmoser, C., Broekhuis, F., . . . & Zimmermann, A. (2013). Conserving large populations of lions - the argument for fences has holes. ***Ecology Letters, 16(11),*** 1413-e3. https://doi.org/10.1111/ele.12145

Creel, S., Spong, G., Becker, M. S., Simukonda, C., Norman, A. J., Schiffthaler, B., & Chifunte, C. (2019). Carnivores, competition and genetic connectivity in the Anthropocene. ***Scientific Reports, 9(1)***. https://doi.org/10.1038/s41598-019-52904-0

Creer, S., Fonseca, V. G., Porazinska, D. L., Giblin-Davis, R. M., Sung, W., Power, D. M., Packer, M., Carvalho, G. R., Blaxter, M., Lambshead, P. J. D., & Thomas, W. K. (2010). Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. ***Molecular Ecology, 19(s1)***, 4–20. https://doi.org/10.1111/j.1365-294x.2009.04473.x

Creer, S., Deiner, K., Frey, S., Porazinska, D., Taberlet, P., Thomas, W. K., Potter, C., & Bik, H. M. (2016). The ecologist's field guide to sequence-based identification of biodiversity. ***Methods in Ecology and Evolution, 7(9),*** 1008–1018. https://doi.org/10.1111/2041-210X.12574

Cresci, G. A., & Bawden, E. (2015). Gut Microbiome. ***Nutrition in Clinical Practice, 30(6),*** 734–746. https://doi.org/10.1177/0884533615609899

Daam, M. A., Teixeira, H., Lillebø, A. I., & Nogueira, A. J. (2019). Establishing causal links between aquatic biodiversity and ecosystem functioning: Status and research needs. ***Science of the Total Environment, 656***, 1145–1156. https://doi.org/10.1016/j.scitotenv.2018.11.413

Dalsgaard, J., St John, M., Kattner, G., Müller-Navarra, D. C., & Hagen, W. (2003). Fatty acid trophic markers in the pelagic marine environment. In Advances in Marine Biology (pp. 225–340). https://doi.org/10.1016/s0065-2881(03)46005-7

Davidson, Z., Valeix, M., Van Kesteren, F., Loveridge, A. J., Hunt, J. E., Murindagomo, F., & Macdonald, D. W. (2013). Seasonal Diet and Prey Preference of the African Lion in a Waterhole-Driven Semi-Arid Savanna. ***PLoS ONE, 8(2)***, e55182. https://doi.org/10.1371/journal.pone.0055182

Davies-Mostert, H. T., Mills, M. J., & Macdonald, D. W. (2013). Hard boundaries influence African wild dogs' diet and prey selection. ***Journal of Applied Ecology, 50(6),*** 1358–1366. https://doi.org/10.1111/1365-2664.12129

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(2),*** 306–323. https://doi.org/10.1111/1755-0998.12188

De Barba, M., Miquel, C., Lobréaux, S., Quenette, P., Swenson, J. E., & Taberlet, P. (2016). High-throughput microsatellite genotyping in ecology: improved accuracy, efficiency, standardization and success with low-quantity and degraded DNA. ***Molecular Ecology Resources, 17(3),*** 492–507. https://doi.org/10.1111/1755-0998.12594

De Barba, M., Baur, M., Boyer, F., Fumagalli, L., Konec, M., Miquel, C., Pazhenkova, E., Remollino, N., Skrbinšek, T., Stoffel, C., & Taberlet, P. (2023). Individual genotypes from environmental DNA: Fingerprinting snow tracks of three large carnivore species. ***Molecular ecology resources,*** e13915. Advance online publication. https://doi.org/10.1111/1755-0998.13915

Deagle, B. E., Tollit, D. J., Jarman, S. N., Hindell, M. A., Trites, A. W., & Gales, N. J. (2005). Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. ***Molecular Ecology, 14(6)***, 1831–1842. https://doi.org/10.1111/j.1365-294x.2005.02531.x

Deagle, B. E., Gales, N., Evans, K., Jarman, S., Robinson, S., Trebilco, R., & Hindell, M. A. (2007). Studying Seabird Diet through Genetic Analysis of Faeces: A Case Study on Macaroni Penguins (Eudyptes chrysolophus). ***PLOS ONE, 2(9),*** e831. https://doi.org/10.1371/journal.pone.0000831

Deagle, B. E., Kirkwood, R., & Jarman, S. N. (2009). Analysis of Australian fur seal diet by pyrosequencing prey DNA in faeces. ***Molecular ecology, 18(9),*** 2022–2038. https://doi.org/10.1111/j.1365-294X.2009.04158.x

Deagle, B. E., Chiaradia, A., McInnes, J. C., & Jarman, S. (2010). Pyrosequencing faecal DNA to determine diet of little penguins: is what goes in what comes out? ***Conservation Genetics, 11(5),*** 2039–2048. https://doi.org/10.1007/s10592-010-0096-6

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(4),*** 620–633. https://doi.org/10.1111/1755-0998.12103

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., Kartzinel, T. R., & Eveson, J. P. (2018). Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? ***Molecular Ecology, 28(2),*** 391–406. https://doi.org/10.1111/mec.14734

Deagle, B. E., Thomas, A. C., McInnes, J. C., Clarke, L. J., Vesterinen, E. J., Clare, E. L., … Eveson, J. P. (2019). Counting with DNA in metabarcoding studies: How should we convert sequence reads to dietary data? ***Molecular ecology, 28(2),*** 391– 406.

Deiner, K., Renshaw, M. A., Li, Y., Olds, B. P., Lodge, D. M., & Pfrender, M. E. (2017). Long-range PCR allows sequencing of mitochondrial genomes from environmental DNA. ***Methods in Ecology and Evolution, 8(12),*** 1888–1898. https://doi.org/10.1111/2041-210x.12836

Del Rio, C. M., Wolf, N., Carleton, S. A., & Gannes, L. Z. (2009). Isotopic ecology ten years after a call for more laboratory experiments. ***Biological Reviews, 84(1),*** 91–111. https://doi.org/10.1111/j.1469-185x.2008.00064.x

Dell'Agnello, F., Natali, C., Bertolino, S., Fattorini, L., Fedele, E., Foggi, B., Martini, M., Pisani, C., Riga, F., Sgarlata, A., Ciofi, C., & Zaccaroni, M. (2019). Assessment of seasonal variation of diet composition in rodents using DNA barcoding and Real-Time PCR. ***Scientific Reports, 9(1).*** https://doi.org/10.1038/s41598-019-50676-1

Di Bernardi, C., Wikenros, C., Hedmark, E., Boitani, L., Ciucci, P., Sand, H., & ÅKesson, M. (2021). Multiple species-specific molecular markers using nanofluidic array as a tool to detect prey DNA from carnivore scats. ***Ecology and Evolution, 11(17)***, 11739–11748. https://doi.org/10.1002/ece3.7918

Dunn, O. (1964). Multiple Comparisons Using Rank Sums. ***Technometrics, 6(3),*** 241-252. doi:10.2307/1266041

Dupuis-Desormeaux, M., Davidson, Z., Pratt, L. A., Mwololo, M., & MacDonald, S. E. (2016). Testing the effects of perimeter fencing and elephant exclosures on lion predation patterns in a Kenyan wildlife conservancy. ***PeerJ, 4,*** e1681. https://doi.org/10.7717/peerj.1681

EFSA, European Food Safety Authority. (2009). Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. ***EFSA Journal, 7(12),*** 1438.

Egeter, B., Bishop, P. J., & Robertson, B. C. (2014). Detecting frogs as prey in the diets of introduced mammals: a comparison between morphological and DNA-based diet analyses. ***Molecular Ecology Resources, 15(2),*** 306–316. https://doi.org/10.1111/1755-0998.12309

Egeter, B., Roe, C. M., 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(9)***, 5032–5048. https://doi.org/10.1002/ece3.4903

Eigaard, O. R., Van Deurs, M., Behrens, J., Bekkevold, D., Brander, K., Plambech, M., Plet-Hansen, K. S., & Mosegaard, H. (2014). Prey or predator—expanding the food web role of sandeel Ammodytes marinus. ***Marine Ecology Progress Series, 516***, 267–273. https://doi.org/10.3354/meps11064

Eisenhauer, N., Schielzeth, H., Barnes, A. D., Barry, K. E., Bonn, A., Brose, U., Bruelheide, H., Buchmann, N., Buscot, F., Ebeling, A., Ferlian, O., Freschet, G. T., Giling, D. P., Hättenschwiler, S., Hillebrand, H., Hines, J., Isbell, F., Koller-France, E., König-Ries, B., . . . Jochum, M. (2019). A multitrophic perspective on biodiversity–ecosystem functioning research. In Advances in Ecological Research (pp. 1–54). https://doi.org/10.1016/bs.aecr.2019.06.001

Elliot, N. B., Bett, A., Chege, M., Sankan, K., Souza, N., Kariuki, L., Broekhuis, F., Omondi, P., Ngene, S., & Gopalaswamy, A. M. (2020). The importance of reliable monitoring methods for the management of small, isolated populations. ***Conservation Science and Practice, 2(7).*** https://doi.org/10.1111/csp2.217

Emmerson, M., Morales, M., Oñate, J., Batáry, P., Berendse, F., Liira, J., Aavik, T., Guerrero, I., Bommarco, R., Eggers, S., Pärt, T., Tscharntke, T., Weisser, W., Clement, L., & Bengtsson, J. (2016). How Agricultural Intensification Affects Biodiversity and Ecosystem Services. ***Advances in Ecological Research***, 43–97. https://doi.org/10.1016/bs.aecr.2016.08.005

EPRS, European Parliamentary Research Service & STOA, Panel for the Future of Science and Technology. (2019). Farming without plant protection products: Can we grow without using herbicides, fungicides and insecticides? https://doi.org/10.2861/05433

Estes, J. A., Terborgh, J., Brashares, J. S., Power, M. E., Berger, J., Bond, W. J., Carpenter, S. R., Essington, T. E., Holt, R. D., Jackson, J. B. C., Marquis, R. J., Oksanen, L., Oksanen, T., Paine, R. T., Pikitch, E. K., Ripple, W. J., Sandin, S. A., Scheffer, M., Schoener, T. W., . . . Wardle, D. A. (2011). Trophic downgrading of planet Earth. Science, 333(6040), 301–306. https://doi.org/10.1126/science.1205106

Estes, R. D., Otte, D., & Wilson, E. O. (2012). The Behavior Guide to African Mammals: Including Hoofed Mammals, Carnivores, Primates, 20th Anniversary Edition (First). University of California Press.

European Food Safety Authority. (2009). Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. ***EFSA Journal, 7(12),*** 1438.

