

ORIGINAL ARTICLE

DNA metabarcoding reveals that African leopard diet varies between habitats

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Abstract

Understanding carnivores' diet is key to understanding their adaptability in a rapidly changing world. However, studying diet of large carnivores is difficult due to their elusive nature. In this study, we performed DNA metabarcoding analyses of 82 putative leopard scats collected from two distinct, but connected, habitat types (rainforest and grassland) in the Udzungwa Mountains, Tanzania. Two mitochondrial markers were used to identify predator and prey. Metabarcoding confirmed that 60 of the collected scats (73%) originated from leopards, and nineteen mammalian prey DNA sequences were identified to species. Using prey size correction factors for leopards, and covariates on habitat type and prey ecology, we investigated whether differences in leopard dietary composition were detectable between habitats. We found that leopards in grassland consumed a larger mean prey size compared with leopards in rainforest. Small prey (<19 kg) constituted >70% of the biomass consumed by leopards in rainforest, while large prey (≥80 kg) were only eaten in grassland. Arboreal species constituted 50% of the biomass consumed by rainforest leopards. Our results highlight the importance of arboreal species in their diet. From a management perspective, we suggest continued protection of all prey species in the protected areas to prevent human–wildlife conflicts.

Keywords

Eastern Arc Mountains, large carnivore, scat analyses, Tanzania, Udzungwa

Résumé

La compréhension du régime alimentaire des carnivores est essentielle pour comprendre leur adaptabilité dans un monde en évolution rapide. Cependant, l'étude du régime alimentaire des grands carnivores est difficile en raison de leur nature insaisissable. Dans cette étude, nous avons effectué des analyses basées sur le métabarcodage de l'ADN de 82 excréments de léopards présumés, collectés dans deux types d'habitats distincts mais liés (forêt tropicale et prairie) au sein des montagnes Udzungwa, en Tanzanie. Deux marqueurs mitochondriaux ont été utilisés pour identifier les prédateurs et les proies. Le métabarcodage a confirmé que 60 des excréments collectés (73%) provenaient de léopards, et dix-neuf séquences d'ADN de proies de mammifères ont été identifiées à l'espèce. En utilisant des facteurs de correction de la taille des proies pour les léopards et des covariables sur le type d'habitat et

l'écologie des proies, nous avons tenté de déterminer si les différences dans la composition du régime alimentaire des léopards étaient détectables entre les différents habitats. Nous avons constaté que les léopards des prairies consommaient des proies dont la taille moyenne était plus importante que celle des proies consommées par les léopards de la forêt tropicale. Les petites proies (<19 kg) constituaient plus de 70% de la biomasse consommée par les léopards de la forêt tropicale, tandis que les grandes proies (≥ 80 kg) étaient uniquement consommées dans les prairies. Les espèces arboricoles constituaient 50% de la biomasse consommée par les léopards de la forêt tropicale. Nos résultats mettent en évidence l'importance des espèces arboricoles dans leur régime alimentaire. En termes de gestion, nous suggérons de continuer à protéger toutes les espèces de proies dans les zones protégées afin de prévenir les conflits entre les hommes et la faune.

1 | INTRODUCTION

Apex predators are on top of the food chain and have been found to control prey communities with cascading effects in the environment where they occur (Atkins et al., 2019; Ripple et al., 2014). Information of the dietary composition of carnivores is important in order to understand their life-history strategies (Miquelle et al., 1996) and how they adapt in a rapidly changing world. However, documenting carnivore diet can be difficult due to their elusive nature and because it is difficult to observe feeding events in the dense habitats where they often reside (Nilsen et al., 2012). Therefore, information on carnivore diet obtained through observations of feeding events is often lacking (Lumetsberger et al., 2017). Morphological identification of remains of prey in scats has offered an attractive alternative to visual observations (Grobler & Wilson, 1972; Hart et al., 1996; Henschel et al., 2005, 2011). The latest advances in dietary studies of elusive species, reviewed in Alberdi et al. (2019), highlight the use of metabarcoding of DNA extracted from scats to identify predator and prey. Doing so, DNA metabarcoding has been used to validate the consumer identity (Shehzad et al., 2015; Taberlet et al., 2012) and to detect prey taxa often missed by classical morphological analysis of undigested prey remains (Berry et al., 2017).

Leopards (*Panthera pardus*) have been recorded to prey on approximately 200 species of vertebrates (Hunter et al., 2013), 110 of which are mammals (Hayward et al., 2006; Shehzad et al., 2015). Leopards seem to prefer prey of a small to medium body size (10–40 kg) (Hayward et al., 2006), which can minimise kleptoparasitism and competition with other large carnivores, such as lions (*P. leo*), tigers (*P. tigris*) and spotted hyaenas (*Crocuta crocuta*) (Hayward & Kerley, 2008; Karanth et al., 2004; Karanth & Sunquist, 1995). However, a recent study that developed prey size correction factors for captive leopards found that the proportion of large-bodied mammals has generally been underestimated (Lumetsberger et al., 2017). These prey size correction factors have so far only been used in one study of a wild leopard population in Iran where they proved to be important in understanding dietary preference and used for conservation planning (Ghoddousi et al., 2017).

Most leopard diet studies have been conducted within a particular landscape type such as rainforest (Hart et al., 1996; Henschel et al., 2005, 2011; Sidhu et al., 2015), mountains (Martins et al., 2011; Norton et al., 1986; Rautenbach, 2010; Rodel et al., 2004; Stuart & Stuart, 1993; Taghdisi et al., 2013), anthropogenic landscapes (Athreya et al., 2016), savannah grasslands (Balme et al., 2017; Kissui, 2008) or arid/semi-arid landscapes (Bothma & Le Riche, 1994; Mondal et al., 2011, 2012; Voigt et al., 2018). Leopard diets differ substantially between the studies as these distinct habitats have different arrays of prey species. Only in one study has leopard diet been studied comparatively in a mixed land-use area (Mann et al., 2020). At the fine scale, in savannah habitat leopards seemingly prefer to hunt in ecotones between habitats with dense and open cover (Balme et al., 2007) and recent studies have found substantial individual dietary niche specialisations (Balme et al., 2020; Voigt et al., 2018). However, little is known about differences in leopard diet in a landscape with large-scale habitat differences (e.g., rainforest and grassland), where they could move unhindered from one habitat to the other.

Loss of habitat and habitat fragmentation has been identified as one of the most important factors in species decline (Tilman et al., 2017). For the majority of leopard subspecies, population isolation that follows from loss of habitat and habitat fragmentation is a main threat to their continued existence (Jacobson et al., 2016). However, leopards in India have adapted to loss and fragmentation of their natural habitats and have adapted to living in anthropogenic landscapes (Athreya et al., 2013, 2016; Kshetry et al., 2017). This phenomenon is less known and poorly understood in Africa (Kuhn, 2014), and due to habitat loss and fragmentation, leopards have disappeared from more than one-third of their historical geographical range (Jacobson et al., 2016).

