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Diet DNA reveals novel African Forest elephant ecology on the grasslands of the Congo Basin

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Abstract

Elephants are essential ecological engineers, creating and maintaining landscape structure and ecosystem function. The recently distinguished and critically endangered forest elephant is currently classified as a selective, non-destructive frugivorous browser that maintains forest diversity, while the savanna elephant is a mixed feeder, often pushing over trees while maintaining grasslands. The presence and diets of forest elephants on grasslands and the potential maintenance of these systems remain largely unexplored. In the ecotone between the Guinea-Congolian forest and Sudanian-Guinean savanna ecosystems in Garamba National Park, DRC, we investigated forest elephant diet selection as a function of sex, age, and habitat using diet DNA (dDNA) metabarcoding of non-invasively collected dung. GPS collar data were used to determine annual habitat use. Dietary niche partitioning was assessed among megaherbivores in the grasslands. Fecal samples represented the diet of individuals within each habitat, providing valuable insight into the plant biodiversity. Ecological patterns of diet were also revealed using a taxonomically free exact sequence variance approach, highlighting useability in a poorly characterized region. In the early wet season, these typically frugivorous forest elephants were consuming mostly grasses in both the woodland and grassland habitats and showing no sexual dimorphism in diet selection when in the same habitats. However, males were greater risk-takers, entering the human-altered landscape to forage on fruit. The forest elephants play a distinctive role within this tropical grassland when compared to other megaherbivores and utilize the unique ecosystem throughout the year. This elephant population is exhibiting behavioral plasticity and shifting their gardening efforts to a novel resource in the grasslands as opposed to their standard role in the forests, which is key to understanding their impact as ecosystem drivers within this landscape. This shift in behavior may result in this recovering elephant population playing a functional role in the restoration and maintenance of these grasslands.

KEYWORDS

Behavioral plasticity, diet DNA (dDNA) metabarcoding, grasslands, *Loxodonta cyclotis* ecology, megaherbivore niche partitioning, non-invasive, sexual dimorphism

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1 | INTRODUCTION

Elephants engineer diverse biomes, from tropical forests to arid savannas, by creating and maintaining landscapes, altering vegetation structure, and contributing to ecosystem function at various scales (Berzaghi et al., 2019; Coverdale et al., 2016; Poulsen et al., 2018). However, the two African elephant species are different in their manner of ecosystem engineering. The recently distinguished forest elephants *Loxodonta cyclotis* (Gobush et al., 2021b) are selective non-destructive browsers, targeting fruits mostly and having little effect on tree size and demography while maintaining the forest diversity (Campos-Arceiz & Blake, 2011; Cardoso et al., 2019). In contrast, savanna elephants *Loxodonta africana* are mixed feeders (grazers and browsers), notably destructive to even large older trees, by pushing them down, browsing them, and thus facilitating open savannas (Morrison et al., 2016; Ripple et al., 2015). As large monogastric hindgut fermenters, elephants cannot digest cellulose as efficiently as ruminants and seeds can pass through their digestive system, facilitating germination (Beirne et al., 2019; Campos-Arceiz & Blake, 2011; Sekar & Sukumar, 2015). As such, the frugivorous forest elephant is considered "forest gardeners" (Campos-Arceiz & Blake, 2011), consuming more seeds from more species and dispersing them longer distances than most mammals in Africa, playing a fundamental role in maintaining forest diversity (Blake et al., 2009; Cardoso et al., 2019).

Although elephants are the quintessential ecosystem engineers of Africa, the forest elephant was recently identified as Critically Endangered and the African savanna elephant as Endangered on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Gobush et al., 2021a, 2021b). The cascading impacts this loss has had and will continue to have across the African continent, and on a global scale through loss of carbon stocks (Berzaghi et al., 2019; Poulsen et al., 2018), is not easy to predict because each species performs very different engineering and foraging roles in their preferred habitat (Bello et al., 2015; Chami et al., 2020; Crooms et al., 2018; Poulsen et al., 2018). Now that the forest and savanna elephants are protected as separate species with very different population doubling times (60 years and 20 years, respectively) (Turkalo et al., 2017), we need to better understand the lesser-known forest elephant ecology to help drive their recovery.