Eurostat. (2020). [apro_cpnh1]. Retrieved from: https://appsso.eurostat.ec.europa.eu/nui/submitViewTableAction.do

Ewen, J. G., Parker, K. J., Richardson, K., Armstrong, D. P., & Smuts-Kennedy, C. (2011). Translocation of hihi Notiomystis cincta to Maungatautari, a mainland reserve protected by a predator-exclusion fence, Waikato, New Zealand. ***Conservation Evidence***. https://www.hihiconservation.com/wp-content/uploads/2011/07/Ewen_etal_2011_Maungatautari-Conservation-Evidence.pdf

Fedriani, J. M., & Kohn, M. H. (2001). Genotyping faeces links individuals to their diet. ***Ecology Letters, 4(5)***, 477–483. https://doi.org/10.1046/j.1461-0248.2001.00250.x

Feit, B., Feit, A., & Letnic, M. (2019). Apex predators decouple population dynamics between mesopredators and their prey. ***Ecosystems, 22(7),*** 1606–1617. https://doi.org/10.1007/s10021-019-00360-2

Fernandes, R., Millard, A. R., Brabec, M., Nadeau, M., & Grootes, P. M. (2014). Food reconstruction using Isotopic transferred signals (FRUITS): a Bayesian model for diet reconstruction. ***PLOS ONE, 9(2***), e87436. https://doi.org/10.1371/journal.pone.0087436

Ferreras, P., & Fernandez-de-Simon, J. (2019). Correction factors for estimating food consumption by red fox Vulpes vulpes from scats. ***Wildlife Biology, 2019(1)***. https://doi.org/10.2981/wlb.00557

Floren, C., Wiedemann, I., Brenig, B., Schütz, E., & Beck, J. (2015). Species identification and quantification in meat and meat products using droplet digital PCR (ddPCR***). Food Chemistry, 173,*** 1054–1058. https://doi.org/10.1016/j.foodchem.2014.10.138

Ford, A. T., Goheen, J. R., Otieno, T. O., Bidner, L. R., Isbell, L. A., Palmer, T. M., Ward, D., Woodroffe, R., & Pringle, R. M. (2014). Large carnivores make savanna tree communities less thorny. ***Science, 346(6207)***, 346–349. https://doi.org/10.1126/science.1252753

Ford, A. T., & Goheen, J. R. (2015). Trophic Cascades by Large Carnivores: A Case for Strong Inference and Mechanism. ***Trends in Ecology and Evolution, 30(12),*** 725–735. https://doi.org/10.1016/j.tree.2015.09.012

Forin-Wiart, M. A., Poulle, M. L., Piry, S., Cosson, J. F., Larose, C., & Galan, M. (2018). Evaluating metabarcoding to analyse diet composition of species foraging in anthropogenic landscapes using Ion Torrent and Illumina sequencing. ***Scientific Reports, 8(1),*** 1–12. https://doi.org/10.1038/s41598-018-34430-7

Galan, M., Pons, J. B., Tournayre, O., Pierre, É., Leuchtmann, M., Pontier, D., & Charbonnel, N. (2018). Metabarcoding for the parallel identification of several hundred predators and their prey: Application to bat species diet analysis. ***Molecular ecology resources, 18(3),*** 474–489. https://doi.org/10.1111/1755-0998.12749

Geiger, F., Bengtsson, J., Berendse, F., Weisser, W. W., Emmerson, M., Morales, M. B., Ceryngier, P., Liira, J., Tscharntke, T., Winqvist, C., Eggers, S., Bommarco, R., Pärt, T., Bretagnolle, V., Plantegenest, M., Clement, L. W., Dennis, C., Palmer, C., Oñate, J. J., . . . Inchausti, P. (2010). Persistent negative effects of pesticides on biodiversity and biological control potential on European farmland. ***Basic and Applied Ecology, 11(2),*** 97–105. https://doi.org/10.1016/j.baae.2009.12.001

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(2),*** 362–369. https://doi.org/10.1002/wsb.749

Gül, M. R., & Griffen, B. D. (2020). Diet, energy storage, and reproductive condition in a bioindicator species across beaches with different levels of human disturbance. ***Ecological Indicators, 117***, 106636. https://doi.org/10.1016/j.ecolind.2020.106636

Han, H., Wei, W., Hu, Y., Nie, Y., Ji, X., Li, Y., Zhang, Z., Shi, X., Zhu, L., Luo, Y., Chen, W., & Wei, F. (2019). Diet evolution and habitat contraction of giant pandas via stable isotope analysis. ***Current Biology, 29(4),*** 664-669.e2. https://doi.org/10.1016/j.cub.2018.12.051

Hart, A., Smith, G. C., Macarthur, R., & Rose, M. (2003). Application of uncertainty analysis in assessing dietary exposure. ***Toxicology Letters, 140–141***, 437–442. https://doi.org/10.1016/s0378-4274(03)00040-7

Hay, C. T., Cross, P. C., & Funston, P. J. (2008). Trade-offs of predation and foraging explain sexual segregation in African buffalo. ***Journal of Animal Ecology, 77(5),*** 850–858. https://doi.org/10.1111/j.1365-2656.2008.01409.x

Hayden, B., Harrod, C., & Kahilainen, K. K. (2014). Dual fuels: intra-annual variation in the relative importance of benthic and pelagic resources to maintenance, growth and reproduction in a generalist salmonid fish. ***Journal of Animal Ecology, 83(6)***, 1501–1512. https://doi.org/10.1111/1365-2656.12233

Hayden, B., Harrod, C., Sonninen, E., & Kahilainen, K. K. (2015). Seasonal depletion of resources intensifies trophic interactions in subarctic freshwater fish communities. ***Freshwater Biology, 60(5),*** 1000–1015. https://doi.org/10.1111/fwb.12564

Hayward, M. W., & Kerley, G. I. H. (2005). Prey preferences of the lion (***Panthera leo***). ***Journal of Zoology, 267(3)***, 309–322. https://doi.org/10.1017/s0952836905007508

Hebert, P. D. N., & Gregory, T. R. (2005). The promise of DNA barcoding for taxonomy. ***Systematic Biology, 54(5)***, 852–859. https://doi.org/10.1080/10635150500354886

Hein, S., & Jacob, J. (2019). Population recovery of a common vole population (***Microtus arvalis***) after population collapse. ***Pest management science, 75(4),*** 908–914. https://doi.org/10.1002/ps.5211

Hernández, M. C., Navarro-Castilla, L., & Barja, I. (2019). Wood mouse feeding effort and decision-making when encountering a restricted unknown food source. ***PLOS ONE, 14(6),*** e0212716. https://doi.org/10.1371/journal.pone.0212716

Hetland, H., & Svihus, B. (2001). Effect of oat hulls on performance, gut capacity and feed passage time in broiler chickens. ***British poultry science, 42(3)***, 354–361. https://doi.org/10.1080/00071660120055331

Hibert, F., Taberlet, P., Chave, J., Scotti-Saintagne, C., Sabatier, D., & Richard-Hansen, C. (2013). Unveiling the diet of elusive rainforest herbivores in next generation sequencing era? The tapir as a case study. ***PloS one, 8(4),*** e60799. https://doi.org/10.1371/journal.pone.0060799

Hilbers, J. P., Hoondert, R. P. J., Schipper, A. M., & Huijbregts, M. A. J. (2018). Using field data to quantify chemical impacts on wildlife population viability. ***Ecological Applications, 28(3),*** 771–785. https://www.jstor.org/stable/26623184

Hoogeveen, M. (2019). naturalis/galaxy-tool-add-metadata-otutable. GitHub. https://github.com/naturalis/galaxy-tool-add-metadata-otutable

Hopcraft, J. 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(3),*** 559–566. https://doi.org/10.1111/j.1365-2656.2005.00955.x

Hopcraft, J. G. C., Olff, H., & Sinclair, A. (2010). Herbivores, resources and risks: alternating regulation along primary environmental gradients in savannas. ***Trends in Ecology & Evolution, 25(2),*** 119–128. https://doi.org/10.1016/j.tree.2009.08.001

Horswill, C., Jackson, J. A., Medeiros, R., Nowell, R. W., Trathan, P., & O'Connell, T. C. (2018). Minimising the limitations of using dietary analysis to assess foodweb changes by combining multiple techniques. ***Ecological Indicators, 94***, 218–225. https://doi.org/10.1016/j.ecolind.2018.06.035

Höss, M., Kohn, M. H., Pääbo, S., Knauer, F., & Schröder, W. P. (1992). Excrement analysis by PCR. ***Nature, 359(6392)***, 199. https://doi.org/10.1038/359199a0

Huqa, T. J. (2019). The impact of climate variability on the ecology of a lion (Panthera leo, Linnaeus 1758) population and lion livestock conflicts in the Amboseli ecosystem, Kenya. Doctoral dissertation, Leiden University, Leiden, The Netherlands). Retrieved from https://scholarlypublications.universiteitleiden.nl/handle/1887/36401

Hyslop, E. J. (1980). Stomach contents analysis-a review of methods and their application. ***Journal of Fish Biology, 17(4)***, 411–429. https://doi.org/10.1111/j.1095-8649.1980.tb02775.x

Iversen, L. L., Kielgast, J., & Sand-Jensen, K. (2015). Monitoring of animal abundance by environmental DNA — An increasingly obscure perspective: A reply to Klymus et al,Monitoring of animal abundance by environmental DNA — An increasingly obscure perspective: A reply to Klymus. *Biological Conservation, 192*, 479–480. https://doi.org/10.1016/j.biocon.2015.09.024

Jacobs, J. (1974). Quantitative measurement of food selection. *Oecologia, 14(4),* 413–417. https://doi.org/10.1007/bf00384581

Jakes, A. F., Jones, P. S., Paige, L. C., Seidler, R. G., & Huijser, M. P. (2018). A fence runs through it: A call for greater attention to the influence of fences on wildlife and ecosystems. *Biological Conservation, 227,* 310–318. https://doi.org/10.1016/j.biocon.2018.09.026

Jardine, T. D., Kidd, K. A., & Fisk, A. T. (2006). Applications, considerations, and sources of uncertainty when using stable isotope analysis in ecotoxicology. *Environmental Science & Technology, 40(24),* 7501–7511. https://doi.org/10.1021/es061263h

Jarman, S., Deagle, B. E., & Gales, N. J. (2004). Group-specific polymerase chain reaction for DNA-based analysis of species diversity and identity in dietary samples. *Molecular Ecology, 13(5),* 1313–1322. https://doi.org/10.1111/j.1365-294x.2004.02109.x

Jones, C. H., McGhee, R., & Wilkie, D. M. (1990). Hamsters (Mesocricetus auratus) use spatial memory in foraging for food to hoard. *Behavioural Processes, 21(2–3)*, 179–187. https://doi.org/10.1016/0376-6357(90)90023-9

Jusino, M. A., Banik, M. T., Palmer, J. M., Wray, A. K., Xiao, L., Pelton, E., … Lindner, D. L. (2019). An improved method for utilizing high-throughput amplicon sequencing to determine the diets of insectivorous animals. *Molecular ecology resources, 19(1),* 176–190. https://doi.org/10.1111/1755-0998.12951

Kallimanis, A. S., Mazaris, A. D., Tsakanikas, D., Dimopoulos, P., Pantis, J. D., & Sgardelis, S. P. (2012). Efficient biodiversity monitoring: Which taxonomic level to study? *Ecological Indicators, 15(1),* 100–104. https://doi.org/10.1016/j.ecolind.2011.09.024

Karasov, W. H., & Douglas, A. E. (2013). Comparative digestive physiology. *Comprehensive Physiology, 3(2)*, 741-783. https://doi.org/10.1002/cphy.c110054

Kartzinel, T. R., Chen, P., 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(26),* 8019–8024. https://doi.org/10.1073/pnas.1503283112

Kassambara, A. (2020). ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0. https://CRAN.R-project.org/package=ggpubr

Kassilly, F. N., Tsingalia, H. M., & Gossow, H. (2008). Mitigating human-wildlife conflicts through wildlife fencing: A Kenyan case study. *Wildlife Biology in Practice, 4(1).* https://doi.org/10.2461/wbp.2008.4.3

Kelly, R. P., Shelton, A. O., & Gallego, R. (2019). Understanding PCR processes to draw meaningful conclusions from environmental DNA studies. *Scientific Reports, 9(1),* 1–14.