The Udzungwa Mountains is a natural mosaic landscape, which contains the largest continuous blocks of Afrotropical rainforests intersected by grass-dominated habitats in East Africa (Rovero et al., 2009). The rainforests in Udzungwa have lower occupancies of large herbivores than the grasslands, and the assemblage of medium-to-large mammals is smaller-bodied compared to the grasslands

(Cavada et al. (2019). Oppositely, the rainforests holds large number of arboreal primates that do not occur in the intersecting grasslands (Araldi et al., 2014; Barelli et al., 2015; Cavada et al., 2016). The Udzungwa Mountains have historically experienced limited anthropogenic pressure due to its remoteness and rugged terrain (Rovero et al., 2009). Although human population density and agriculture have recently intensified at its surroundings, the area still holds a relatively intact fauna and flora of the region.

Leopards are the most abundant apex predator in the area, a mean of 4.22 leopards/100 km² and no significant difference in densities between habitat types (Havmøller et al., 2019). The second most abundant species is the spotted hyaena (*Crocuta crocuta*) yet only half the number of individuals were identified across the same landscape (Havmøller et al., 2019). Leopards thus have little competition from other large carnivores across the Udzungwa landscape and therefore represent an opportunity to profile a baseline for leopard diet in one of the few naturally complex landscapes in Tanzania.

Dietary baselines for leopards living in natural habitats are necessary in order to understand how they may cope with future

human population growth and habitat fragmentation. In this study, we aim to assess how leopard diet differs between rainforest and grassland habitats in Udzungwa. Because prey assemblages in Udzungwa are different, we predict that the diet of leopards in rainforest would include a higher proportion of small-bodied and arboreal prey, compared to the diet of leopard in grasslands. To assess the differences, we studied leopard diet in relation to prey ecology, body size and biomass consumed. To achieve this, we collected scats from the Udzungwa Mountains National Park (UMNP) and the adjoining Kilombero Nature Reserve (KNR) and determined as well as validated the taxonomic identity of predator and prey using DNA metabarcoding of two mitochondrial markers. We then use prey size correction factors to estimate biomass and number of individuals consumed of each prey taxa as well as prey species ecology (arboreal/terrestrial). We used these three variables to assess whether composition of diet taxa differed between rainforest and grassland habitats. To our knowledge, our assessment is the first to use DNA metabarcoding of two mitochondrial markers in combination with size correction factors to characterise the diet of any large carnivore.

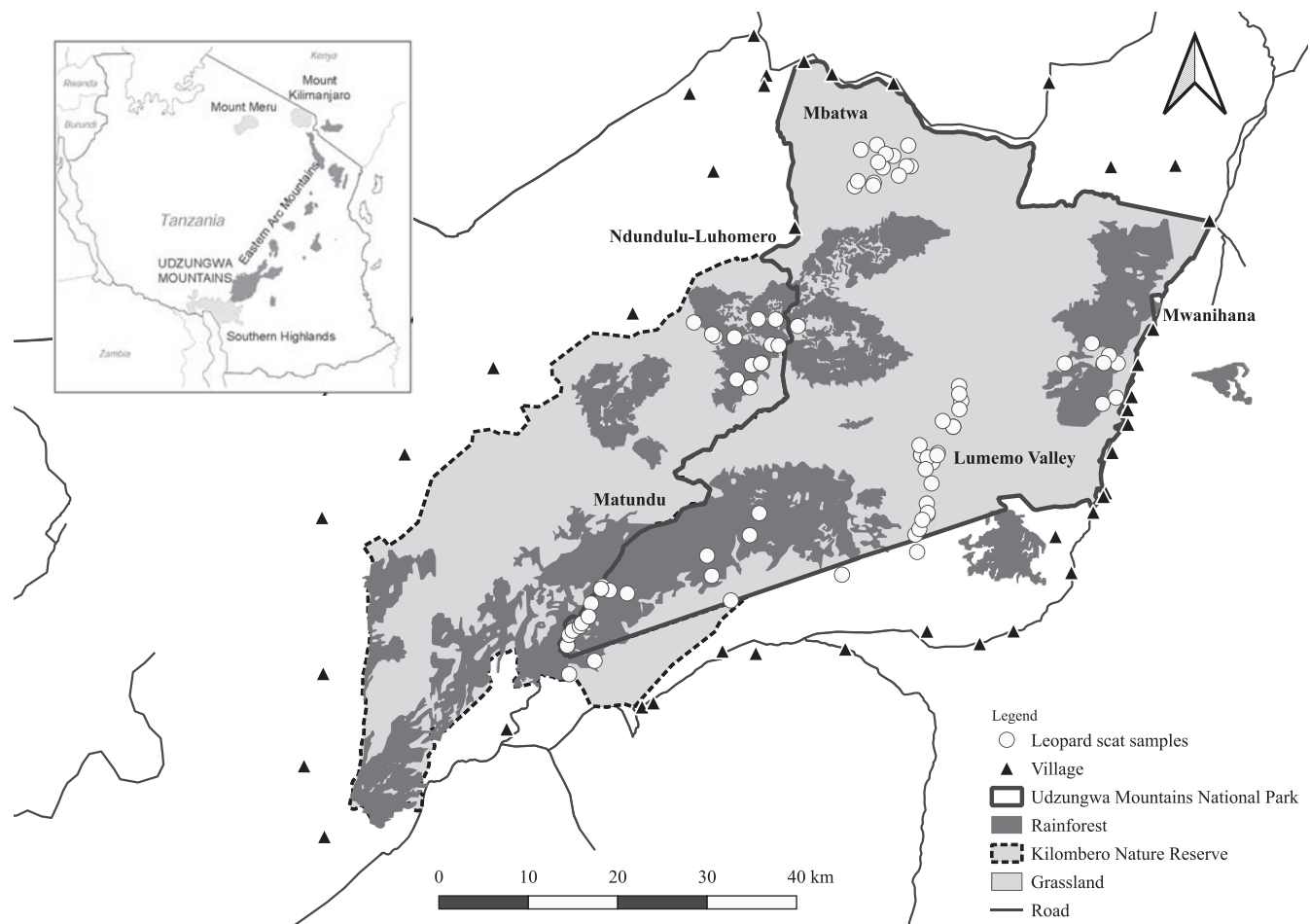


FIGURE 1 Locations of leopard scat samples collected in different habitat types of the Udzungwa Mountains. Note that open grassland habitats (Mbatwa and Lumemo valley) are only coloured light grey, whereas the rainforest habitats (Matundu, Mwanihana and Ndundulu-Luhomero) are coloured dark grey)