While the diet of savanna elephants has been well documented (Codron et al., 2006; Greyling, 2004), that of the forest elephant is less known across different habitats. Almost all forest elephant diet studies have been conducted in West and Western-Central Africa and found them to only consume browse and fruit (Blake et al., 2009; Campos-Arceiz & Blake, 2011; Danquah & Oppong, 2006; White et al., 1993). One study reports grass in all fecal samples of forest elephant in Cameroon; however, only visually identifying content (Tchamba & Seme, 1993), while another in the Ivory Coast, found only one grass species out of 147 food plants their diet, showing active avoidance of grass (Theuerkauf et al., 2000). Thus, further work and a more robust method, such as DNA metabarcoding, may aid in exploring the diet of forest elephants across its geographic range.

Diet DNA (dDNA) metabarcoding (i.e., environmental DNA approach that uses DNA extracted from feces (de Sousa et al., 2019)) is a powerful tool to infer ingested plants and prey (Clare, 2014; Ji et al., 2013) and to assess biodiversity and species interactions (Meyer et al., 2020) as it provides higher resolution, is more accurate, and reduces time and costs. Although used in other herbivores, including savanna elephants (Czernik et al., 2013; Erickson et al., 2017; Iwanowicz et al., 2016; Kartzin et al., 2015; Soininen et al., 2009), this technique has not been used for a forest elephant diet analysis. Forest elephants reside in difficult to study regions, and the representation of fauna and flora species in the database is most likely limited (Elbrecht et al., 2017). However, we can still identify ecological patterns through the taxonomic-free assessment (Tapolczai et al., 2021) and the diversity of species captured in the database.

Better understanding the forest elephant's relationship with its ecosystem and drivers of behavior will strengthen efforts in protection. The tropical biomes of Africa have shaped, and in turn been shaped by, the social structures of forest and savanna elephants (Goldenberg et al., 2020). The co-dependence of plants and animals is strikingly clear when observing the distribution of the two African elephant species, most likely playing an adaptive role on parental care and social structure (Royle et al., 2012). While savanna elephants move in large groups (35–100+) (Archie et al., 2006; Poole & Moss, 2008), the social structure of the forest elephant rarely exceeds the core unit of a mother and one to two of her calves (Goldenberg et al., 2020), ranging from two to 11 individuals (Turkalo & Fay, 2001). The small groups create well-worn trails through the dense forests, where they repeatedly browse from large fruiting trees and shrubs, converging occasionally in forest openings called *bais* (natural forest clearings) (Turkalo & Fay, 1995). Unlike the matriarchal savanna elephant, in which only males disperse from their natal herd, both sexes disperse; forest elephant males were at a similar age to savanna elephants (13 to 17 years old), while females leave their maternal group at first pregnancy, around 20 to 23 years of age (Fishlock et al., 2008; Turkalo et al., 2013, 2018). These differences might also lead to dissimilarities in ecological segregation such as habitat use and diet selection. Understanding the effect of sex, family group size, and preferred diet on the vegetation occupied by the lesser-studied forest elephant, and vice versa is important and under-investigated.

Different energy requirements based on sex-related differences in body size and reproductive demands, that is, gestation, lactation, and musth, often lead to distinctions in plant selection and habitat use between the sexes of sexually dimorphic species. Only one study of forest elephants looked at the sexual difference in movement patterns and found males to have a much larger home range than females, as expected; however, the study revealed no difference in habitat use (Mills et al., 2018). The mechanisms driving sexual segregation are important for the conservation of sexually dimorphic elephants (Shannon et al., 2008). However, to date, this has only been assessed in savanna elephants showing differences in diet and habitat use (Greyling, 2004; Shannon et al., 2008, 2013; Stokke & Du Toit, 2000; Woolley et al.,

2009) and all diet studies of forest elephants have ignored the distinctions in food choice arising from differences in sex and age.