Kesch, M. K., Bauer, D., & Loveridge, A. J. (2015). Break on Through to the Other Side: The Effectiveness of Game Fencing to Mitigate Human—Wildlife Conflict. *South African Journal of Wildlife Research - 24-month Delayed Open Access, 45(1),* 76. https://doi.org/10.3957/056.045.0109

King, R. A., Read, D. S., Traugott, M., & Symondson, W. O. (2008). Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular ecology, 17(4),* 947–963. https://doi.org/10.1111/j.1365-294X.2007.03613.x

Klare, U., Kamler, J. F., & Macdonald, D. W. (2011). A comparison and critique of different scat-analysis methods for determining carnivore diet. *Mammal Review, 41(4)*, 294–312. https://doi.org/10.1111/j.1365-2907.2011.00183.x

Koepfli, C., Nguitragool, W., Hofmann, N. E., Robinson, L. J., Ome-Kaius, M., Sattabongkot, J., … Mueller, I. (2016). Sensitive and accurate quantification of human malaria parasites using droplet digital PCR (ddPCR*). Scientific reports, 6*, 39183. https://doi.org/10.1038/srep39183

Kohn, M. H., Knauer, F., Stoffella, A., Schröder, W. P., & Pääbo, S. (1995). Conservation genetics of the European brown bear - a study using excremental PCR of nuclear and mitochondrial sequences. *Molecular Ecology, 4(1)*, 95–104. https://doi.org/10.1111/j.1365-294x.1995.tb00196.x

Kratina, P., LeCraw, R. M., Ingram, T., & Anholt, B. R. (2012). Stability and persistence of food webs with omnivory: Is there a general pattern? *Ecosphere, 3(6),* 1–18. https://doi.org/10.1890/es12-00121.1

Kröschel, M., Reineking, B., Werwie, F., Wildi, F., & Storch, I. (2017). Remote monitoring of vigilance behavior in large herbivores using acceleration data. *Animal Biotelemetry, 5(1).* https://doi.org/10.1186/s40317-017-0125-z

Lambert, J.E. (1998). Primate digestion: Interactions among anatomy, physiology, and feeding ecology. *Evolutionary Anthropology, 7(1),* 8-20.

Laszlo, A. H., Derrington, I. M., Ross, B., Brinkerhoff, H., Adey, A., Nova, I. C., Craig, J. M., Langford, K. W., Samson, J. M., Daza, R. M., Doering, K., Shendure, J., & Gundlach, J. H. (2014). Decoding long nanopore sequencing reads of natural DNA. *Nature Biotechnology, 32(8),* 829–833. https://doi.org/10.1038/nbt.2950

Lehmann, M. B., Funston, P. J., Owen, C. R., & Slotow, R. (2008). Feeding behaviour of lions (Panthera leo) on a small reserve. *African Journal of Wildlife Research, 38(1)*, 66–78. https://doi.org/10.3957/0379-4369-38.1.66

Lesilau, F. L. (2019). Human-Lion conflict around Nairobi National Park: Lion (Panthera leo melanochaita, Hamilton Smith, 1842) Population Structure, Landscape Use and Diet, in a Semi-Fenced Park. (Doctoral dissertation, Leiden University, Leiden, The Netherlands). Retrieved from https://scholarlypublications.universiteitleiden.nl/handle/1887/81380

Letelier, R. M., Bidigare, R. R., Hebel, D. V., Ondrusek, M., Winn, C. D., & Karl, D. M. (1993). Temporal variability of phytoplankton community structure based on pigment analysis. *Limnology and Oceanography, 38(7),* 1420–1437. https://doi.org/10.4319/lo.1993.38.7.1420

Li, L., Victoria, J., Wang, C., Jones, M. S. M., Fellers, G. M., Kunz, T., & Delwart, E. (2010). Bat Guano Virome: Predominance of Dietary Viruses from Insects and Plants plus Novel Mammalian Viruses. *Journal of Virology, 84(14),* 6955–6965. https://doi.org/10.1128/jvi.00501-10

Libralato, S., Christensen, V., & Pauly, D. (2006). A method for identifying keystone species in food web models. *Ecological Modelling, 195(3–4),* 153–171. https://doi.org/10.1016/j.ecolmodel.2005.11.029

Lindsey, P. A., Petracca, L. S., Funston, P. J., Bauer, H., Dickman, A., Everatt, K. T., … Hunter, L. T. B. (2017). The performance of African protected areas for lions and their prey. *Biological Conservation, 209*, 137–149. https://doi.org/10.1016/j.biocon.2017.01.011

Liu, G., Zhang, S., Zhao, X., Li, C., & Gong, M. (2021). Advances and Limitations of next generation sequencing in animal diet analysis. *Genes, 12(12),* 1854. https://doi.org/10.3390/genes12121854

Loarie, S. R., Tambling, C. J., & Asner, G. P. (2013). Lion hunting behaviour and vegetation structure in an African savanna. ***Animal Behaviour, 85(5),*** 899–906. https://doi.org/10.1016/j.anbehav.2013.01.018

Louw, J. A., Funston, P. J., Greeff, A., & Kloppers, H. (2012). The Applicability of Lion Prey Selection Models to Smaller Game Reserves in South Africa. ***South African Journal of Wildlife Research, 42(2),*** 73–81. https://doi.org/10.3957/056.042.0211

Løvschal, M., Bøcher, P. K., Pilgaard, J., Amoke, I., Odingo, A. A., Thuo, A. D. M., & Svenning, J. (2017). Fencing bodes a rapid collapse of the unique Greater Mara ecosystem. ***Scientific Reports, 7(1).*** https://doi.org/10.1038/srep41450

Magoč, T., & Salzberg, S. L. (2011). FLASH: Fast length adjustment of short reads to improve genome assemblies. ***Bioinformatics, 27(21),*** 2957–2963. https://doi.org/10.1093/bioinformatics/btr507

Mangiafico, S. (2021). rcompanion: Functions to Support Extension Education Program Evaluation. R package version 2.4.1. https://CRAN.R-project.org/package=rcompanion

Martin, M. (2013). Cutadapt removes adapter sequences from high-throughput sequencing reads. ***EMBnet.Journal, 17(1),*** 10–12. https://doi.org/https://doi.org/10.14806/ej.17.1.200

Massart, L.M, Vandenginste, B.G.M., Buydens, L.M.C., De Jong, S., Lewi, P.J., Smeyers-Verbeke, J. (1997) ***Handbook of Chemometrics and Qualimetrics: Part A,*** p. 200.

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(2)***, 165–175. https://doi.org/10.1111/mec.14779

Matley, J. K., Maes, G. E., Devloo-Delva, F., Huerlimann, R., Chua, G., Tobin, A. J., Fisk, A. T., Simpfendorfer, C. A., & Heupel, M. R. (2018). Integrating complementary methods to improve diet analysis in fishery-targeted species. ***Ecology and Evolution, 8(18),*** 9503–9515. https://doi.org/10.1002/ece3.4456

McCann, K. S. (2007). Protecting biostructure. ***Nature, 446(7131),*** 29. https://doi.org/10.1038/446029a

McCracken, G. F., Westbrook, J. K., Brown, V. A., Eldridge, M., Federico, P., & Kunz, T. H. (2012). Bats track and exploit changes in insect pest populations. ***PloS one, 7(8),*** e43839. https://doi.org/10.1371/journal.pone.0043839

McCutchan, J. H., Lewis, W. M., Kendall, C., & McGrath, C. C. (2003). Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. ***Oikos, 102(2),*** 378–390. https://doi.org/10.1034/j.1600-0706.2003.12098.x

McInnes, J. C., Alderman, R., Deagle, B. E., Lea, M., Raymond, B., & Jarman, S. (2016). Optimised scat collection protocols for dietary DNA metabarcoding in vertebrates. ***Methods in Ecology and Evolution, 8(2),*** 192–202. https://doi.org/10.1111/2041-210x.12677

McInturff, A., Xu, W., Wilkinson, C. F., Dejid, N., & Brashares, J. S. (2020). Fence Ecology: Frameworks for understanding the ecological effects of fences. ***BioScience.*** https://doi.org/10.1093/biosci/biaa103

McMeans, B. C., McCann, K. S., Humphries, M. M., Rooney, N., & Fisk, A. T. (2015). Food web structure in Temporally-Forced ecosystems. ***Trends in Ecology and Evolution, 30(11),*** 662–672. https://doi.org/10.1016/j.tree.2015.09.001

Mesa-Cruz, J. B., Brown, J. L., Waits, L. P., & Kelly, M. J. (2016). Non-invasive genetic sampling reveals diet shifts, but little difference in endoparasite richness and faecal glucocorticoids, in Belizean felids inside and outside protected areas. ***Journal of Tropical Ecology, 32(3),*** 226–239. https://doi.org/10.1017/s0266467416000213

Meyer, J. M., Leempoel, K., Losapio, G., & Hadly, E. A. (2020). Molecular Ecological Network Analyses: an effective conservation tool for the assessment of biodiversity, trophic interactions, and community structure. ***Frontiers in Ecology and Evolution,*** 8. https://doi.org/10.3389/fevo.2020.588430

Michener, R. H., Kaufman, L., & Lajtha, K. (1994). Stable isotope ratios as tracers in marine aquatic food webs. ***Stable Isotopes in Ecology and Environmental Science, 7***, 138–157. https://ci.nii.ac.jp/naid/10026140602

Miller, K. M., & McEwen, L. C. (1995). Diet of nesting Savannah Sparrows in interior Alaska. ***Journal of Field Ornithology, 66(1),*** 152–158.

Miotke, L., Lau, B. T., Rumma, R. T., & Ji, H. P. (2014). High sensitivity detection and quantitation of DNA copy number and single nucleotide variants with single color droplet digital PCR. ***Analytical chemistry, 86(5)***, 2618–2624. https://doi.org/10.1021/ac403843j

Mitchell-Jones, A.J., Amori, G., Bogdanowicz, W., Krystufek, B., Reijnders, P.J.H., Spitzenberger, F., … Zima, J., (1999). The atlas of European mammals. London, UK: T.Poyser & A.D.Poyser.

Montague T. L., & Cullen, J. M. (1985). Comparison of techniques to recover stomach contents from penguins. ***Australian Wildlife Research, 12(2),*** 327–330.

Monterroso, P., Godinho, R., De Oliveira, T. M., Ferreras, P., Kelly, M. J., Morin, D. J., Waits, L. P., Alves, P. C., & Mills, L. S. (2018). Feeding ecological knowledge: the underutilised power of faecal DNA approaches for carnivore diet analysis. ***Mammal Review, 49(2)***, 97–112. https://doi.org/10.1111/mam.12144

Moon, K. (2015). R statistics and graphs for medical writing (Korean Edition). Hannarae Academy.