2 | MATERIAL AND METHODS

Leopard scats were collected opportunistically between September 2013 and December 2014 in five study sites in the Udzungwa Mountains, Tanzania: Matundu, Mbatwa, Lumemo Valley, Mwanihana and Ndundulu-Luhomero. The Udzungwa Mountains are surrounded by pastoral and agricultural development but have virtually no infrastructure in the form of roads or lodges within it and can only be explored by foot (Figure 1). The rainforest blocks in Udzungwa are intersected by open grass-dominated habitats with little canopy cover, such as *Brachystegia* woodland, high-altitude grassland, alpine heathland and *Acacia-Commiphora* woodland (Rovero & De Luca, 2007) (Figure 1). The UMNP (1990 km²) and connecting KNR (1345 km²) are recorded to hold some 118 species of mammals, including thirteen near-endemic and five endemic species (Rovero & De Luca, 2007). All sample locations were categorised as either rainforest (closed-canopy evergreen forest) or grasslands (little or no canopy and interspersed deciduous trees). Morphological criteria for selection of scats as potential leopard scats included visual evidence of a high content of hair and bone, as well as sizes of >10 cm in length and >20 mm in circumference (Hunter et al., 2013). Scats with very little bone and hair content and white appearance were ignored as they were presumed to originate from spotted hyaenas (Hofer & East, 2013). No other large carnivores than leopards and spotted hyaenas are considered resident in UMNP (Cavada et al., 2019). Scats were kept in 96% ethanol for twelve hours before the ethanol was removed and the sample desiccated with sterile silica gel (Sigma-Aldrich) and stored frozen at -18°C (Nsubuga et al., 2004). DNA was extracted from ~200 mg of central material from each scat sample. Since DNA from scats is often degraded, especially when collected in moist tropical regions (Brinkman et al., 2011), we attempted to improve prey and predator species identification through DNA metabarcoding using two mitochondrial markers of which one was relatively short. Specifically, two primer sets were used: one mammal mitochondrial 16S rRNA primer set (16smam1/16smam2) amplifying a ca. 95-bp fragment (excluding primers), from here on referred to as 16S (Taylor, 1996);

and one metazoan COI primer set (mlCOLintF/jgHCO2198) amplifying a partial fragment (313 bp excl. primers) of the COI barcode region, from here on referred to as COI (Geller et al., 2013; Leray et al., 2013). Metabarcoding principally followed methods described in Bohmann et al. (2018). See details for DNA extraction, amplification, library preparation and taxon assignment in Appendix 1 and bioinformatic analyses in Appendix 2.

Predator taxa were assigned to samples through comparisons to the NCHBI GenBank (16S) and/pr Barcode of Life (BOLD) (COI) reference databases. If no predator could be assigned to a sample, it was discarded from further analyses. Several potential prey species in Udzungwa did not have publicly available DNA references for the two employed markers. Therefore, assignments of some operational taxonomical units (OTUs) could not be made to taxonomic species level and there were instead assigned to higher taxonomic levels. Assignment criteria for taxon assignment can be found in Appendix 3. Only samples for which taxonomic species assignments of both predator and prey could be made were included in the statistical analyses. Prey species weights derived from Kingdon (2015) consisted of three-fourths of female body weights, which have been found to be a reliable mean when estimating consumed biomass for leopards (Jooste et al., 2013) (see Table 2). For species too large to be killed by a leopard (e.g., African buffalo [*Syncerus caffer*] and African bush elephant [*Loxodonta africana*]), the weights of infants were used as realistic alternatives for the analyses (Table 2). We applied two correction factors: CF1_{leopard}, to correct for small-bodied prey species when estimating total biomass consumed; and CF2_{leopard}, when estimating the relative proportion for each prey weight class (Lumetsberger et al. (2017). We calculated the correction factors following the parameters from Lumetsberger et al. (2017)

$$CF1 = \frac{2.242W}{4.976 + W}$$

and

$$CF2 = 13.004 - 11.601e^{-0.078W}$$

Study site	Botanical classification	Habitat category	# Scats collected	Collection period
Matundu	Lowland afro-tropical rainforest	Rainforest	14	Sep.–Nov. 2013, Dec. 2014
Mbatwa	<i>Acacia-Commiphora</i> grassland	Open grassland	8	Jun.–Jul. 2014
Lumemo Valley	Miombo woodland grassland	Open grassland	17	Aug.–Sep. 2014
Ndundulu-Luhomero	Afromontane rainforest	Rainforest	13	Sep.–Oct. 2014
Mwanihana	Rainforest escarpment	Rainforest	8	Nov.–Dec. 2014
Total			60	

TABLE 1 Locality information and number of leopard scats collected and collection periods in the Udzungwa Mountains, Tanzania

