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

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

Published in:
Environmental DNA

DOI:
10.1002/edn3.457

Publication date:
2023

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
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 (Atkins et al., 2019; Vogel et al., 2018), a comprehensive insight into apex predator diets is of utmost importance to designing appropriate conservation and management strategies to preserve them (van der Heyde et al., 2021; Xiong et al., 2017).
Lion (Panthera leo), an apex predator, prefers prey species within the weight range 96–632 kg—for example, gemsbok (Oryx gazella), African buffalo (Syncerus caffer), blue wildebeest (Connochaetes taurinus), giraffe (Giraffa camelopardalis), and plains zebra (Equus quagga)—with an optimum prey weight around 350 kg (Clements et al., 2014; Hayward & Kerley, 2005). 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 (Barnardo et al., 2020; Carbone et al., 1999). 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; Lesilau, 2019; Sogbohossou et al., 2011) 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)), which regularly (68.7%) resulted in a hunt (Hayward & Kerley, 2005). Encounter rates increase when small prey occur in high densities, reducing the costs of actively pursuing small prey (Clements 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 (Hayward & Kerley, 2005; Power, 2002). Diet composition estimates based on carcass counts include a bias toward large 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 (Rubmark 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 (Beng & Corlett, 2020; Forin-Wiart et al., 2018; Lesilau, 2019; Shehzad et al., 2012; Symondson, 2002; Thuo et al., 2019; Xiong et al., 2017). 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 toward 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.

2 | MATERIALS AND METHODS

2.1 | 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 1, see Appendix S1 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′ to 0°10′ S; 38°0′ to 38°25′ E) (Sitienei et al., 2014). Vegetation and ecosystem types within MNP predominantly consist of thorny Acacia-Commiphora bushland and Acacia wood 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°32′ to 02°44′ S; 37°04′ to 37°25′ E), a non-fenced savannah grassland with seasonal flooding (Okello et al., 2008). Lions here have the opportunity to disperse to other reserves and 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′ to 01°26′ S; 36°50′ to 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′ to 0°30′ S; 36°2′ to 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 (Kassilly 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 (Elliot et al., 2020; Lindsey et al., 2017).

2.2 | Sample collection and DNA extraction

Lion scat samples were collected between the February 4 and the April 16, 2019 (Figure 1). Scat samples were collected opportunistically along transects during patrols thrice a week. Stuart and 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; and 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, and 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.

2.3 | 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 (P. leo)-specific primers (specificity checks were done in silico) that targeted the cytochrome b gene (5′→3′: forward primer: TCCAC CTCTTGTTCCCTCAT and reverse primer: AGGATGGCGGGGGG TATAGT). The PCR reaction (25 μL) consisted of 1 μL DNA extract and 16.75 μL Milli-Q 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 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 vertebrates taxa can be found in Appendix S1. Since lions tend to eat only once a day, 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., 2013). 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), 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 C. aperea f. capensis, 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.) at the NGS facility of BaseClear, Leiden, the Netherlands.

2.4 | 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 (Branch, 2014; Stevenson & Fanshawe, 2002; Stuart & Stuart, 2013). Fish were not taken into account as, to our knowledge,
there is not any scientific literature suggesting active hunting behavior of lions toward fish. Bird sequences were selected at family level instead of genus level, since the identification of multiple taxa is needed to increase the taxonomic identification. In total, 194 sequences (Appendix S1) 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).

2.5 | 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 using FLASH (Magoc & Salzberg, 2011) with a minimum overlap of 10bp and a maximum overlap of 300bp, 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 240–260bp 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 amounts of contamination is inevitable with NGS technologies, especially when using universal primers, despite strict conformance to good laboratory practices for minimizing contamination risks (De Barba et al., 2014; Pompanon et al., 2012), we used the reads in the control samples (POS, NTC, and NEG) to filter out any contamination by subtracting the number of sequences 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.

2.6 | 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 (5–50kg) and very small (<5kg) 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 S1): medium-to-large prey taxa (>50kg), small prey taxa (5–50kg), and very small prey taxa (<5kg). No distinction was made between large- (>200kg) and medium-sized (50–200kg) 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 of 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 Di Bernardi et al., 2021; Lesilau, 2019; Wikenros et al., 2017). 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 S2 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 taxa) within a fecal sample was counted as an occurrence, where the 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, 2020) and statistical calculations were done using the FSA package (Ogle et al., 2021) in R (R Core Team, 2021).