The climate and human-induced merging of the unusual ecotone between the Guinea-Congolian forest and Sudanian-Guinean savanna ecosystems in the Democratic Republic of Congo (DRC) makes the Garamba National Park Complex a unique study area to address diet selection of forest elephants in different habitats, as they spend time in both the woodlands and open savanna grasslands (de Merode et al., 2007). Unfortunately, poaching has shaped this unique population of elephants, declining from an estimated 22,000 elephants in 1976 (Hillman-Smith et al., 2014) to the current population of ~800–1000 (African Parks aerial survey report 2019). With the support from the NGO African Parks Network and Congo government, biodiversity in Garamba NP is finally starting to stabilize and recover from its lowest point in history, having lost more than >95% of its megaherbivore biomass (see Figure S1). Not only did this unusual savanna–tropical forest ecotone contain the highest density of elephants in the Congo Basin historically, but it is also unique for the savanna-forest elephant hybrids that were recently identified there (Groves & Grubb, 2000; Roca et al., 2001). Subsequent work (Ishida et al., 2011a, 2011b) did not identify F_1 hybrid genotypes between savanna and forest elephants, suggesting that no recent hybridization (past ~40 years) has been occurring and that gene flow between the two species is limited. It is not known when the last savanna elephants were present in Garamba NP, possibly in the 1950s (Backhaus, 1958), and the few genetic samples taken were primarily forest elephant genotypes with a few hybrids, suggesting that savanna elephants are locally extinct and this population is majority forest elephants (Roca et al., 2015). Defaunation of elephants across non-forested African landscapes often leads to the transformation of grasslands to woodland savannas (Campos-Arceiz & Blake, 2011), as we are seeing in Garamba NP with a reduced grassland cover from 80% of the park in the 1950s to less than 30% in recent years (De Merode et al., 2000; Hillman Smith et al., 2014). The approximate 91,438,000–150,702,000 kg (~180–300 kg/ha) reduction in megaherbivore biomass (elephant, giraffe, buffalo, and white rhino) since the 1970s on this savanna grassland would definitely be contributing to this change, along with the changes in fire regimes (Landu et al., 2016). Therefore, can the “frugivorous” forest elephant actively contribute to maintaining these grasslands?

To better understand the presence of elephants in this transitional zone, this study aims to investigate the influence of sex, age, family groupings, and habitat type (including human-occupied areas) on diet diversity and composition within this recovering elephant population. To ascertain whether there is redundancy in the role of elephants and other large herbivore species in vegetation choice, we also tested for dietary niche partitioning across the savanna grassland of the study area. Although we were only able to assess diet in the wet season, we used elephant GPS collar data to understand the use of the grasslands throughout the year. Our study is the first to investigate forest elephant diet selection strategies as a function of sex, age, and habitat using dDNA metabarcoding. Information attained from this study will help inform elephant and protected area management strategies, such as the possible need for the

reintroduction of savanna elephants for the conservation/maintenance of the unique grassland in this system.

2 | MATERIALS & METHODS

2.1 | Study site

Garamba National Park (GNP) (~500,000 ha) is in the Haut-Uele Province in the north-eastern Democratic Republic of the Congo (DRC), established in 1938 and designated a UNESCO World Heritage Site (1984). The Park is continuous with three community reserves around most of its boundary (est. 1938); Azandes in the west, Mondo Missa in the East, and Gangalana Bodio in the south covering an overall area of 1,400,000 ha and managed by the African Parks Network (APN) on behalf of the Congo government as the GNP Complex (GNPC; Figure 1). GNPC is within a tropical climate receiving an average of 1500-mm annual rainfall during 9 months of the rainy season (March–November) and 3–4-month dry season.

Garamba NPC lies in the transition zone between two centers of endemism: the Guinea-Congolian forest and Sudanian-Guinean savanna and is one of the WWF/IUCN Centers of Plant Diversity. The Park hosts three main biomes: gallery forest with forest clumps and marshland, aquatic and semi-aquatic associations, and savannas ranging from dense deciduous woodlands to treeless grasslands. The vegetation within the park boundary has been modified through man-made fires and high densities of large herbivores in the distant past, forming a unique long grass savanna (De Merode et al., 2000), surrounded by the three reserves consisting of a mosaic of deciduously wooded and bushed savanna and forest. GNPC is one of the last strongholds of Sudano-Guinean species. It contains the largest number of elephants in the DRC (*L. cyclotis*; est. pop. 800–1200), the endemic and critically endangered Kordofan giraffe (*Giraffa camelopardalis antiquorum*; est. pop 55 (D'haen et al., 2019)) and recently lost the last viable natural population of northern white rhinoceros (*Ceratotherium simum cottoni*) in 2005.

