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

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

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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 km²), 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 km², 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 km², 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 km², 0°18' - 0°30' S; 36°2' - 36°9' E) which is located in the Rift Valley Province, like ANP (Kassily 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; TCCACCTCCTGTTCTCCAT and reverse primer; AGGATTGGCGGGGTATAGT). 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

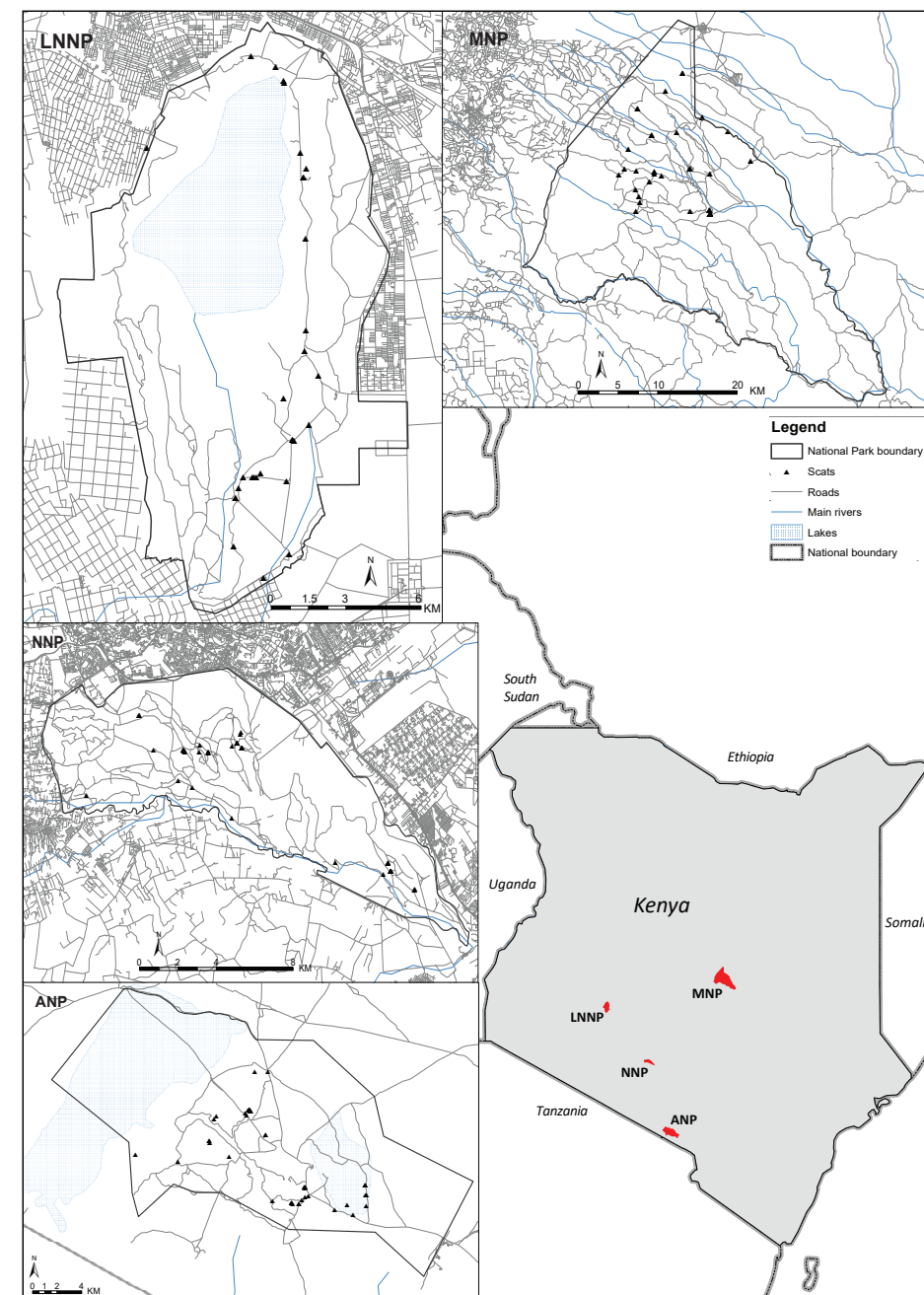


Figure 4.1 | Maps of the study sites where scat samples were collected: Amboseli National Park (ANP), Lake Nakuru National Park (LNNP), Meru National Park (MNP), and Nairobi National Park (NNP). Locations for the scat collection sites are indicated with black triangles. The map on the bottom right corner 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 $\geq 1\%$ 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.

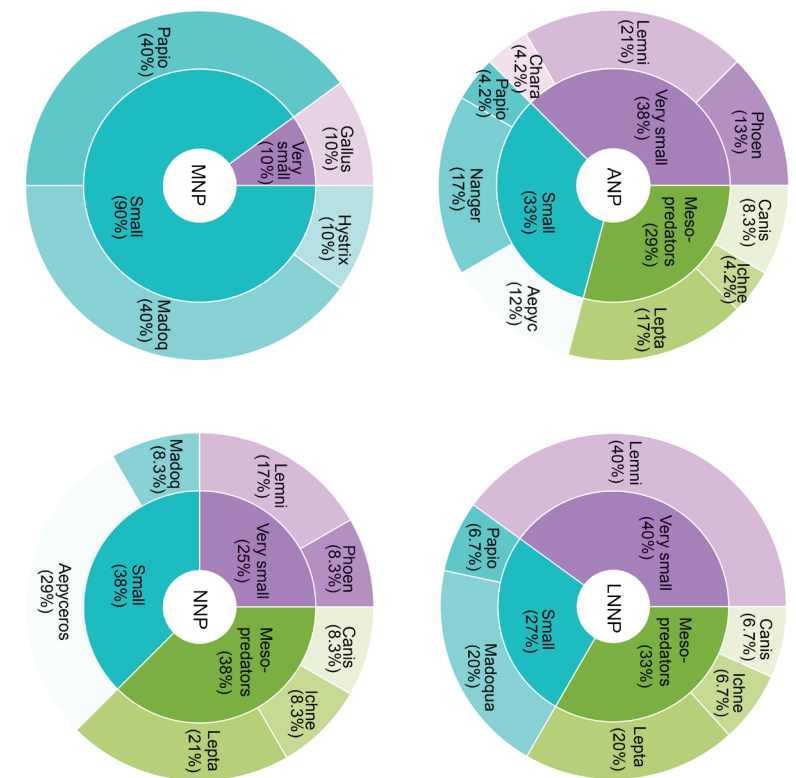
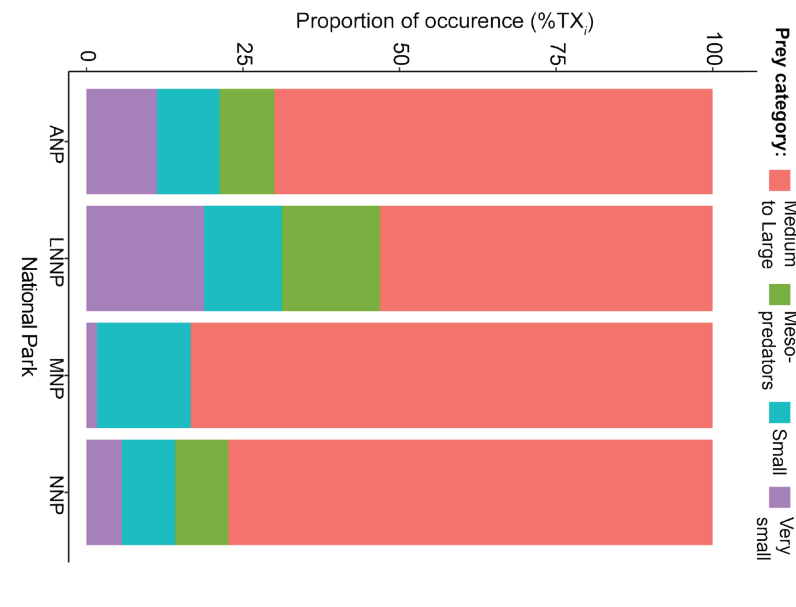


