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

Groen, K.

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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).

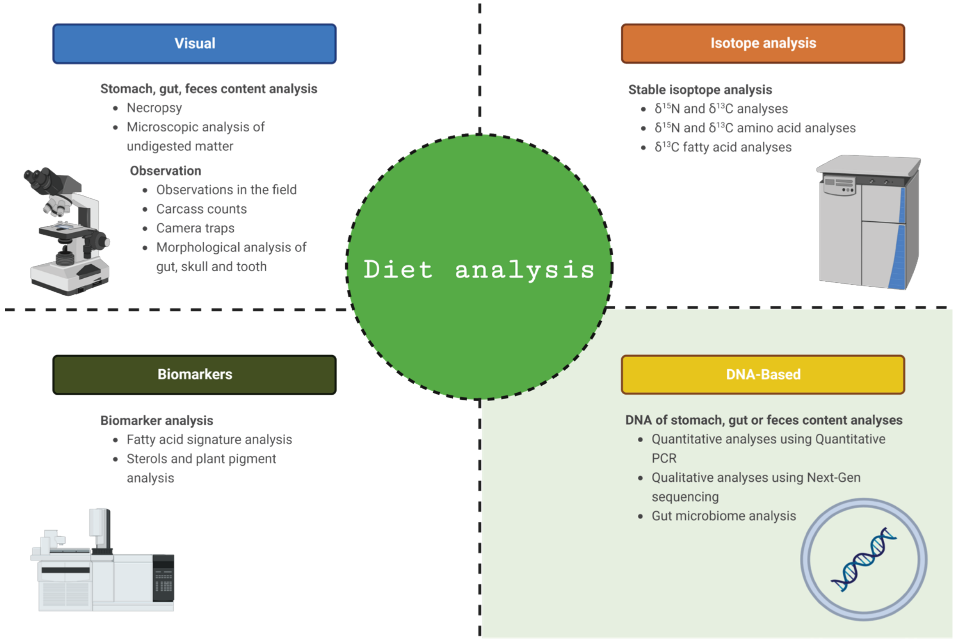

Figure 1.1 | Conceptual overview of common non-genetic and genetic techniques (shaded green) used in animal diet analysis.

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 δ^{13}C or δ^{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.

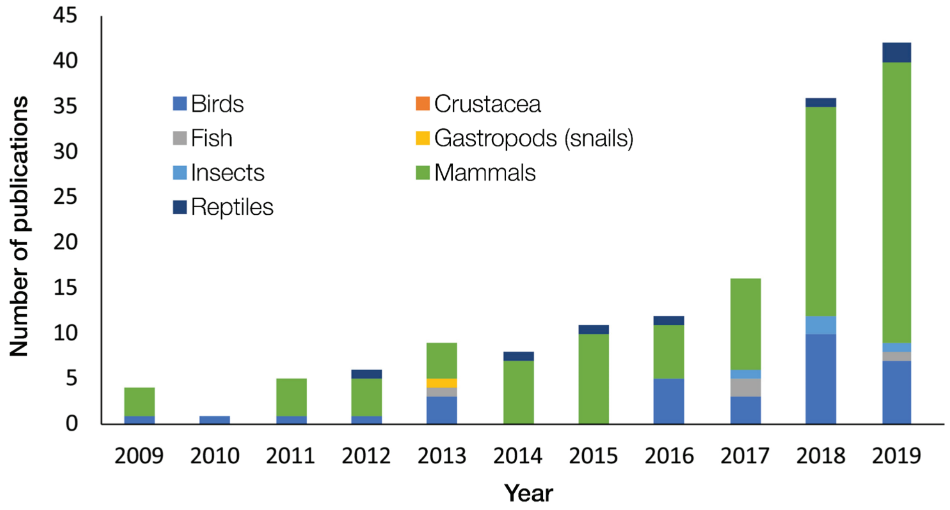

Figure 1.2 | The bar chart depicts the yearly count of original articles utilizing DNA metabarcoding of fecal samples for dietary assessment published in international journals up to 2019. A similar trend is expected for 2020 and onwards. Each distinct color on the chart corresponds to a specific target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