Moon, K. (2020). CRAN - Package webr. Retrieved October 13, 2022, from

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(4),*** 651–660. https://doi.org/10.1002/wsb.1021

Morisset, D., Štebih, D., Milavec, M., Gruden, K., & Žel, J. (2013). Quantitative analysis of food and feed samples with droplet digital PCR. ***PloS one, 8(5),*** e62583. https://doi.org/10.1371/journal.pone.0062583

Mumma, M. A., Adams, J. R., Zieminski, C., Fuller, T. K., Mahoney, S. P., & Waits, L. P. (2016). A comparison of morphological and molecular diet analyses of predator scats, ***Journal of Mammalogy, 97(1),*** 112–120, doi: 10.1093/jmammal/gyv160

Murray, D. C., Bunce, M., Cannell, B., Oliver, R., Houston, J., White, N. E., Barrero, R. A., Bellgard, M., & Haile, J. (2011). DNA-Based Faecal Dietary Analysis: A comparison of QPCR and high throughput sequencing approaches. ***PLOS ONE, 6(10),*** e25776. https://doi.org/10.1371/journal.pone.0025776

Naha, D., Périquet, S., Kilian, J. W., Kupferman, C. A., Hoth-Hanssen, T., & Beasley, J. C. (2023). Fencing affects movement patterns of two large carnivores in Southern Africa. ***Frontiers in Ecology and Evolution***, 11. https://doi.org/10.3389/fevo.2023.1031321

Nakahara, F., Ando, H., Ito, H., Murakami, A., Morimoto, N., Yamasaki, M., Takayanagi, A., & Isagi, Y. (2015). The applicability of DNA barcoding for dietary analysis of sika deer. ***DNA Barcodes, 3(1).*** https://doi.org/10.1515/dna-2015-0021

Nielsen, J. M., Clare, E. L., Hayden, B., Brett, M. T., & Kratina, P. (2017). Diet tracing in ecology: Method comparison and selection. ***Methods in Ecology and Evolution, 9(2),*** 278–291. https://doi.org/10.1111/2041-210x.12869

Notz, E., Imholt, C., Reil, D., & Jacob, J. (2017). Testing automated sensor traps for mammal field studies. ***Wildlife Research, 44(1),*** 72. https://doi.org/10.1071/wr16192

Ogle, D. H., Wheeler, P., & Dinno, A. (2021). FSA: Fisheries Stock Analysis. R package version 0.9.0, https://github.com/droglenc/FSA.

Oja, R., Soe, E., Valdmann, H., & Saarma, U. (2017). Non-invasive genetics outperforms morphological methods in faecal dietary analysis, revealing wild boar as a considerable conservation concern for ground-nesting birds. ***PLOS ONE, 12(6),*** e0179463. https://doi.org/10.1371/journal.pone.0179463

Okello, M. M., Manka, S. G., & D'Amour, D. E. (2008). The relative importance of large mammal species for tourism in Amboseli National Park, Kenya. ***Tourism Management, 29(4),*** 751–760. https://doi.org/10.1016/j.tourman.2007.08.003

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Sólymos, P., Stevens, M. H. H., Wagner, H. H., . . . & Weedon, J. (2022). vegan: Community Ecology Package. R Package Version 2.6-4. https://CRAN.R-project.org/package=vegan

Owen-Smith, N., & Mills, M. G. L. (2008). Predator-prey size relationships in an African large-mammal food web. ***Journal of Animal Ecology, 77(1),*** 173–183. https://doi.org/10.1111/j.1365-2656.2007.01314.x

Packer, L., Gibbs, J., Sheffield, C. S., & Hanner, R. (2009). DNA barcoding and the mediocrity of morphology. ***Molecular Ecology Resources, 9(s1),*** 42–50. https://doi.org/10.1111/j.1755-0998.2009.02631.x

Packer, C., Loveridge, A., Canney, S. M., Caro, T., Garnett, S. T., Pfeifer, M., Zander, K. K., Swanson, A., MacNulty, D. R., Balme, G., Bauer, H., Begg, C. M., Begg, K. S., Bhalla, S., Bissett, C., Bodasing, T., Brink, H., Burger, A., Burton, A., . . . & Polasky, S. (2013a). Conserving large carnivores: dollars and fence. ***Ecology Letters, 16(5),*** 635–641. https://doi.org/10.1111/ele.12091

Packer, C., Swanson, A., Canney, S. M., Loveridge, A., Garnett, S. T., Pfeifer, M., Burton, A., Bauer, H., & MacNulty, D. R. (2013b). The case for fencing remains intact. ***Ecology Letters, 16(11),*** 1414-e4. https://doi.org/10.1111/ele.12171

Pedersen, A. B., Jones, K. E., Nunn, C. L., & Altizer, S. (2007). Infectious diseases and extinction risk in wild mammals. ***Conservation Biology, 21(5),*** 1269–1279. https://doi.org/10.1111/j.1523-1739.2007.00776.x

Pelz, H.J., 1986. Damage by wood mice (Apodemus sylvaticus) to sugarbeet seeds in relation to food availability. ***Control of mammal pests (A supplement to tropical pest management) 32,*** 5-14.

Pennycuick, C. J., & Rudnai, J. (1970). A method of identifying individual lions Panthera leo with an analysis of the reliability of identification. ***Journal of Zoology, 160(4***), 497–508. https://doi.org/10.1111/j.1469-7998.1970.tb03093.x

Pereira, A., Xavier, R., Perera, A., Salvi, D., & Harris, D. J. (2019). DNA metabarcoding to assess diet partitioning and feeding strategies in generalist vertebrate predators: a case study on three syntopic lacertid lizards from Morocco. ***Biological Journal of the Linnean Society, 127(4),*** 800–809. https://doi.org/10.1093/biolinnean/blz061

Phelps, M. (2019). Increasing eDNA capabilities with CRISPR technology for real-time monitoring of ecosystem biodiversity. ***Molecular Ecology Resources, 19(5),*** 1103–1105. https://doi.org/10.1111/1755-0998.13084

Pierce, G. J., & Boyle, P. R. (1991). A review of methods for diet analysis in piscivorous marine mammals. ***Oceanography and Marine Biology, 29(29),*** 409–486. https://abdn.pure.elsevier.com/en/publications/a-review-of-methods-for-diet-analysis-in-piscivorous-marine-mamma

Piñol, J., Andrés, V. S., Clare, E. L., Mir, G., & Symondson, W. O. C. (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(1),*** 18–26. https://doi.org/10.1111/1755-0998.12156

Piñol, J., Senar, M. A., & Symondson, W. O. (2018). The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative. ***Molecular Ecology, 28(2),*** 407-419. doi:10.1111/mec.14776

Piñol, J. (2021). Genotype by sequencing: An alternative new method to amplicon metabarcoding and shotgun metagenomics for the assessment of eukaryote biodiversity. ***Molecular Ecology Resources, 21(4),*** 1001–1004. https://doi.org/10.1111/1755-0998.13320

Pompanon, F., Deagle, B. E., Symondson, W. O. C., Brown, D., Jarman, S. N., & Taberlet, P. (2012). Who is eating what: diet assessment using next generation sequencing. ***Molecular Ecology, 21(8),*** 1931–1950. https://doi.org/10.1111/j.1365-294x.2011.05403.x

Power, R. J. (2002). Prey selection of lions Panthera leo in a small, enclosed reserve. ***Koedoe, 45(2).*** https://doi.org/10.4102/koedoe.v45i2.32

Pringle, R. M., & Hutchinson, M. C. (2020). Resolving Food-Web Structure. ***Annual Review of Ecology, Evolution, and Systematics, 51(1),*** 55–80. https://doi.org/10.1146/annurev-ecolsys-110218-024908

Pujolar, J. M., Limborg, M. T., Ehrlich, M., & Jaspers, C. (2022). High throughput SNP chip as cost effective new monitoring tool for assessing invasion dynamics in the comb jelly ***Mnemiopsis leidyi. Frontiers in Marine Science, 9.*** https://doi.org/10.3389/fmars.2022.1019001

Quy, R., Chandler-Morris, S., Watkins, R., & Thompson, H. (2005, May 22–26). Guidance on Risk Assessment for Birds and Mammals: Hoarding of pesticide-treated seed extends the exposure period. [Poster session]. SETAC EU, Lille, France.

R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

R Core Team. (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Ranke, J. (2022). chemCal: Calibration Functions for Analytical Chemistry. R package version 0.2.3. https://CRAN.R-project.org/package=chemCal

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The Barcode of Life Data System: Barcoding. ***Molecular Ecology Notes, 7(3),*** 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Razgour, O., Clare, E. L., Zeale, M. R., Hanmer, J., Schnell, I. B., Rasmussen, M., Gilbert, M. T. P., & Jones, G. (2011). High-throughput sequencing offers insight into mechanisms of resource partitioning in cryptic bat species. ***Ecology and Evolution, 1(4),*** 556–570. https://doi.org/10.1002/ece3.49

Reed, J. Z., Tollit, D. J., Thompson, P. M., & Amos, W. (1997). Molecular scatology: the use of molecular genetic analysis to assign species, sex and individual identity to seal faeces. ***Molecular Ecology, 6(3),*** 225–234. https://doi.org/10.1046/j.1365-294x.1997.00175.x

Reeves, L. E., Gillett-kaufman, J. L., Kawahara, A. Y., & Kaufman, E. (2018). Barcoding blood meals: New vertebrate-specific primer sets for assigning taxonomic identities to host DNA from mosquito blood meals. ***PLoS Neglected Tropical Diseases, 12(8),*** 1–18.

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(21***), 1–11. https://doi.org/10.1093/nar/gkr732

Richly, E., & Leister, D. (2004). NUPTs in sequenced eukaryotes and their genomic organization in relation to NUMTs. ***Molecular biology and evolution, 21(10),*** 1972–1980. https://doi.org/10.1093/molbev/msh210

Rindorf, A., & Lewy, P. (2004). Bias in estimating food consumption of fish by stomach-content analysis. Canadian ***Journal of Fisheries and Aquatic Sciences, 61(12),*** 2487–2498. https://doi.org/10.1139/f04-200

Ripple, W. J., Estes, J. A., Beschta, R. L., Wilmers, C. C., Ritchie, E. G., Hebblewhite, M., Berger, J., Elmhagen, B., Letnic, M., Nelson, M. P., Schmitz, O. J., Smith, D. W., Wallach, A. D., & Wirsing, A. J. (2014). Status and Ecological Effects of the World's Largest Carnivores. ***Science, 343(6167)***. https://doi.org/10.1126/science.1241484

Rizzi, A., Raddadi, N., Sorlini, C., Nordgrd, L., Nielsen, K. M., & Daffonchio, D. (2012). The Stability and Degradation of Dietary DNA in the Gastrointestinal Tract of Mammals: Implications for Horizontal Gene Transfer and the Biosafety of GMOs. ***Critical Reviews in Food Science and Nutrition, 52(2),*** 142–161. https://doi.org/10.1080/10408398.2010.499480

Robeson, M. S., Khanipov, K., Golovko, G., Wisely, S. M., White, M. D., Bodenchuck, M., Smyser, T. J., Fofanov, Y., Fierer, N., & Piaggio, A. J. (2017). Assessing the utility of metabarcoding for diet analyses of the omnivorous wild pig (Sus scrofa). ***Ecology and Evolution, 8(1),*** 185–196. https://doi.org/10.1002/ece3.3638

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. ***PeerJ, 2016(10),*** 1–22. https://doi.org/10.7717/peerj.2584

Roslin, T., & Majaneva, S. (2016). The use of DNA barcodes in food web construction-terrestrial and aquatic ecologists unite!. ***Genome, 59(9),*** 603–628. https://doi.org/10.1139/gen-2015-0229

Roy, C. L., Coy, P. L., Chen, D., Ponder, J., & Jankowski, M. D. (2019). Multi-scale availability of neonicotinoid-treated seed for wildlife in an agricultural landscape during spring planting. ***Science of the Total Environment, 682***, 271–281. https://doi.org/10.1016/j.scitotenv.2019.05.010

Rubbmark, O. R., Sint, D., Horngacher, N., & Traugott, M. (2018). A broadly applicable COI primer pair and an efficient single- tube amplicon library preparation protocol for metabarcoding. ***Ecol Evol***. 2018; 8: 12335–12350. https://doi.org/10.1002/ece3.4520

Sayers, E. W., Bolton, E. E., Brister, J. R., Canese, K., Chan, J., Comeau, D. C., Connor, R., Funk, K., Kelly, C., Kim, S., Madej, T., Marchler-Bauer, A., Lanczycki, C., Lathrop, S., Lu, Z., Thibaud-Nissen, F., Murphy, T., Phan, L., Skripchenko, Y., Tse, T., … Sherry, S. T. (2022). Database resources of the national center for biotechnology information. ***Nucleic acids research, 50(D1),*** D20–D26. https://doi.org/10.1093/nar/gkab1112

Schaller, G. B. (1972). The Serengeti Lion: A Study of Predator-Prey Relations (Wildlife Behavior and Ecology series) (1st ed.). University of Chicago Press.