Figure 4.3 | Proportions of occurrences (%TX) of the four prey categories in the diet of the lion given for four National Parks (NPs) in Kenya on the left. On the right, small, very small and mesopredator proportions of occurrences (%TX) are further specified per taxa for each NP. Total occurrences in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

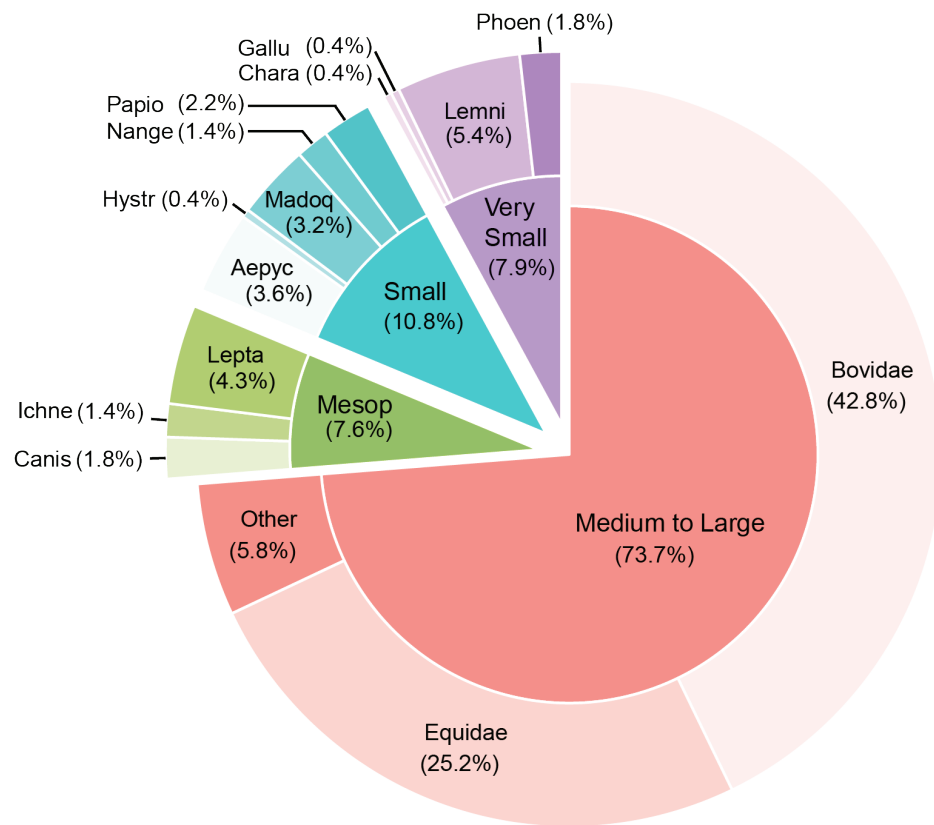


Figure 4.2 | Proportions of occurrences (%TX) of the four prey categories in the diet of the lion in four National Parks (NPs) in Kenya. Prey taxa identified and their contribution in percentages are given, and the diet contribution of medium to large, small, very small taxa are depicted, as well as 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* (Hyst), *Ichneumia* (Ichne), *Lemniscomys* (Lemni), *Leptailurus* (Lepta), *Madoqua* (Madoq), *Nanger* (Nange) and *Phoenicopterus roseus* (Phoen).

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 (Ferrerias & 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>.