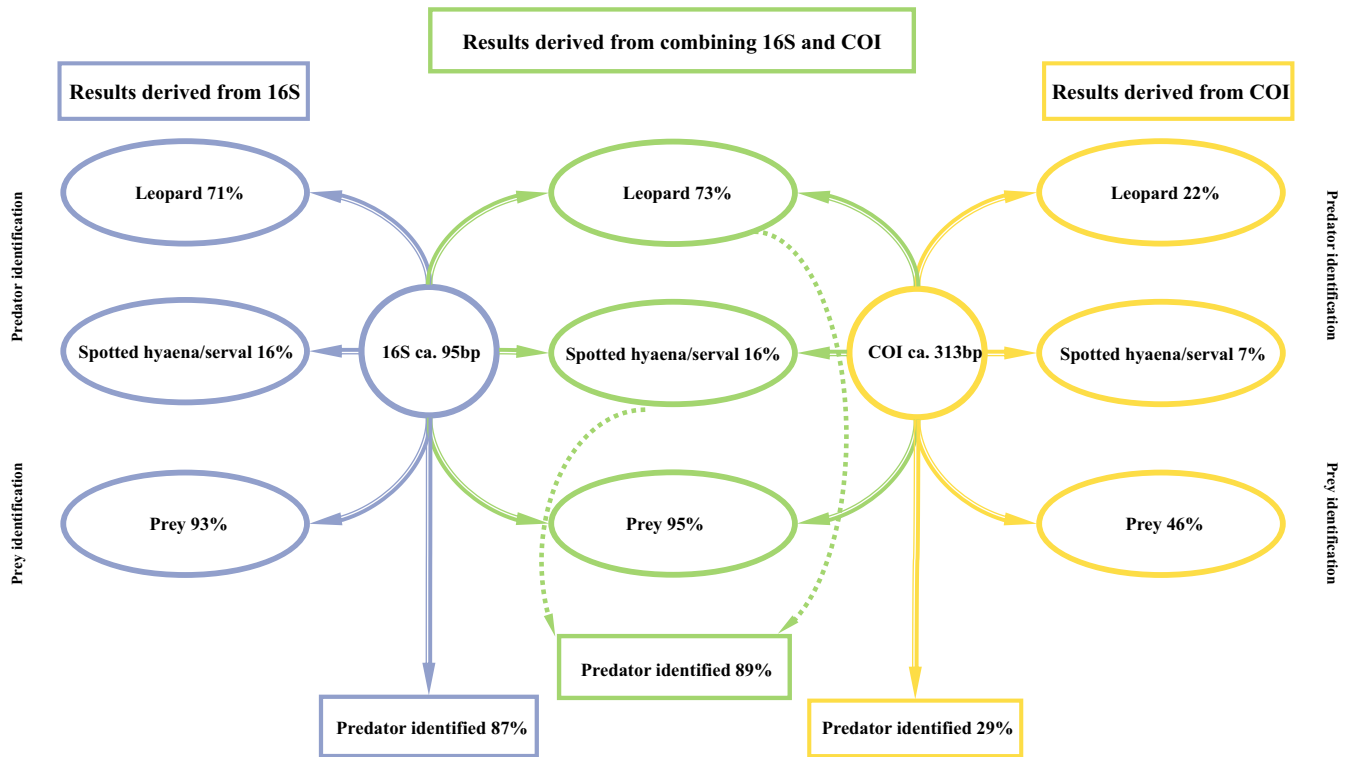


FIGURE 2 Comparison on primer performance for the 82 scats collected, thus percentages shown are of $n = 82$, where in nine samples (11%) carnivore DNA was not detected. Results derived from the longer (313 bp) COI fragment (in yellow) are outperformed by the shorter (95 bp) 16S fragment (in blue), yet the combined use of the COI and 16S increased the overall identification of predators and prey (in green), albeit only by a few per cent. However, for discrepancies in closely related species with identical 16S operational taxonomic unit sequences (e.g., red forest duiker [*Cephalophus natalensis*] and Harvey's duiker [*Cephalophus harveyi*]), the COI was invaluable for species determination [Colour figure can be viewed at wileyonlinelibrary.com]

where W is the weight of the prey in grams.

We defined prey class size as small <19 kg, medium ≥19 kg and large ≥80 kg, according to the classification by Pitman et al. (2012). Additionally, identified prey species were categorised as terrestrial or arboreal, depending on their ecology. Arboreal species in this case also include semi-arboreal species that will sleep and/or take refuge in trees when threatened (e.g., yellow baboon [*Papio cynocephalus*]).

A Welch two-sample t test was performed to investigate difference in means between habitats. A Pearson's chi-squared with simulated p -values (2000 replicates) was performed to investigate the difference in distribution of prey sizes between the two habitats. Finally, a Pearson's chi-squared test with Yates' correction for continuity was used to investigate difference in frequencies of arboreal and terrestrial species consumed by leopards in each habitat.

To test for the impact of sample sizes on the means between habitats, we resampled the data from the prey distributions within rainforest and grassland habitats and performed a Welch two-sample t test, given the generated sample data from the population. We resampled by randomly picking between 5 and 100 samples 5000 times and compared the distribution of t test p -values for each combination (Figure S1). The test indicated that at least 24 samples per area were needed for an adequate representation of the p -value given the dataset from rainforest and grassland habitats.

3 | RESULTS

From the two habitat categories, a total of 82 scats were collected, of which 60 were confirmed to be from leopards through DNA metabarcoding analyses (Table 1, Figure 2, details in Data Table SD1). Eight scats were found to originate from spotted hyaena and five from serval (*Leptailurus serval*) (Data Table SD2). Identification of predator was not possible in the remaining nine analysed samples (11%) (Figure 2).

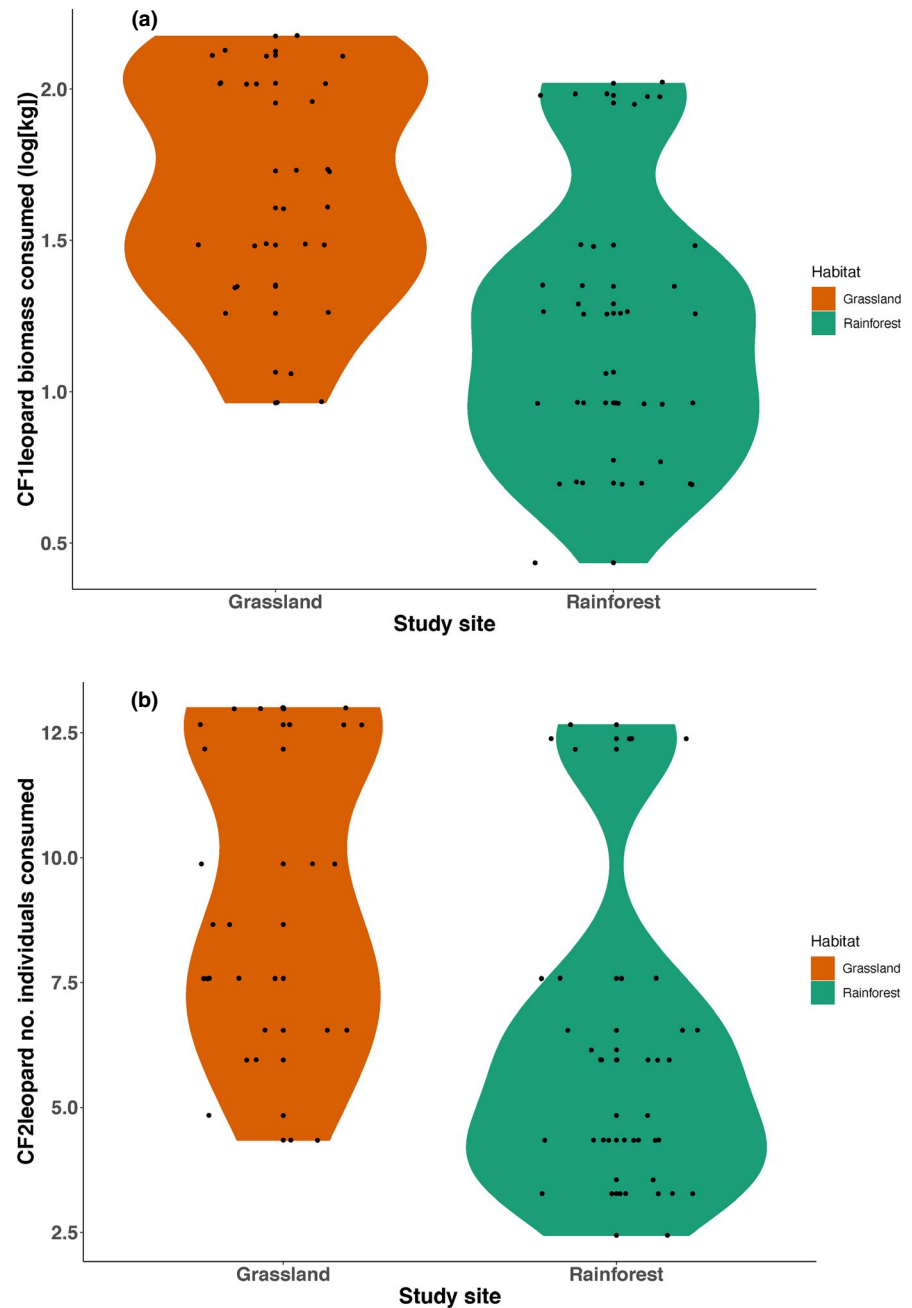
From the 60 scats confirmed to originate from leopards, twenty different taxa of mammals were confirmed from DNA metabarcoding; none of these were domestic species. Ten of the leopard scat samples contained DNA from two prey species; thus, the overall number of prey detections through DNA metabarcoding from leopard scats was 70. One of the twenty prey taxa could only be assigned to genus (*Genetta* spp.) and was therefore excluded, bringing the total number of species detections used in our analyses to 69 (Table 2). Of the 69 prey detections, 32 (46.4%; nine species) were ungulates, 26 were primates (37.7%; five species), eight were Afrotherians (11.6%; two species), and three were rodents (4.3%; three species) (Table 2). The two most commonly detected prey species were blue monkey (*Cercopithecus mitis*) and Harvey's duiker (*Cephalophus harveyi*), which both was detected in nine samples each. The third most commonly detected species was Angolan pied colobus (*Colobus angolensis*) which was detected in eight