Schmieder, R., & Edwards, R. (2011). Quality control and preprocessing of metagenomic datasets. ***Bioinformatics, 27(6),*** 863–864. https://doi.org/10.1093/bioinformatics/btr026

Schoener, T. W. (1974). Resource partitioning in ecological communities. ***Science, 185(4145),*** 27–39. https://doi.org/10.1126/science.185.4145.27

Schwarz, D., Spitzer, S. M., Thomas, A. C., Kohnert, C. M., Keates, T. R., & Acevedo-Gutiérrez, A. (2018). Large-scale molecular diet analysis in a generalist marine mammal reveals male preference for prey of conservation concern. ***Ecology and Evolution, 8(19),*** 9889–9905. https://doi.org/10.1002/ece3.4474

Scott, M. (1988). The Impact of infection and disease on Animal Populations: Implications for Conservation Biology. ***Conservation Biology, 2(1),*** 40–56. https://doi.org/10.1111/j.1523-1739.1988.tb00334.x

Sethi, S. A., Larson, W. A., Turnquist, K., & Isermann, D. (2018). Estimating the number of contributors to DNA mixtures provides a novel tool for ecology. ***Methods in Ecology and Evolution, 10(1),*** 109–119. https://doi.org/10.1111/2041-210x.13079

Shashank, P. R., Parker, B., Rananaware, S. R., Plotkin, D., Couch, C., Yang, L. G., Nguyễn, L. T., Prasannakumar, N. R., Braswell, W. E., Jain, P., & Kawahara, A. Y. (2023). CRISPR-based diagnostics detects invasive insect pests. ***Molecular Ecology Resources, 24(1)***. https://doi.org/10.1111/1755-0998.13881

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(8),*** 1951–1965. https://doi.org/10.1111/j.1365-294X.2011.05424.x

Shelton, A. O., Gold, Z., Jensen, A. J., D'Agnese, E., Allan, E. A., Van Cise, A., Gallego, R., Ramón-Laca, A., Garber-Yonts, M., Parsons, K. M., & Kelly, R. P. (2022). Toward quantitative metabarcoding. ***Ecology, 104(2)***. https://doi.org/10.1002/ecy.3906

Shum, P., & Palumbi, S. R. (2021). Testing small-scale ecological gradients and intraspecific differentiation for hundreds of kelp forest species using haplotypes from metabarcoding. ***Molecular Ecology, 30(13),*** 3355–3373. https://doi.org/10.1111/mec.15851

Sitienei, A. J., Jiwen, G., & Ngene, S. M. (2014). Assessing the cost of living with elephants (Loxodonta africana) in areas adjacent to Meru National Park, Kenya. ***European Journal of Wildlife Research, 60(2),*** 323–330. https://doi.org/10.1007/s10344-013-0789-5

Slobodian, V., Rizzato, P. P., & Sobral, G. (2021). Vertebrates (Chordata). In Springer eBooks (pp. 1–28). https://doi.org/10.1007/978-3-319-47829-6_1426-2

Sogbohossou, E. A., de Iongh, H. H., Sinsin, B., de Snoo, G. R., & Funston, P. J. (2011). Human–carnivore conflict around Pendjari Biosphere Reserve, northern Benin. ***Oryx, 45(4),*** 569–578. https://doi.org/10.1017/s0030605310001109

Soininen, E. M., Gauthier, G., Bilodeau, F., Berteaux, D., Gielly, L., Taberlet, P., … Yoccoz, N. G. (2015). Highly overlapping winter diet in two sympatric lemming species revealed by DNA metabarcoding. ***PloS one, 10(1),*** e0115335. https://doi.org/10.1371/journal.pone.0115335

Sousa, L. L., Silva, S., & Xavier, R. (2019). DNA metabarcoding in diet studies: Unveiling ecological aspects in aquatic and terrestrial ecosystems. ***Environmental DNA, 1(3),*** 199–214. https://doi.org/10.1002/edn3.27

Spaulding, R. L., Krausman, P. R., & Ballard, W. B. (2000). Observer bias and analysis of gray wolf diets from scats. ***Wildlife Society Bulletin, 28(4),*** 947–950. https://www.cabdirect.org/abstracts/20013035381.html

Steenweg, R., Gillingham, M. P., Parker, K. L., & Heard, D. C. (2015). Considering sampling approaches when determining carnivore diets: the importance of where, how, and when scats are collected. ***Mammal Research, 60(3),*** 207–216. https://doi.org/10.1007/s13364-015-0222-4

Stevenson, T., & Fanshawe, J. (2002). The Birds of East Africa: Kenya, Tanzania, Uganda, Rwanda, Burundi (Princeton Field Guides, 14) (1st Edition). Princeton University Press.

Stoate, C., Báldi, A., Beja, P., Boatman, N. D., Herzon, I., van Doorn, A., de Snoo, G. R., Rakosy, L., & Ramwell, C. (2009). Ecological impacts of early 21st century agricultural change in Europe--a review. ***Journal of environmental management, 91(1),*** 22–46. https://doi.org/10.1016/j.jenvman.2009.07.005

Storck, V., Karpouzas, D. G., & Martin-Laurent, F. (2017). Towards a better pesticide policy for the European Union. ***Science of The Total Environment, 575***, 1027–1033. https://doi.org/10.1016/j.scitotenv.2016.09.167

Stuart, C., & Stuart, M. (2013). Pocket Guide - Mammals of East Africa. Struik Nature.

Stunkard, A. J. (1983). Nutrition, aging and obesity: a critical review of a complex relationship. ***International Journal of Obesity, 7(3),*** 201–220. https://pubmed.ncbi.nlm.nih.gov/6885229/

Symondson, W. O. C. (2002). Molecular identification of prey in predator diets. ***Molecular ecology, 11(4),*** 627–641. https://doi.org/10.1046/j.1365-294x.2002.01471.x

Symondson, W. O. C. (2012). The Molecular Revolution: Using Polymerase Chain Reaction based Methods to Explore the Role of Predators in Terrestrial Food Webs. In Gurr, G. M., Wratten, S. D., Snyder, W.E., & Read, D.M.Y. (Eds.), Biodiversity and Insect Pests: Key Issues for Sustainable Management, (pp. 166-184). New Jersey, USA: Wiley-Blackwell.

Tercel, M. P. T. G., Symondson, W. O. C., & Cuff, J. P. (2021). The problem of omnivory: A synthesis on omnivory and DNA metabarcoding. ***Molecular Ecology, 30(10),*** 2199–2206. https://doi.org/10.1111/mec.15903

Thalinger, B., Oehm, J., Zeisler, C., Vorhauser, J., & Traugott, M. (2018). Sex-specific prey partitioning in breeding piscivorous birds examined via a novel, noninvasive approach. ***Ecology and Evolution, 8(17),*** 8985–8998. https://doi.org/10.1002/ece3.4421

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(15),*** 3706–3718. https://doi.org/10.1111/mec.12523

Thomas, A. C., Deagle, B. E., Eveson, J. P., Harsch, C. H., & Trites, A. W. (2015). Quantitative DNA metabarcoding: improved estimates of species proportional biomass using correction factors derived from control material. ***Molecular Ecology Resources, 16(3),*** 714–726. https://doi.org/10.1111/1755-0998.12490

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

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(4),*** 889–905. https://doi.org/10.1890/07-1701.1

Traugott, M., Kamenova, S., Rueß, L., Seeber, J., & Plantegenest, M. (2013). Empirically characterising trophic networks. In Advances in Ecological Research (pp. 177–224). https://doi.org/10.1016/b978-0-12-420002-9.00003-2

Traugott, M., Thalinger, B., Wallinger, C., & Sint, D. (2020). Fish as predators and prey: DNA-based assessment of their role in food webs. ***Journal of Fish Biology, 98(2),*** 367–382. https://doi.org/10.1111/jfb.14400

Trinkel, M., Fleischmann, P., & Slotow, R. (2016). Electrifying the fence or living with consequences? Problem animal control threatens the long-term viability of a free-ranging lion population. ***Journal of Zoology, 301(1),*** 41–50. https://doi.org/10.1111/jzo.12387

Trites, A. W. & Joy, R. (2005). Dietary Analysis from Fecal Samples: How Many Scats are Enough? ***Journal of Mammalogy, 86(4),*** 704–712, https://doi.org/10.1644/1545-1542(2005)086[0704:DAFFSH]2.0.CO;2

Tscharntke, T., Klein, A. M., Kruess, A., Steffan-Dewenter, I., & Thies, C. (2005). Landscape perspectives on agricultural intensification and biodiversity - ecosystem service management. Ecology Letters, 8(8), 857–874. https://doi.org/10.1111/j.1461-0248.2005.00782.x

Tsuji, Y., Miura, S., Kotoge, T., Shiraishi, T., & Murai, H. (2015). Effects of Food Intake on Digesta Passage Time in Captive Japanese Martens (***Martes melampus***) and Implications for Endozoochorous Seed Dispersal. ***Mammal Study, 40(1),*** 13-18.