2.2 | GPS collar data

We used GPS satellite collar location data (hourly) provided by African Parks to assess the proportion of time elephants (Female = 27, Male = 3) spent in the different habitat types throughout twelve months in 2019. We identified the land cover type using the Land Cover Classification System (LCCS) at hourly GPS locations of the collars. Following the Land Cover Climate Change Initiative categorizations, we classified habitats into three main categories, Forest, Grasslands, and Agriculture.

2.3 | Fecal collection

Forest elephant *L. cyclotis*, buffalo *Syncerus caffer caffer*, and giraffe *Giraffa camelopardalis antiquorum* fecal samples were systematically

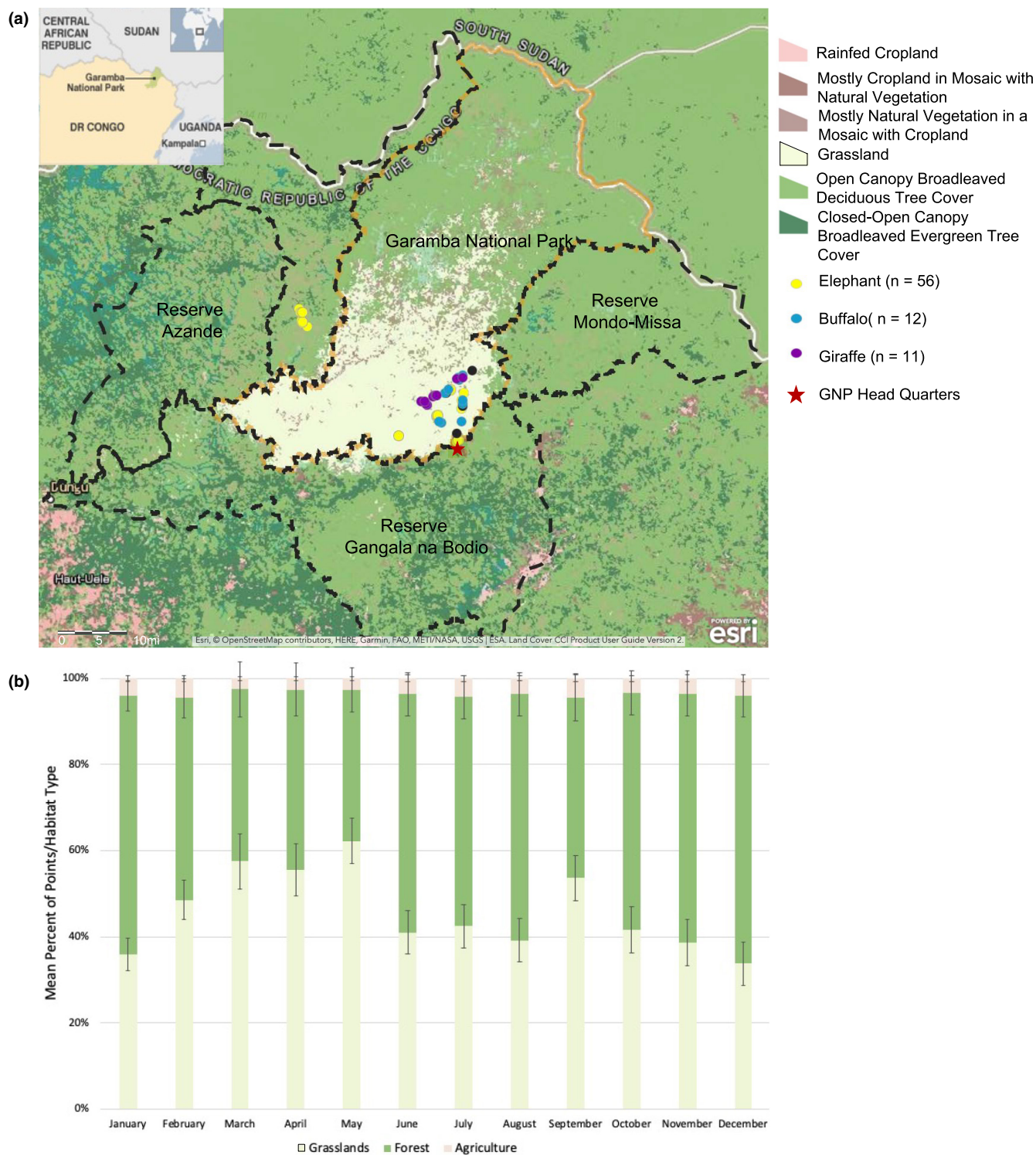


FIGURE 1 Fecal sample collection and Forest elephant habitat use. a) Map of fecal samples collected in Garamba National Park, DRC, surrounded by three reserves that make up the greater complex (GNPC). The vegetation type is color-coded along with the samples that were successfully sequenced for elephant, buffalo, and giraffe. b) The bar graph represents the mean proportion of hourly GPS points per habitat type \pm SE from GPS satellite collars on forest elephants ($n = 30$)

collected along road transects, and wildlife trails during a single field season between May and June 2019 (Table 1). Elephant satellite collar GPS coordinates were used to determine elephant group locations before traveling into the field for targeted fresh fecal collection

and to avoid non-independent sampling. The samples collected around a 25m radius of collar point were labeled as possible “family” group; however, we did not confirm relatedness with genetic tests. All elephants are being assessed as forest elephants for this

TABLE 1 GNP large mammal species fecal samples were sequenced for dietary analyses with the mean number of reads, Shannon's diversity, and richness \pm standard error (SE) and ESVs per sample for the 2,215 taxonomic-free ESVs and 188 unique plant OTUs across all habitats and within the grasslands only