TABLE 2 List of leopard prey identified to species recorded from scats in grassland and rainforest in the Udzungwa Mountains, Tanzania

Species	Common name	Ecology	3/4 ♀ weight (kg)	CF1 _{leopard} conversion	CF2 _{leopard} conversion	Size category	No. records rainforest	No. records grassland	Total no. records
¹ <i>Cephalophus harvey</i>	Harvey's duiker	Terrestrial	9.8	1.48	7.58	Small	3	6	9
² <i>Cephalophus spadix</i>	Abbott's duiker	Terrestrial	37.5	1.98	12.38	Medium	5	–	5
³ <i>Cercopithecus mitis</i>	Blue monkey	Arboreal	3.8	0.96	4.35	Small	7	2	9
⁴ <i>Colobus angolensis</i>	Angolan pied colobus	Arboreal	6.4	1.26	5.95	Small	6	2	8
⁵ <i>Cricetomys gambianus</i>	Giant pouched rat	Terrestrial	1.2	0.44	2.44	Small	1	–	1
⁶ <i>Dendrohyrax validus</i>	Eastern tree hyrax	Arboreal	2.3	0.70	3.27	Small	7	–	7
⁷ <i>Hystrix africaeaustralis</i>	African porcupine	Terrestrial	7.5	1.35	6.54	Small	1	–	1
⁸ <i>Loxodonta africana</i>	African bush elephant	Terrestrial	90	2.12	12.99	Large	–	1	1
⁹ <i>Neotragus moschatus</i>	Suni	Terrestrial	4.5	1.06	4.84	Small	1	1	2
¹⁰ <i>Papio cynocephalus</i>	Yellow baboon	Arboreal	12.6	1.61	8.66	Small	–	2	2
¹¹ <i>Philantomba monticola</i>	Blue duiker	Terrestrial	6.8	1.29	6.15	Small	1	–	1
¹² <i>Ptilocolobus gordonorum</i>	Udzungwa red colobus	Arboreal	3.8	0.96	4.35	Small	2	–	2
¹³ <i>Potamochoerus larvatus</i>	Bushpig	Terrestrial	33.8	1.95	12.17	Medium	1	1	2
¹⁴ <i>Rungwecebus kipunji</i>	Kipunji	Arboreal	7.5	1.35	6.54	Small	2	3	5
¹⁵ <i>Sylvicapra grimmia</i>	Bush duiker	Terrestrial	16.8	1.73	9.88	Small	–	3	3
¹⁶ <i>Syncerus caffer</i>	African buffalo	Terrestrial	80	2.11	12.98	Large	–	3	3
¹⁷ <i>Thryonomys swinderianus</i>	Marsh cane rat	Terrestrial	2.6	0.77	3.55	Small	1	–	1
¹⁸ <i>Tragelaphus scriptus</i>	Bushbuck	Terrestrial	45	2.02	12.66	Medium	1	5	6
¹⁹ <i>Tragelaphus streptoceros</i>	Greater kudu	Terrestrial	161.3	2.17	13.00	Large	–	1	1
Totals							39	30	69

Note: Weights are three-fourths of reported for females derived from Kingdon (2015). Numbers for CF1_{leopard} and CF2_{leopard} are the ¼ prey weights converted using the methodology by Lumetsberger et al. (2017). Note that for African bush elephant and African buffalo, infant weights have been used as a realistic body size that a leopard could kill.

FIGURE 3 Prey consumed by leopards in the Udzungwa Mountains, Tanzania. (a) Differences in biomass ($CF1_{\text{leopard}}$) consumed in grassland and rainforest habitats, with a higher number of biomass consumed per scat sample in grassland. (b) Differences in number of individuals consumed ($CF2_{\text{leopard}}$) in grassland and rainforest habitats, with a higher number of prey consumed per scat in grassland [Colour figure can be viewed at wileyonlinelibrary.com]



samples (Table 2). Large (>80 kg) species, including African bush elephant (*Loxodonta africana*), greater kudu (*Tragelaphus strepticeus*) and African buffalo (*Syncerus caffer*), were only detected in scats collected in grassland (Table 2). Most forest-dwelling species (e.g., Abbott's duiker [*Cephalophus spadix*], blue duiker [*Philantomba monticola*] and eastern tree hyrax [*Dendrohyrax validus*]) were only detected in scats collected in rainforests. The kipunji (highland mangabey) (*Rungwecebus kipunji*) was detected in five separate scats from two different years, two in rainforest and three in grassland well outside the known range of the kipunji in Udzungwa. Despite the close proximity to villages (Figure 2), we did not detect any domestic species.

Consumed prey biomass for all 69 prey detections that were confirmed to originate from leopard was calculated using a leopard-specific correction factor $CF1_{\text{leopard}}$ by Lumetsberger et al. (2017). Estimation

of consumed number of individuals was calculated using correction factor $CF2_{\text{leopard}}$ (Lumetsberger et al., 2017). The mean biomass of prey was significantly higher in open grassland (1.66 kg) than in rainforest habitats (1.21 kg) (Welch two-tailed *t* test, *p*-value < 0.001) (Figure 3a). The mean estimated number of individuals consumed per scat was significantly higher in grassland (9.32 individuals) than in rainforest (6.28 individuals) (Welch two-tailed *t* test, *p*-value = <0.001) (Figure 3b).