Tunney, T. D., McCann, K. S., Lester, N. P., & Shuter, B. J. (2014). Effects of differential habitat warming on complex communities. ***Proceedings of the National Academy of Sciences of the United States of America, 111(22),*** 8077–8082. https://doi.org/10.1073/pnas.1319618111

UNEP-WCMC and IUCN (2022), Protected Planet: The World Database on Protected Areas (WDPA) and World Database on Other Effective Area-based Conservation Measures (WD-OECM) [Online], October 2022, Cambridge, UK: UNEP-WCMC and IUCN. Available at: www.protectedplanet.net

Vamos, E. E., Elbrecht, V., & Leese, F. (2017). Short COI markers for freshwater macroinvertebrate metabarcoding. ***Metabarcoding and Metagenomics, 1,*** e14625. https://doi.org/10.3897/mbmg.1.14625

van der Heyde, M., Bateman, P. W., Bunce, M., Wardell-Johnson, G., White, N. E., & Nevill, P. (2021). Scat DNA provides important data for effective monitoring of mammal and bird biodiversity. ***Biodiversity and Conservation, 30(12),*** 3585–3602. https://doi.org/10.1007/s10531-021-02264-x

Van Loco, J., Elskens, M., Croux, C., & Beernaert, H. (2002). Linearity of calibration curves: use and misuse of the correlation coefficient. ***Accreditation and Quality Assurance, 7(7),*** 281–285. https://doi.org/10.1007/s00769-002-0487-6

Vander Zanden, M. J., Clayton, M. K., Moody, E. K., Solomon, C. T., & Weidel, B. C. (2015). Stable isotope Turnover and Half-Life in Animal Tissues: a literature synthesis. ***PLOS ONE, 10(1),*** e0116182. https://doi.org/10.1371/journal.pone.0116182

Verhaegen, B., De Reu, K., De Zutter, L., Verstraete, K., Heyndrickx, M., & Van Coillie, E. (2016). Comparison of Droplet Digital PCR and qPCR for the Quantification of Shiga Toxin-Producing Escherichia coli in Bovine Feces. ***Toxins, 8(5),*** 157. https://doi.org/10.3390/toxins8050157

Vestheim, H., & Jarman, S. (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),*** 12. https://doi.org/10.1186/1742-9994-5-12

Vijver, M.G., Hunting, E.R., Nederstigt, T.A., Tamis, W.L., van den Brink, P.J. and van Bodegom, P.M. (2017), Postregistration monitoring of pesticides is urgently required to protect ecosystems. ***Environ Toxicol Chem, 36***, 860-865. https://doi.org/10.1002/etc.3721

Vogel, J. T., Somers, M. J., & Venter, J. A. (2018). The foraging ecology of reintroduced African wild dog in small protected areas. ***Wildlife Biology, 2018(1).*** https://doi.org/10.2981/wlb.00424

Wachter, B., Blanc, A. S., Melzheimer, J., Höner, O. P., Jago, M., & Hofer, H. (2012). An Advanced Method to Assess the Diet of Free-Ranging Large Carnivores Based on Scats. ***PLoS ONE, 7(6),*** e38066. https://doi.org/10.1371/journal.pone.0038066

Wagemaker, C. a. M., Mommer, L., Visser, E. J. W., Weigelt, A., Van Gurp, T. P., Postuma, M., Smit-Tiekstra, A. E., & De Kroon, H. (2020). msGBS: A new high-throughput approach to quantify the relative species abundance in root samples of multispecies plant communities. ***Molecular Ecology Resources, 21(4),*** 1021–1036. https://doi.org/10.1111/1755-0998.13278

Wang, D., Trimbos, K. B., Gomes, S. I. F., Jacquemyn, H., & Merckx, V. (2023). Metabarcoding read abundances of orchid mycorrhizal fungi are correlated to copy numbers estimated using ddPCR. ***New Phytologist.*** https://doi.org/10.1111/nph.19385

Wang, Y., Zhao, Y., Bollas, A., Wang, Y., & Au, K. F. (2021). Nanopore sequencing technology, bioinformatics and applications. ***Nature Biotechnology, 39(11),*** 1348–1365. https://doi.org/10.1038/s41587-021-01108-x

Wanniarachchi, S., Swan, M., Nevill, P., & York, A. (2022). Using eDNA metabarcoding to understand the effect of fire on the diet of small mammals in a woodland ecosystem. ***Ecology and Evolution, 12(11).*** https://doi.org/10.1002/ece3.9457

Ward, E. J., Levin, P. S., Lance, M. M., Jeffries, S. J., & Acevedo-Gutiérrez, A. (2011). Integrating diet and movement data to identify hot spots of predation risk and areas of conservation concern for endangered species. ***Conservation Letters, 5(1),*** 37–47. https://doi.org/10.1111/j.1755-263x.2011.00210.x

Weitemier, K., Penaluna, B. E., Hauck, L., Longway, L. J., Garcia, T. S., & Cronn, R. (2021). Estimating the genetic diversity of Pacific salmon and trout using multigene eDNA metabarcoding. ***Molecular Ecology, 30(20),*** 4970–4990. https://doi.org/10.1111/mec.15811

Westoby, M., Rost, G. R., & Weis, J. A. (1976). Problems with Estimating Herbivore Diets by Microscopically Identifying Plant Fragments from Stomachs. ***Journal of Mammalogy, 57(1),*** 167–172. https://doi.org/10.2307/1379521

Wickham, H. (2009). ggplot2: Elegant Graphics for Data Analysis. http://ndl.ethernet.edu.et/bitstream/123456789/60263/1/107.pdf

Wikenros, C., Jarnemo, A., Frisén, M., Kuijper, D. P. J., & Schmidt, K. (2017). Mesopredator behavioral response to olfactory signals of an apex predator. ***Journal of Ethology, 35(2),*** 161–168. https://doi.org/10.1007/s10164-016-0504-6

Wilcoxon, F. (1945). Individual comparisons by ranking methods. ***Biometrics Bulletin, 1(6),*** 80–83.

Willerslev, E., Davison, J., Moora, M., Zobel, M., Coissac, É., Edwards, M. E., Lorenzen, E. D., Vestergård, M., Gussarova, G., Haile, J., Craine, J. M., Gielly, L., Boessenkool, S., Epp, L. S., Pearman, P. B., Cheddadi, R., Murray, D. F., Bråthen, K. A., Yoccoz, N. G., . . . Taberlet, P. (2014). Fifty thousand years of Arctic vegetation and megafaunal diet. ***Nature, 506(7486)***, 47–51. https://doi.org/10.1038/nature12921

Williams, M., O'Grady, J., Ball, B., Carlsson, J., De Eyto, E., McGinnity, P., Jennings, E., Regan, F., & Parle-McDermott, A. (2019). The application of CRISPR-Cas for single species identification from environmental DNA. ***Molecular Ecology Resources, 19(5),*** 1106–1114. https://doi.org/10.1111/1755-0998.13045

Wilson, D. E., & Reeder D. M. (editors). (2005). Mammal Species of the World. A Taxonomic and Geographic Reference (3rd ed). Baltimore, Maryland: Johns Hopkins University Press.

Woodroffe, R., Hedges, S., & Durant, S. M. (2014). To Fence or Not to Fence. ***Science, 344(6179),*** 46–48. https://doi.org/10.1126/science.1246251

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

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. ***BMC Bioinformatics, 13(1),*** 134.

Yirga, G., De Iongh, H. H., Leirs, H., Gebrihiwot, K., Deckers, J., & Bauer, H. (2012). Adaptability of large carnivores to changing anthropogenic food sources: diet change of spotted hyena (Crocuta crocuta) during Christian fasting period in northern Ethiopia. ***Journal of Animal Ecology, 81(5),*** 1052–1055. https://doi.org/10.1111/j.1365-2656.2012.01977.x

Zarzoso-Lacoste, D., Bonnaud, E., Corse, E., Gilles, A., Meglecz, E., Costedoat, C., … Vidal, E. (2016). Improving morphological diet studies with molecular ecology: An application for invasive mammal predation on island birds. ***Biological Conservation, 193,*** 134-142.

Zeale, M. R., Butlin, R. K., Barker, G. L., Lees, D. C., & Jones, G. (2011). Taxon-specific PCR for DNA barcoding arthropod prey in bat faeces. ***Molecular ecology resources, 11(2),*** 236–244. https://doi.org/10.1111/j.1755-0998.2010.02920.x

Zhou, X., Li, Y., Liu, S., Yang, Q., Su, X., Zhou, L., Tang, M., Fu, R., Li, J., & Huang, Q. (2013). Ultra-deep sequencing enables high-fidelity recovery of biodiversity for bulk arthropod samples without PCR amplification. ***GigaScience, 2(1).*** https://doi.org/10.1186/2047-217x-2-4

Summary

The study of animal diets plays a pivotal role in ecology, offering insights into trophic interactions, energy flow, and nutrient cycling within ecosystems. By analyzing what animals eat, researchers can understand species specialization and predator-prey dynamics, all of which are crucial for conservation and ecosystem management. Diet analyses are also vital for managing invasive species, predicting environmental changes, and assessing ecosystem health.

Ecologists use various techniques to analyze animal diets, including non-genetic and genetic methods. Non-genetic approaches, such as visual examination of stomach contents, feces, and field observations, provide direct insights but can be labor-intensive and biased by digestion rates. Genetic approaches, like DNA sequencing of scat or stomach contents, allow precise identification of consumed species, facilitating both qualitative and quantitative dietary assessments.

This thesis delves into DNA-based techniques used to study animal diets, and investigates its strengths and limitations. DNA-based approaches, particularly DNA metabarcoding, have revolutionized dietary studies by offering non-invasive and precise identification of consumed species. However, these advanced methods face challenges like incomplete reference databases, non-standardized protocols and issues with quantitative accuracy. In this thesis I focused on developing standardized protocols for quantitative analysis using digital droplet PCR (ddPCR) to improve the accuracy of rare diet constituent quantification and address contamination and secondary consumption in qualitative genetic dietary assessments, thereby enhancing our understanding of complex dietary interactions in ecological research.

Chapter 1 begins by outlining the diverse applications of diet studies across various ecological research fields. It then delves into the methodologies of diet analysis, categorizing them into genetic and non-genetic approaches. The chapter proceeds to discuss the challenges associated with non-genetic methods, highlighting how genetic (DNA-based) qualitative and quantitative techniques can address these issues. The introduction concludes with a discussion of the current limitations and knowledge gaps in DNA-based diet studies and outlines my research aims to address these challenges.

In Chapter 2, a robust method for quantification of seed DNA in the diet of wood mice was developed. Here, we especially looked into the methodological biases of DNA extraction, PCR inhibition and marker choice (PCR assay and sensitivity) on target DNA quantification in wood mouse feces. Biological biases, as the effects of digestion, age and sex of the wood mouse, and the effect of other diet components in their diet on target DNA quantities were additionally investigated. Furthermore, gut transition times for target DNA were obtained for the wood mouse, observing a gut transition time between 8 and 24 hours for 95% of target DNA. We observed that DNA extraction needed optimization, as standard extraction kits prevented reliable quantification. Additionally, by using droplet digital PCR we prevented PCR inhibition as far as possible. Next, different DNA markers, targeting different parts of the chloroplast, influenced target DNA detectability. However, all markers showed higher target DNA content for higher seed numbers. Although we tried to prevent methodological biases as far as possible, DNA degradation, due to random digestive processes, still complicated robust quantification somewhat as the target DNA quantities showed large variation within mice fed the same number of seeds. However, we did find that biological factors; age, sex and other diet constituents did not alter the digestion patterns and thus quantification. Overall, the newly developed, sensitive DNA-based approach allowed for minimally invasive and robust quantification of small diet constituents in feces, which would otherwise be undetectable with traditional non-genetic methods.