3 | 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, 95% CI [38,749–46,051] reads for samples collected in LNNP; 45,790, 95% CI [40,264–51,314] reads for samples collected in MNP; and 47,346, 95% 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, 95% CI [9815–12,734] for LNNP; 11,488, 95% CI [10,477–12,498] for MNP; and 14,469, 95% CI [13,559–15,378] reads for NNP (see Appendix S2 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 Section 2), these reads were assigned to 2 families, 22 genera, and 1 species (Figure S1). Controls (POS, NTC, and NEG) contained little contamination (Table S1). 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 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%, and 1% of total occurrences, respectively.

Differences in the lion diet composition among ANP, LNNP, MNP, and NNP were found (Figure 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.

**FIGURE 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, and very small taxa are depicted, as well as the 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 seven 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).
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 S2, \(H=37.1, N=171\), \(p<0.0005\)). 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 (\(Phoenicopterus roseus\), \(Charadrius\), \(Gallus\)) as part of the medium- to- large prey taxa. The other 9% of the lion diet in ANP was contributed to three mesopredator genera, with the highest occurrence of \(Leptailurus\). In NNP, 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.

### DISCUSSION

This study specifically addressed the role of small (5–50 kg) and very small (<5 kg) prey in the diet of lions across multiple Kenyan NPs without a bias toward larger prey species. We found 24 prey taxa and 278 prey occurrences in 171 fecal samples. 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% (of...
all prey occurrences) to the diet of lions in Kenya. A total of 7.6% of all occurrences were contributed to mesopredator taxa.

4.1 | 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%–9%. However, the primers (12SV5F/12SV5R) used in the research of Lesilau (2019) are known to be biased toward amplifying only mammal DNA which explains the lack of birds in his dataset (Riaz et al., 2011; Shehzad 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 (Barnardo et al., 2020; Davidson et al., 2013; Lehmann et al., 2008), meaning that lion predation is likely more robust compared to Lesilau’s (2019) study as we used his dataset (Riaz et al., 2011).

4.2 | 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 et al. (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.

4.3 | 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)
state 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 1–3 species per sample (Figure S2). Smaller prey probably constitutes 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 Groen et al., 2022). 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? However, Carbone et al. (1999) and Owen-Smith and 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.

AUTHOR CONTRIBUTIONS
KG, HI, FL, MC, LN, PMB, and KBT designed the study. KG, SB, HI, FL, MC, and LN performed the field research and collected data. LDB advised on genetic approaches and KG performed the genetic analyses. KG and SB performed the bioinformatic analyses. KG, SB, MV, PMB, and KBT interpreted the results. KG and SB wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

ACKNOWLEDGMENTS
We would like to express our profound gratitude to Amy Montanje, Sam Boerlijst, Nir Noordermeer, Dionne Jacobs, Megan Verhagen, Josine Meijer, Mateo Bal, Gert-Jan Goeminne, and Kennedy Ole Kariuki for their help with data collection in the field and the laboratory of KWS and Leiden University. Of extremely great help were our skillful drivers Julius Saiyianka Kiooi Shonko, Michael Letaloi, and Kasaine Sompot without whom no data could have been collected. We would like to thank the Senior Warden K. Nashuu and senior scientist C. Mwini of KWS Amboseli NP for receiving us and letting us use the staff compound and aiding us with our research during our stay. Special thanks go to Alice Bett and Catherine Wambani of KWS for their guidance and cooperation in the fieldwork in Lake Nakuru National Park. We also want to thank the local scientist in Meru National Park, Geoffrey Bundotch, for helping with the data collection and letting us stay in his house during our fieldwork, and Peter Gitonga Njeru and Meshak K. for being our guides in Meru National Park. This study was funded by the Ecology Fund of the Royal Netherlands Academy of Arts and Sciences and the National Geographic Society (grant number EC-51438R-18) to Kevin Groen.

CONFLICT OF INTEREST STATEMENT
The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT
The raw output of the NGS run is available at Dryad: doi:10.5061/dryad.zs7h44jfm.

ORCID
Kevin Groen https://orcid.org/0000-0001-6049-6325
Michiel Veldhuis https://orcid.org/0000-0001-5940-039X
Peter M. van Bodegom https://orcid.org/0000-0003-0771-4500
Krijn B. Trimbos https://orcid.org/0000-0001-5280-6434

REFERENCES


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


SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.