Latin Name	Common Name	Samples Collected (N)	Samples Sequenced (N)	Shannon's Diversity (H)		Niche Breadth	
				Mean \pm SE	Mean \pm SE	Mean no. ESVs per sample \pm SE ³	Mean no. OTUs per sample \pm SE ⁴
L. cyclotis (All habitats)	Elephant	58	56	13,707 \pm 290	3.49 \pm 0.05 ^a	2.23 \pm 0.05 ^a	114.25 \pm 2.65 ^a
L. cyclotis (Grasslands)	Elephant	32	32	14,091 \pm 440	3.56 \pm 0.06 ^a	2.31 \pm 0.04 ^a	116 \pm 3.12 ^a
Syncerus caffer caffer	Buffalo	13	12	17,795 \pm 665	3.13 \pm 0.18 ^a	1.72 \pm 0.1 ^b	106 \pm 6.35 ^a
G. camelopardalis antiatorum	Giraffe	13	11	10,314 \pm 2475	2.25 \pm 0.27 ^b	1.12 \pm 0.16 ^c	51.64 \pm 11.02 ^b
Total		84	79	13,857 \pm 462			7.55 \pm 1.25 ^c

¹Each comparison revealed significant differences (ANOVA, all habitats: $F_{2,76} = 27.9, p < 0.001$; Grasslands: $F_{2,52} = 24.7, p < 0.001$). Superscript letters denote significant differences (Tukey's HSD, $p < 0.05$)

²All habitats: $F_{2,76} = 43.2, p < 0.001$; Grasslands: $F_{2,52} = 52.97, p < 0.001$

³All habitats: $F_{2,76} = 33.5, p < 0.001$; Grasslands: $F_{2,52} = 31.5, p < 0.001$

⁴All habitats: $F_{2,76} = 60.2, p < 0.001$; Grasslands: $F_{2,52} = 69.04, p < 0.001$

study and not hybrids, as hybrids are estimated to be in low numbers. Collection efforts were tracked and mapped with GPS, and all relevant ecogeographical variables were recorded in an ESRI mobile application (Survey123). Collection areas were grouped into three categories: Grasslands defined as land covered with grasses and herbaceous plants with few trees scattered throughout (Southern section of GNP), Woodlands defined as land covered with a mix of grassland and 50% percent tree cover (Azande Hunting Reserve), and Human-Altered Landscape (HAL) that consisted of the GNP Headquarters and village along riverine habitat housing some unique riparian species.