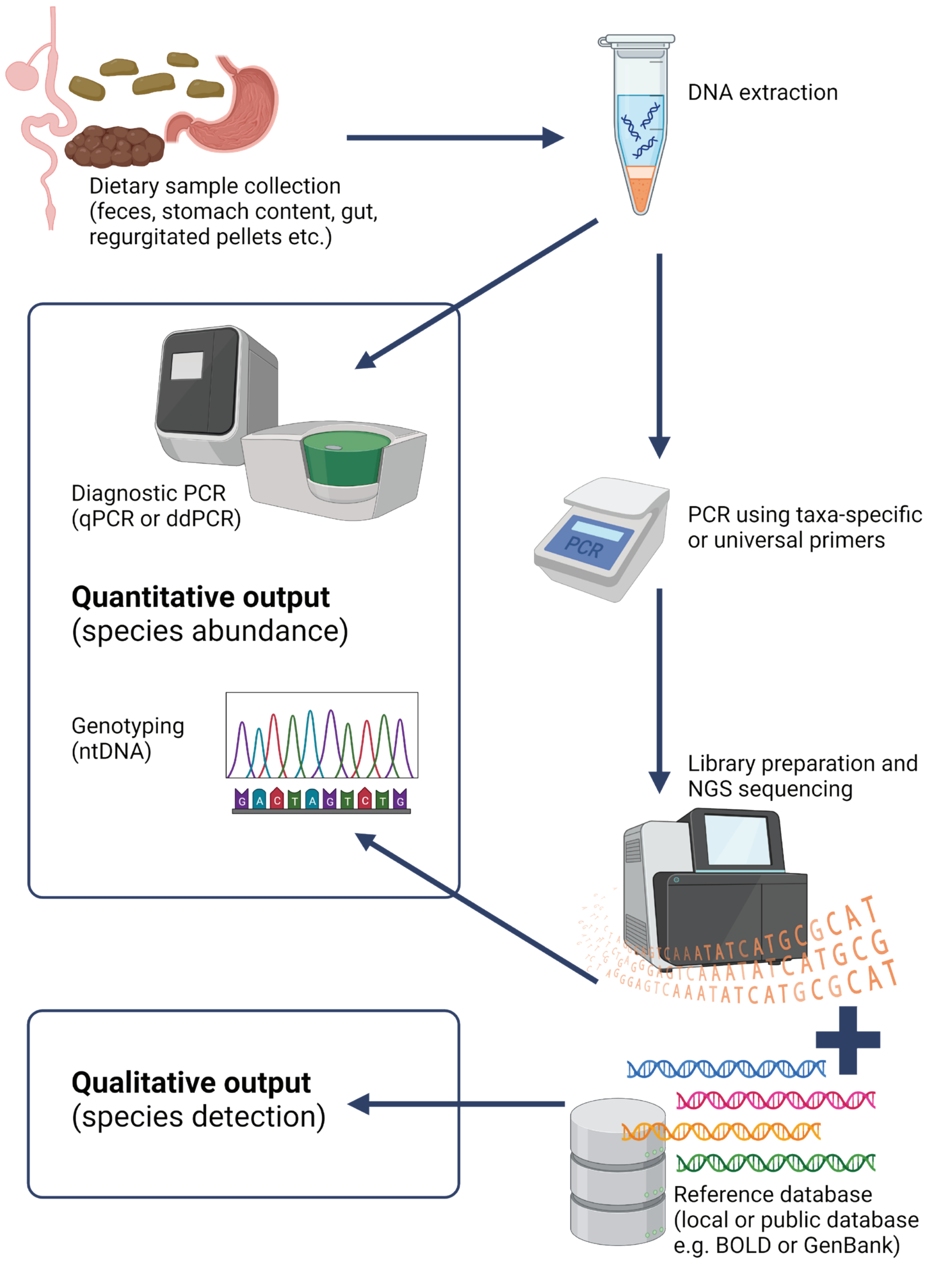

Figure 1.3 | A conceptual framework of DNA-based diet analysis using quantitative PCR (qPCR) and droplet digital PCR (ddPCR) or Next Generation Sequencing (NGS).

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.