There was a significant difference in the distribution of prey weights between the two habitats (Pearson's chi-squared test for count data with simulated *p*-values and 5000 replicates, $X^2 = 33.33$, *p* = <0.001). The difference was driven by a higher proportion of biomass ($CF1_{\text{leopard}}$) consumed (Figure 4a), as well as a higher number of individuals ($CF2_{\text{leopard}}$) consumed (Figure 4b) by leopards in rainforest of small prey (<19 kg) compared to grassland. Large prey (>80 kg) was

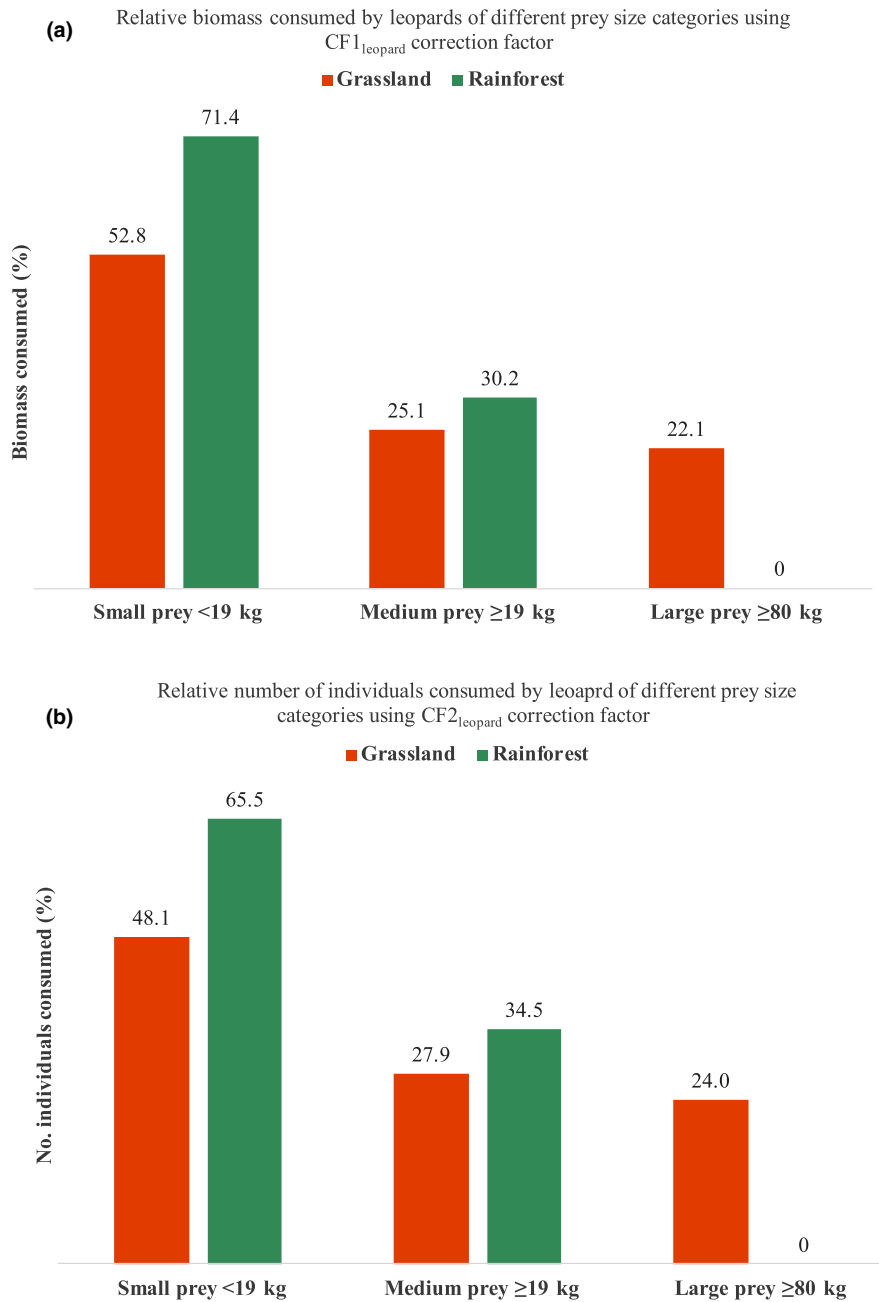


FIGURE 4 (a) Differences in biomass consumed by leopards in rainforest and grassland habitats in the Udzungwa landscapes within three different size categories of prey: Small (<19 kg), medium (≥19 kg) and large (≥80 kg) prey. (b) Difference in relative number of individuals consumed by leopards per habitat of three different size categories of prey [Colour figure can be viewed at wileyonlinelibrary.com]

exclusively recorded from grassland (Figure 4). There was also a significantly different proportion of terrestrial/arboreal species eaten by leopards between grassland and rainforest habitats (Pearson's chi-squared test with Yates' correction for continuity, $X^2 = 4.55$ p -value = 0.033). Here, a higher proportion in biomass ($CF1_{\text{leopard}}$) of terrestrial prey was detected in scats collected in grassland (Figure 5a), while the proportion of individuals consumed ($CF2_{\text{leopard}}$) of arboreal species was twice as high in rainforest compared to grassland (Figure 5b).

4 | DISCUSSION

Our results showed that leopard diet composition differed significantly in relation to prey ecology, body size and biomass consumed

between grassland and rainforest in the Udzungwa Mountains of Tanzania. The results supported our prediction that diet of leopards in rainforest and grassland habitats had different compositions. We did not detect any domestic species in leopard scats despite the proximity to agriculture and pastoral lands. This could be due to our relative low sample size and/or that Udzungwa holds sufficient prey to discourage leopards from venturing outside the protected area. An alternative explanation could be that leopards preying on domestic animals outside protected areas are removed from the population. Supporting evidence for this was found in the study by Havmøller et al. (2019), where distance to protected area boundary was found to be the main factor affecting leopard densities in the Udzungwa Mountains. The implications are likely that leopards venturing outside the protected area are in high risk of human induced