In Chapter 3, the abovementioned method was tested in a realistic field scenario. Biological factors as meal size and digestibility and their effect on target DNA quantity in wood mice feces were further studied here. Additionally, we investigated the possibilities of relating and converting target DNA to actual number of diet items eaten. We observed that increasing meal size also increased the variability of target DNA found in the feces of wood mouse. This further illustrated that, although robust quantification techniques were established, using feces as starting material which is always subject to digestive processes, DNA quantification can only be reliable when sufficient number of feces samples are analyzed to accommodate for random DNA degradation. Doing so, we succeeded to obtain statistically significant differences of target DNA when mice were fed varying seed intake numbers. This enabled the development of calibration curves relating target DNA numbers to seed number fed. Subsequently, these curves were applied in a field trial to quantify seed intake in a wild wood mouse population. We detected seed DNA in the fecal samples of the wood mice caught in the field, which resembled a seed intake of up to 1 seed (although with large confidence intervals due to digestion variation). Nevertheless, this was the first-ever study to quantify seed intake in a realistic field scenario using a DNA-based analysis, showing that accurate seed intake estimates can be obtained given a high enough sample number.

In Chapter 4, the aim was to look at the contribution of small prey species to the diet of lions. Moreover, we discussed the bias that is introduced by non-genetic methods towards larger prey species compared to diet estimation with qualitative DNA metabarcoding. We inferred that the sensitivity of DNA-based diet analysis aids detection of small taxa compared to non-genetic methods, but this sensitivity also means that it will detect species that may have not been eaten by the animal of focus. In our study, we found substantial presence (8% of all prey items) of mesopredator DNA in the fecal samples of lions, which we interpret as over-marking by mesopredators. Furthermore, ecological transfer by scats attracting coprophagous taxa (mostly insects) which in turn attract small predators, such as insectivores, that feast on these prey species additionally scats may attract osteophagous animals. Via saliva or feeding remains or directly by touching the fecal samples, these small predators can transmit their DNA into the scat of lions. We concluded that field contamination (via overmarking, ecological transfer or secondary predation) is likely to happen very often in terrestrial studies of DNA-based carnivore diet and should be accounted for. A combination of DNA and morphologically based methods which are less prone to field contamination (such as microscopic analysis of undigested matter, e.g. hairs) might be complementary in such cases.

In Chapter 5, after taken knowledge of the biases and considerations in previous chapters and applying the lessons learned we steered away from methodological questions towards an applied ecological question. We harnessed the power of being able to analyze large sample sizes using DNA metabarcoding to enable a direct comparison of lion diet composition and prey preference between four different National Parks (NPs) as a means to study the effect of fencing. We found changing lion diet composition and prey preference over a gradient from fully fenced NP's to completely unfenced NP's. Strikingly, the fenced park also showed almost an opposite pattern in terms of prey preferences for specific prey body weights compared to the unfenced NP. The lions in this small and fenced reserve

showed high preference for smaller prey and avoidance for prey weights that are generally preferred in other NPs. Partly fenced NPs generally showed varying diet results, but were nearly always situated within the preference boundaries set by the prey preferences of the fenced and unfenced NP and showed no particular outliers for certain prey species.

Chapter 6 addresses the challenges and opportunities associated with DNA-based analysis for qualitatively and quantitatively assessing animal diets. Here, I explore the interpretation of DNA-derived diet data and highlight the potential of combining high-throughput sequencing and diagnostic PCR techniques to enhance accuracy. Furthermore I emphasize the need to address methodological, biological, and environmental biases to better match the estimated diet with the true diet. Additionally, I discuss the complementarity of genetic and non-genetic methods, concluding that integrating these approaches can improve dietary assessments. Furthermore, the results of this thesis underscores the importance of continued technological advancements and multidisciplinary methods for accurate diet reconstruction and ecological research, stressing the potential of genetic techniques to provide comprehensive insights into trophic interactions and ecosystem dynamics.

Samenvatting

Het bestuderen van diëten van dieren speelt een cruciale rol binnen de ecologie door inzicht te bieden in trofische interacties, energiestromen en nutriëntenkringlopen binnen ecosystemen. Door te analyseren wat dieren eten, kunnen onderzoekers specialisatie van soorten en roofdier-prooi dynamieken begrijpen, wat van groot belang is voor natuurbescherming en ecosysteembeheer. Dieetanalyses zijn ook essentieel voor het beheersen van invasieve soorten, het voorspellen van milieuveranderingen en het beoordelen van de gezondheid van ecosystemen.

Ecologen gebruiken verschillende technieken om diëten van dieren te analyseren, waaronder niet-genetische en genetische methoden. Niet-genetische benaderingen, zoals visuele inspectie van maaginhoud, uitwerpselen en veldobservaties van jacht, bieden directe inzichten maar kunnen arbeidsintensief zijn en worden beïnvloed door verschillen in vertering van bepaalde prooidiersoorten. Genetische benaderingen, zoals DNA-sequencing van uitwerpselen of maaginhoud, maken een nauwkeurige identificatie van geconsumeerde soorten mogelijk, wat zowel kwalitatieve als kwantitatieve dieetstudies vergemakkelijkt. Deze geavanceerde methoden hebben echter te maken met uitdagingen zoals onvolledige referentiedatabases, niet-gestandaardiseerde protocollen en problemen met kwantitatieve nauwkeurigheid.

Deze scriptie heeft als doel enkele van de kritieke uitdagingen die gepaard gaan met DNA-gebaseerde analyses te onderzoeken door middel van kwalitatieve en kwantitatieve beoordeling van het dieet van dieren. Het verkent de rijkdom aan mogelijkheden en uitdagingen die deze innovatieve benadering biedt en biedt een uitgebreide studie naar de voordelen, beperkingen en toepassingen voor ecologisch onderzoek waardoor ons begrip van complexe dieetinteracties in ecologisch onderzoek wordt verbeterd.

Hoofdstuk 1 begint met het schetsen van de diverse toepassingen van dieetstudies in verschillende ecologische onderzoeksvelden. Vervolgens wordt ingegaan op de methoden van dieetanalyses, waarbij ze worden gecategoriseerd in genetische en niet-genetische benaderingen. Het hoofdstuk bespreekt vervolgens de uitdagingen die gepaard gaan met niet-genetische methoden en benadrukt hoe genetische (DNA-gebaseerde) kwalitatieve en kwantitatieve

technieken deze problemen kunnen oplossen. De inleiding sluit af met een bespreking van de huidige beperkingen en kennishiaten in DNA-gebaseerde dieetstudies en schetst mijn onderzoeksdoelen om deze uitdagingen aan te pakken.

In Hoofdstuk 2 werd een robuuste methode ontwikkeld voor de kwantificering van DNA (hierna target DNA genoemd) afkomstig van geconsumeerde zaden in het dieet van bosmuizen. Hier hebben we vooral gekeken naar de methodologische biases van DNA-extractie, PCR-inhibitie en markerkeuze (PCR-assay en gevoeligheid) op de kwantificering van target DNA in de uitwerpselen van bosmuizen. Biologische biases, zoals de effecten van vertering, leeftijd en geslacht van de bosmuis, en het effect van andere dieetcomponenten in hun dieet op de hoeveelheden target DNA werden daarnaast onderzocht. Verder werden de darmpassagetijden voor het target DNA verkregen voor de bosmuis, waarbij een darmpassagetijd tussen 8 en 24 uur werd waargenomen voor 95% van het target DNA. We observeerden daarnaast dat standaard extractiekits betrouwbare kwantificering verhinderden en optimalisatie van de DNA extractie methode noodzakelijk was. PCR inhibitie werd voorkomen door het gebruik van droplet digital PCR, een inhibitie vrije manier van DNA kwantificeren. Verschillende DNA-markers, die op verschillende delen van het chloroplast gericht waren, beïnvloedden de detecteerbaarheid van target DNA. Echter, alle markers toonden een hogere hoeveelheid target DNA voor hogere zaadaantallen. Hoewel we methodologische biases zoveel mogelijk probeerden te voorkomen, bemoeilijkte DNA-afbraak door willekeurige verteringsprocessen een robuuste kwantificering enigszins, aangezien de hoeveelheden target DNA grote variatie vertoonden bij muizen die hetzelfde aantal zaden hadden geconsumeerd. We vonden echter dat biologische factoren zoals leeftijd, geslacht en andere dieetcomponenten de verteringspatronen en dus de kwantificering niet veranderden. Over het algemeen maakte de nieuw ontwikkelde, DNA-gebaseerde benadering een minimaal invasieve en robuuste kwantificering van kleine dieetcomponenten in uitwerpselen mogelijk, die anders niet detecteerbaar zouden zijn met traditionele niet-genetische methoden.

In Hoofdstuk 3 werd de bovengenoemde methode getest in een realistisch veldscenario. Biologische factoren zoals maaltijdgrootte en verteerbaarheid en hun effect op de hoeveelheid target DNA in de uitwerpselen van bosmuizen werden hier verder bestudeerd. Daarnaast onderzochten we de mogelijkheden om de hoeveelheden target DNA in de uitwerpselen te relateren aan het daadwerkelijke aantal geconsumeerde zaden. We observeerden dat een grotere maaltijdgrootte ook de variabiliteit van target DNA in de uitwerpselen van bosmuizen vergrootte. Dit illustreerde verder dat, hoewel robuuste kwantificatietechnieken waren vastgesteld, het gebruik van uitwerpselen als startmateriaal, dat altijd onderhevig is aan verteringsprocessen, alleen betrouwbaar kan zijn wanneer voldoende uitwerpselenmonsters worden geanalyseerd om voor willekeurige DNA-afbraak te corrigeren. Door dit te doen, slaagden we erin statistisch significante verschillen in target DNA te verkrijgen wanneer muizen verschillende zaadaantallen gevoerd kregen. Dit stelde ons in staat kalibratiecurves te ontwikkelen die target DNA hoeveelheden relateren aan het aantal gevoerde zaden. Vervolgens werden deze curven toegepast in een veldproef om zaadinname in een wilde bosmuispopulatie te kwantificeren. We detecteerden zaad DNA in de fecale monsters van de in het veld gevangen bosmuizen, wat een zaad consumptie van maximaal 1 zaad aangaf (hoewel met grote betrouwbaarheidsintervallen). Dit was echter de allereerste studie die zaadinname in een realistisch veldscenario kwantificeerde met behulp van een DNA-gebaseerde analyse, wat aantoonde dat nauwkeurige schattingen van zaadinname kunnen worden verkregen mits een voldoende groot aantal monsters wordt geanalyseerd.

In Hoofdstuk 4 was het doel om te kijken naar de bijdrage van kleine prooisoorten aan het dieet van leeuwen. We concludeerden dat de gevoeligheid van DNA-gebaseerde dieetanalyses helpt bij het detecteren van kleine taxa in vergelijking met niet-genetische methoden, maar dat deze gevoeligheid ook betekent dat het soorten zal detecteren die mogelijk niet zijn gegeten door het dier in kwestie. In onze studie vonden we een substantiële aanwezigheid (8% van het dieet) van mesopredator DNA in de fecale monsters van leeuwen, wat we interpreteerden als overmarkering door mesopredatoren. Daarnaast kan er ook sprake zijn van ecologische overdracht; uitwerpselen trekken insecten aan (coprofagische taxa), deze insecten trekken op hun beurt weer kleine roofdieren aan (zoals insectivoren). Via speeksel of eetrestanten of direct door het aanraken van de fecale monsters kunnen deze kleine roofdieren hun DNA overbrengen op de uitwerpselen van leeuwen. We concludeerden dat veldbesmetting (via overmarkering, ecologische overdracht of secundaire predatie) waarschijnlijk zeer vaak voorkomt in terrestrische studies van DNA-gebaseerde carnivoordiëten en moet voor worden gecorrigeerd in de analyses. Een combinatie van DNA- en morfologisch gebaseerde methoden, die minder gevoelig zijn voor veldbesmetting

(zoals microscopische analyse van onverteerd materiaal, bijvoorbeeld haren), kan complementair zijn in dergelijke gevallen.