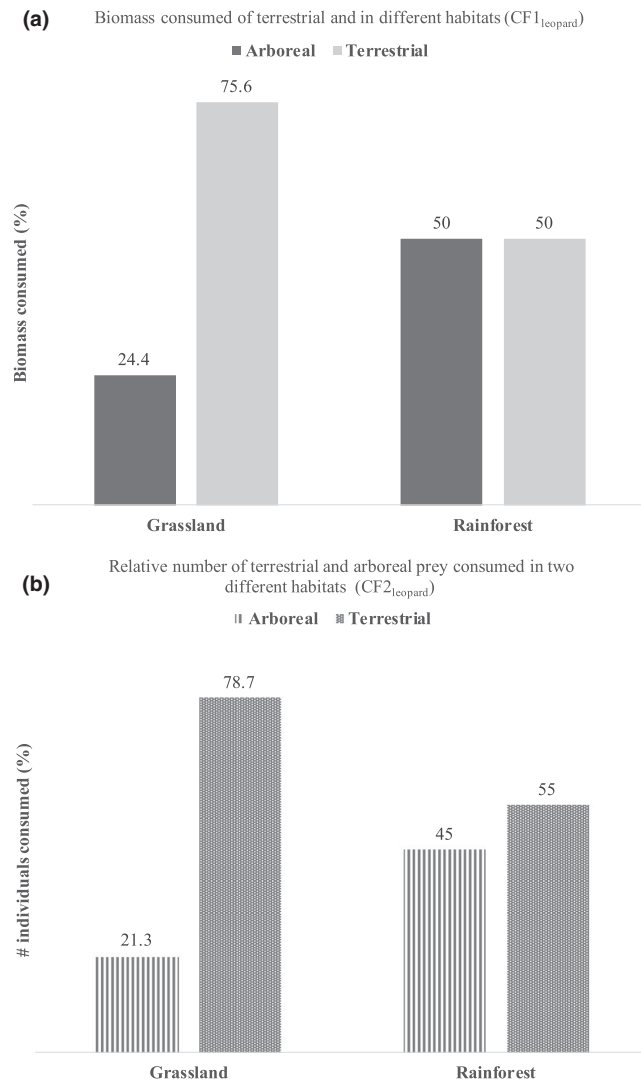


FIGURE 5 (a) Difference in biomass of arboreal and terrestrial species consumed by leopards in grassland and rainforest habitats in the Udzungwa landscape. (b) Difference in number of arboreal and terrestrial individuals consumed by leopards in grassland and rainforest habitats

mortalities, as has been found to be the case for leopards in South Africa (Balme et al., 2020; Naude et al., 2020).

Regardless of habitat types, small-bodied mammals (<19 kg) were found to make up the majority of both biomass consumed, and number of individuals eaten by leopards in Udzungwa regardless of habitat type. Large prey (≥80 kg) was recorded only in scats collected in grassland (Figure 4). This supports the findings of a camera trap study in the Udzungwa landscape where the average estimated body mass of all species tended to be lower in rainforest than in grasslands (Cavada et al., 2019).

A higher proportion of small and arboreal species constituted the prey base for leopards in rainforest, whereas a higher proportion of medium- and large-bodied terrestrial species was consumed by leopards in grassland. However, the number of individuals of terrestrial species consumed was still higher in both grassland and rainforest

habitats, albeit near to equal with arboreal species in rainforests. This skewed ratio is likely linked to prey availability and allometric constraints of leopards. Ungulates can easily access nutrient-rich vegetation in open habitats and can quickly flee from a predator unable to engage in long pursuits. On the rainforest floor, food resources are comparably more limited for ungulates, but more abundant and accessible for tree-dwelling species, which also use trees as a refuge from predators.

In our study, 50% of the consumed biomass found in leopard scats from the rainforest originated from arboreal species: five primate species (40%) and the eastern tree hyrax (10%). This is, to our knowledge, the first insight into leopard diet from an East African rainforest that indicates a high frequency of arboreal prey species. The importance of primates in leopard diet is debated and has previously been regarded as exaggerated (Hunter, 2015). In the savannahs of East and Southern Africa, two species of primate make up just 5%–6% of leopard diet (Norton et al., 1986; Radloff & Du Toit, 2004). In the moist savannah of Comoé National Park, the diversity of primates is higher, and six species were found to constitute 14.2%–15.3% of leopard diet (Bodendorfer et al., 2006). For leopards in the rainforests of Central and West Africa, primates constituted 26%–41% of their diet (Hart et al., 1996; Hayward et al., 2006; Henschel et al., 2005, 2011; Zuberbühler & Jenny, 2002). Small primates could have been missed in the above-mentioned studies, as they used morphology of hairs and not DNA markers to identify prey species. In Udzungwa, primates appear to make up the same proportion of leopard diet as has been found in West and Central African rainforests.

No small rodents, insectivores or birds were detected in leopard scats in our study (but were detected in serval scats; see Data Table SD2). This is in direct contrast to a recent leopard diet study from the nearby Mount Rungwe Nature Reserve and Kitulo National Park in the Southern Highlands of Tanzania, where rodents comprised ~60% and small carnivores ~30% of leopard diet, yet no ungulates were detected from a total of 76 scats (De Luca & Mpunga, 2018). Due to hunting, the Southern Highlands and western KNR have experienced defaunation of ungulates and primates (De Luca & Mpunga, 2018; Havmøller et al., 2014; Topp-Jørgensen et al., 2009). In a defaunated landscape in Iran, wild boar (*Sus scrofa*) constituted the majority of leopard diet (Ghoddousi et al., 2017), while Dwarf forest buffalos (*Syncerus nanus*) constituted 14% of leopard diet in West Africa (Henschel et al., 2005). Suids and buffalo are large aggressive ungulates that pose a high risk of injury for the predator hunting them (Hayward et al., 2006). Despite their high occupancy in both rainforest and grassland habitats, we only had five detections of bushpig (*Potamochoerus larvatus*) and African buffalo (Cavada et al., 2019). The reason for this could be that prey that pose low risk of injury is still sufficient in numbers to maintain the current leopard population in Udzungwa. In other regions, leopards may alter their hunting behaviour towards dangerous species because the preferred prey species have been depleted by humans (Ghoddousi et al., 2017). Loss of medium-sized ungulates and primates may force leopards to switch to smaller and more dangerous prey which could influence

their survival. This highlights the importance of sufficient protection of ungulates and primates in the UMNP.

It is well established that leopards have a preferred size range of prey (Hayward et al., 2006). The majority of leopard prey in our study fell within this known preferred size range of leopards; however, one scat contained DNA from African bush elephant and three contained African buffalo—species not normally preyed upon by leopards (Hayward et al., 2006). It is currently unknown if leopards in UMNP actively hunt African bush elephants and adult African buffalo, or if they scavenge on these large preys killed by large social carnivores, such as spotted hyaenas. The elephant that was detected could also have been from a scavenging event on a poached individual, which were observed on thirteen occasions during our study period. Other potential scavenging events are the records of buffalo that were only found in scats from grassland where both spotted hyaena and other large prey are comparably more abundant than in rainforest (Cavada et al., 2019).

While the occurrence of ground-dwelling mammals and differences between rainforest and grassland habitat has been documented by camera trap surveys (Cavada et al. (2019), comparable data are not available for arboreal species such as most primates. Reliable density estimates on group sizes for several of the primate species are available for most of UMNP (Cavada et al., 2016; Rovero et al., 2009), yet they were estimated from line transects and are thus not directly comparable to camera trap data. The development of an accurate conversion of primate group sizes and camera trap data into a body mass index would be necessary to further study prey preference of leopards in Udzungwa.

On-site identification of carnivore scats using visual cues is challenging and involves a wide margin of error because of the often high variation and overlap in scat morphology (Morin et al., 2016). Regurgitated remnants of undigested hairs and bones by spotted hyaenas have a similar appearance to older leopard scats (Hofer & East, 2013), and such scats were mistakenly collected on at least eight occasions in this study. However, we found molecular identification to be an invaluable method for identification of scat predator origin. This suggests that studies that have used morphological methods may have potentially overestimated the presence of small-bodied mammals in the diet, if scats were mistakenly assumed to originate from leopards, but were actually from smaller carnivores like serval, caracal (*Caracal caracal*) or golden cat (*Caracal aurata*). Hence, our study highlights the benefits of using metabarcoding to identify both predator and prey species as the method also captures DNA sequences of prey that could have been overlooked when only using morphological methods. However, it does not provide any information on age or size of prey, which traditional morphological studies could potentially do. The method presented in this study is replicable and time efficient; using metabarcoding coupled with second-generation sequencing can be applied to any cryptic carnivore. However, a paucity of relevant reference sequences in GenBank and BOLD databases prevented unequivocal identification of some prey species in our study. This applied to three species of *Genetta* spp. recorded in the Udzungwa Mountains (Rovero & De Luca, 2007; Rovero, Martin,

et al., 2014; Rovero, Menegon, et al., 2014) and one species of tree hyrax (*Dendrohyrax* sp.). However, only *Dendrohyrax validus* has been reported from Udzungwa and therefore its weight record was therefore used for our analyses. Additionally, five samples yielded 16S OTUs that had 96% similarity to DNA reference sequence published for yellow baboon. Three of these samples were subsequently found to contain BOLD database COI sequences that have 100% similarity to those published for the near-endemic and endangered kipunji monkey (Davenport et al., 2008). This example underlines the benefit of using two genetic markers in systems with few or no DNA reference sequences available. However, the overall gain in species determination for this study only increased by a few per cent overall (Figure 2). On the other hand, the DNA sequences discovered and published in this study contribute to addressing the DNA reference sequence paucity for mammals in Udzungwa (Bowkett et al., 2015). We recommend that future studies investigate the availability of reference DNA sequences or reference material as an important prerequisite from, e.g. natural history museum collections.

The majority of prey detections in this study were of species within their known distributional ranges in Udzungwa. However, the five detections of kipunji were inconsistent with their known distributional range. The kipunji is thought to be extremely range restricted in Udzungwa and has only been recorded to occur in 7.18 km² of the montane forest of Ndundulu (Bracebridge et al., 2011), with less than 100 individuals estimated to persist there (Davenport et al., 2008). The detections of kipunji outside their known range suggest that leopards likely move between habitats, as the possibility that these monkeys have unknown pockets of presence is low (Bracebridge et al., 2011).

Eisenberg and Lockhart (1972) reported that leopards eating large prey defecated in the nearby vicinity of the kill. However, male leopards in Kalahari have been found to move between 10.9 and 15 km between successive defecations, while females move 15.6–22.6 km (Bothma & Le Riche, 1994). Leopards in arid habitat are known to have higher daily distances moved (Bothma & Le Riche, 1994) than rainforest dwelling leopards that have been found to travel 1.2–9.5 km/day (Jenny, 1996). The limited movement of leopards between habitat types in Udzungwa is supported by results from a camera trap study by Havmøller et al. (2019), where none of the 62 leopards identified during camera trapping was recaptured in another habitat (but individual spotted hyaenas where). Udzungwa leopards also had low mean maximum daily distance travelled (1.2–2.5 km) (Havmøller et al., 2019). Based on this knowledge, we assume that the majority of our samples were from predation events within the same habitat that they were collected in. Yet, we do acknowledge our study has a limited sample size and may not provide full insights into the diet of leopards in Udzungwa. Another limitation of our study was that sample collection only occurred in the dry season. However, our sample size was above the minimum previously found to be necessary to identify principle prey (Trites & Joy, 2005). While Trites and Joy (2005) state that 94 scats are necessary for comparisons of species composition (discrete) between habitats, our study investigates size composition of the scats (continuous), and is therefore not directly comparable to species compositions. Nevertheless, additional samples to confirm our findings

would be beneficial to better understand leopard diets in various habitats. Additionally, further studies are needed to determine the extent to which leopards move between different habitats in the landscape. Moreover, a better assessment of the selective predation of kipunji over more abundant prey species would contribute to determining whether leopards represent a potential threat to conservation of the highly endangered kipunji in Udzungwa.

Perhaps just as surprising as finding kipunji was the absence of Sanje mangabey (*Cercocebus sanjei*) in leopard scats. Sanje mangabey is much more abundant than kipunji and they spend large parts of their day on the ground foraging. A total of 1750–2100 individuals are currently estimated to live within the Mwanihana escarpment, which was part of our study site (Rovero et al., 2009). However, we only collected eight scats from this site so scats containing remains of Sanje mangabey could have been missed.

Our study is the first to investigate dietary differences related to connected but distinct habitats in East Africa. One of the next questions that presents itself is whether there is a difference in diet between male and female leopards in Udzungwa as has recently been found in Southern Africa (Balme et al., 2020; Voigt et al., 2018). Male and female leopards in Udzungwa have already been found to have sexually segregated temporal patterns (Havmøller et al., 2020), so dietary niche specialisation as a result thereof is also possible (Balme et al., 2020). Ecological segregation has been observed in coyotes (*Canis latrans*) in the western United States, where genetic and behavioural distinctions are detectable in populations across different habitat types (Sacks et al., 2004). Similarly, ecology has also been observed to restrict gene flow in lions (*Panthera leo*) in southern Africa (Dures et al., 2019). Further study of the ecology of leopards and their adaptability in Africa would require a population genetics and GPS study of leopard movements.

In this study, we have shown that arboreal species constitute a substantial part of the diet of rainforest leopards in Udzungwa. Leopards in Udzungwa eat even uncommon prey species such as the kipunji monkey and to a very small extent potentially dangerous species. We found no evidence of predation on domestic species, despite the close proximity to livestock. This makes it hard to gauge how African leopards will adapt in the coming decades, which are likely to see accelerating rates of habitat fragmentation and human population growth. We recommend continued high-intensity protection of UMNP and KNR to maintain prey populations in Udzungwa as key to sustained management of these protected areas. Considering the challenges to observing leopards directly, especially in forested habitats, this study provides a valuable insight into understanding leopard diet and its variation between habitats.

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CONFLICT OF INTEREST

We declare that we do not have any conflict of interest in connection with the work submitted.

DATA AVAILABILITY STATEMENT


The data that support the findings of this study are available from GenBank. Please see the Data Tables SD1 and SD2 for species-specific GenBank Accession numbers.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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