In Hoofdstuk 5, na kennis te hebben genomen van de biases en overwegingen in eerdere hoofdstukken en de geleerde lessen toe te passen, stapten we af van methodologische vragen en richtten we ons op een toegepaste ecologische vraag. We maakten gebruik van het vermogen om grote steekproefgroottes te analyseren met DNA-metabarcoding om een directe vergelijking van het dieet en de prooivoorkeur van leeuwen tussen vier verschillende Nationale Parken (NP's) mogelijk te maken om het effect van omheiningen te bestuderen. We vonden veranderende dieetcomposities en prooivoorkeuren van leeuwen over een gradient van volledig omheinde NP's tot volledig niet-omheinde NP's. Opvallend was dat het omheinde park ook een bijna tegenovergesteld patroon vertoonde in termen van prooivoorkeuren voor specifieke prooi lichaamsgewichten in vergelijking met het niet-omheinde NP. De leeuwen in dit kleine en omheinde reservaat toonden een hoge voorkeur voor kleinere prooien en vermeden prooi gewichten die over het algemeen worden geprefereerd in andere NP's. Gedeeltelijk omheinde NP's vertoonden over het algemeen wisselende dieetresultaten, maar bevonden zich bijna altijd binnen de voorkeur grenzen die werden vastgesteld door de prooivoorkeuren van het omheinde en niet-omheinde NP en vertoonden geen bijzondere uitschieters voor bepaalde prooisoorten.

Hoofdstuk 6 behandelt de uitdagingen en kansen die gepaard gaan met DNA-gebaseerde analyses voor kwalitatieve en kwantitatieve beoordeling van diëten van dieren. Hier verken ik de interpretatie van DNA-afgeleide dieetdata en benadruk ik het potentieel van het combineren van high-throughput sequencing en diagnostische PCR-technieken om de nauwkeurigheid te verbeteren. Verder benadruk ik de noodzaak om methodologische, biologische en omgevingsbiases aan te pakken om een betere overeenstemming te bereiken tussen het geschatte dieet en het werkelijke dieet. Bovendien bespreek ik de complementariteit van genetische en niet-genetische methoden, waarbij ik concludeer dat het integreren van deze benaderingen dieetstudies kan verbeteren. De resultaten van deze scriptie onderstrepen verder het belang van voortdurende technologische vooruitgang en multidisciplinaire methoden voor nauwkeurige reconstructie van diëten en ecologisch onderzoek, en benadrukken het potentieel van genetische technieken om uitgebreide inzichten te bieden in trofische interacties en ecosysteem dynamiek.

Acknowledgments

My fantastic journey, through the depths and highs of my Ph.D, has brought me in contact with many interesting, intelligent and rather wonderful people. Here, I will try to thank all of you who made my scientific life more fantastic. If I missed someone I am terribly sorry.

First of all, Krijn Trimbos was not only my supervisor but also my hero. I would say that without you getting my Ph.D would have been far less likely. Thank you for all your patience, encouragement and perseverance. Your guidance has made my journey very enjoyable, and I am still very grateful to have you as my supervisor. Although I started as your first supervised PhD I finished as your second (which probably says enough about my pace) nevertheless I always hoped that I would be your first (sorry about that).

Second, Peter van Bodegom, my promotor, taught me the tools of the trade (or should I say science). Your absolutely spot on feedback when I handed in a new manuscript caused me to be a better scientist every time. I know that sometimes it must have been hard to see me say yes to something new but never finish something old. Well, thank you for believing in me and sitting this one out. I am truly grateful for your guidance and sharing your wisdom and intellect. I hope one day I might find to acquire these great qualities in myself too.

I would like to express my gratitude to the members of the reading committee: Prof. Dr. Ing. M. G. Vijver, Prof. Dr. G. R. de Snoo, Prof. Dr. B. Gravendeel, Dr. M. P. Veldhuis, and Dr. R. Brys. Thank you for taking the time to review my work and provide invaluable feedback, which has significantly improved the quality of this dissertation. My gratitude also extends to the members of the opposition committee for their contributions.

A huge thank you goes out to Hans de Iongh, I always called him my (senior) advisor. But you sometimes felt a little bit like family. You are a great person with so much joy in life and a heart for conservation. Your amazing spirit made not only my time in Kenia very memorable but the whole of my Ph.D. I am very grateful for all your advice, great networking skills and (super) adventurous stories. It is and was my pleasure to work with you.

Within CML there have been many great colleagues and I would like to thank all of you who contributed to the nice atmosphere at the office. Especially I would like to extend my gratitude towards my former colleagues Leon Hauser and Ricardo Mancinelli. I had a wonderful time with you guys. And I do miss you at CML. I really enjoyed my time giving GIS workshops with Maarten van ‘t Zelfde. The fieldwork trips to Schiermonnikoog and the Polderlab with Maarten Schrama and Marco Roos were always great fun. I hope to join many more years to come. The secretariat at the CML, thank you, you are the best! I would also like to thank Jan Boersema for our little thought-provoking conversations at the coffee machine. Keep doing that. My sincere gratitude goes out to Emilie Didaskalou and Kornelia Serwatowska for managing the CML DNA labs so well.

Next, I would like to thank my collaborators at JKI; Ralf Schlieper, Engelbert Kampling, Hendrik Ennen, Diana Below, Mechthild Budde, Emilia Wojcik, and last but certainly not least Jens Jacob. I would also like to express my profound gratitude to Amy Montanje, Sam Boerlijst, Iris Noordermeer, Dionne Jacobs, Megan Verhagen, Josine Meijer, Mateo Bal, Gert-Jan Goeminne, and the late Kennedy Ole Kariuki (rest in peace) for their help with data collection for the lion diet chapters. Here, also of extremely great help were our skillful drivers Julius Saiyianka Kiooi Shonko, Michael Letaloi, and Kasaine Sompet without whom no data could have been collected. I would like to thank the staff at Kenya Wildlife Service; K. Nashuu, C. Mwinzi, Alice Bett, Catherine Wambani, Geoffrey Bundotich, Peter Gitonga Njeru and Meshak K. A very special thank you goes out to Luka Narisha, Monica Chege and Dr. Francis Lesilau for which no research in Kenia could have been conducted without them. A very very warm thank you!

Als laatste bedank ik graag mijn familie, Aad, Corrie, Nik, Sylvia, Viv én natuurlijk mijn tweelingbroer Marvin. Ook al heeft het merendeel waarschijnlijk geen flauw idee wat ik onderzoek, bedankt voor jullie onvoorwaardelijke steun. Ik bof met jullie! Suusie Heesbeen ook al werkte ik op onmogelijke tijden en uren om het allemaal voor mekaar te krijgen. You were always there to support me. Thanks for being so great.

Curriculum Vitae

Kevin Groen was born on March 8, 1991, in Schiedam, The Netherlands. He has established himself as an environmental biologist with a strong focus on molecular ecology. He is currently a postdoctoral researcher at the Institute of Environmental Sciences (CML) at Leiden University, where he explores the use of DNA-based methods to assess human impacts on biodiversity. His research includes leading projects on the diet composition of wolves recolonizing the Netherlands and contributing to studies on plant-pollinator networks, as well as the diet of carps and bats through DNA analysis and assessing fish diversity through environmental DNA (eDNA) analysis.

Kevin started his Ph.D. in Environmental Biology at the same institute in 2017. His doctoral research was centered on using DNA analysis to quantify seed intake for improving pesticide risk assessment in small mammals and investigating the effects of fencing on lion diets in Kenyan national parks. He holds a Master's degree in Biology with a specialization in Evolution, Biodiversity & Conservation from Leiden University (obtained in December 2016), where he conducted research in both the Western Himalayas and the Netherlands, focusing on bird diversity, macroinvertebrate abundance, and landscape ecology. He also holds a Bachelor's degree in Life Science & Technology from Delft University of Technology and Leiden University (obtained in September 2013).

Beyond his academic pursuits, Kevin is actively involved in environmental conservation through his role as a board member of the Leo Foundation, which focuses on conserving large carnivores and volunteering at a bird rehabilitation center. He has also been involved in science communication through various roles, including as a TV presenter for BNNVARA and founder of 'Groen Voorziening', an initiative promoting nature education. His work has been recognized with several awards, including the Stans Prize for best student thesis and outreach, and he has secured grants from organizations such as National Geographic and the KNAW Academy. He has also authored a book, "The Wild Wild East," which explores East-European nature areas.

List of publications

Peer-reviewed

Groen, K., Jacob, J., Hein, S., Didaskalou, E. A., Van Bodegom, P. M., Hahne, J., & Trimbos, K. B. (2023). DNA-based seed intake quantification for enhanced ecological risk assessment of small mammals. *Ecotoxicology and Environmental Safety*, *259*, 115036. https://doi.org/10.1016/j.ecoenv.2023.115036

Groen, K., Beekenkamp, S., De Iongh, H. H., Lesilau, F., Chege, M., Narisha, L., Veldhuis, M., Bertola, L. D., Van Bodegom, P. M., & Trimbos, K. B. (2023). DNA metabarcoding illuminates the contribution of small and very small prey taxa to the diet of lions. *Environmental DNA*, *5*(6), 1321–1331. https://doi.org/10.1002/edn3.457

Groen, K., Trimbos, K. B., Hein, S., Blaauw, A. I., Van Bodegom, P. M., Hahne, J., & Jacob, J. (2022). Establishment of a faecal DNA quantification technique for rare and cryptic diet constituents in small mammals. *Molecular Ecology Resources*, *22*(6), 2220–2231. https://doi.org/10.1111/1755-0998.13609

Miller, J. A., Schilthuizen, M., Burmester, J. L., Van Der Graaf, L., Merckx, V., Jocqué, M., Kessler, P., Fayle, T. M., Breeschoten, T., Broeren, R., Bouman, R. W., Chua, W. J., Feijen, F., Fermont, T., **Groen, K.**, Groen, M., Kil, N. J. C., De Laat, H. A., Moerland, M. S., . . . Goossens, B. (2014). Dispatch from the field: ecology of ground-web-building spiders with description of a new species (Araneae, Symphytognathidae). *Biodiversity Data Journal*, *2*, e1076. https://doi.org/10.3897/bdj.2.e1076

Outreach

Groen, K. (24 december 2022). Een gezonde honger naar kennis. door Myrthe Prins. *National Geographic Magazine.*

Groen, K. & Groen M. (2022). The Wild Wild East. Uitgeverij Fjord. ISBN 9789083014890.

Groen, K. & Trimbos, K. B. (2022). Alle muizenkeutels verzamelen. *Mare*.

Groen, K. & Groen, M. (22 juni 2020). Gewapend met stoepkrijt willen broers Marvin en Kevin zorgen dat we 'onkruid' meer waarderen. *EenVandaag (NPO | AVROTROS)*. [interview].

Groen, K. (16 september 2017). 'Groene tweeling' krijgt eigen tv-show over stadsnatuur. *Algemeen Dagblad*. [interview].