To collect dDNA for elephant and buffalo, multiple fecal boluses from the same individual were broken open and small samples (~25 g) were taken from inside of each bolus (to avoid environmental contamination on the outside of the feces) using a gloved hand and placed in 35 ml of DESS (20% DMSO (dimethyl sulfoxide), 250 mM EDTA, Tris, saturated with NaCl) buffer for storage (Wasser et al., 2007). For giraffe fecal samples, multiple pellets were collected and homogenized together (fecal pellets were too small to remove feces from the inside) and stored using the same method. All samples were collected from fresh fecal samples, noted visually from appearance, or observed defecation, except for half of the giraffe samples (all samples were collected due to the small population size). These samples were transported to our laboratory in the United States with appropriate permissions from the Institut Congolais pour la Conservation de la Nature (ICCN).

2.4 | Fecal dDNA extraction

All laboratory work was conducted at Stanford University in designated eDNA and PCR rooms, and surfaces were sterilized with a 10% bleach solution to avoid contamination. Fecal samples that were stored in DESS were mixed thoroughly, centrifuged and the DESS removed to concentrate the fecal samples to create a "sludge" of fecal material and stored at -40°C in triplicates for later extraction. dDNA was extracted from fecal sludge (~0.2g) utilizing the Zymo Quick-DNA Fecal/Soil Miniprep Kit (Kartzin et al., 2015), adding an extra Ethanol cleaning step to remove the DESS buffer. We conducted all dDNA extractions in a sterilized laminar airflow hood to avoid contamination. Samples were processed in small batches (~14) with an extraction blank to monitor for potential cross-contamination in the laboratory. The eluted DNA was quantified using a Nanodrop 2000 (Thermo Fisher Scientific Inc.) and stored at -20°C until it was used for PCR experiments.

2.5 | Elephant sex and age

To identify the sex of each elephant fecal sample, we amplified the X-Y homologs of Amelogenin (AMEL) gene using three primers: the common forward primer AMELXY 5'-TTCTGGAATCTGGTTTGAGGCT-3', and the X-specific AMELX-R 5'-ATCTTTACAACAAAA

CAATTGTTAACCATGCTC-3' and Y-specific AMELY-R 5'-TCAGATTCAGAGTTTCCTTCA TGCAGTAG-3' reverse primers, following the Lohay, (2020) protocol. These primers were found to be more successful than the previous methods (Ahlering et al., 2011). We had elephant samples from known sexes to test these primers.

A suitable measurement for estimating age in elephants using dung was developed for African savanna (Morrison et al., 2005) and Sumatran elephants (Reilly, 2002). The mean bolus diameter of dung increases with age, up to a point. We used the Morrison et al. (2005) predicted growth curves based on dung bolus diameter for male and female elephants to estimate age. Because forest elephants are smaller in height and weight than savanna elephants (2–5 tones vs 4–7 tones, respectively), we used the female curve. We distinguished two age categories: juvenile (mean dung bolus diameter <11 cm) and adult (>11 cm).

2.6 | dDNA Metabarcoding

We used metabarcoding on the extracted DNA to characterize the host diet by quantifying large numbers of a short, unique region of DNA extracted from individual fecal samples (Pompanon et al., 2012). We targeted 200–387 bp amplicons of the second internal transcribed spacer of nuclear ribosomal DNA (ITS2) region for the amplification of plants in the diet using primers UniPlantF: 5'-TGTGAATTGCARRATYCMG-3' and UniPlantR: 5'-CCCGHYTGAYYTGRGGTDC-3', as they have a taxonomic resolution of 86.1%, 99.4% and 99.9% at the species, genus and family levels, respectively (Meyer et al., 2020; Moorhouse-Gann et al., 2018). All primers were modified with an Illumina adaptor preceding the target primers and separated by 6-N spacers as designed by Ushio et al. (2017). The first PCR comprised of 20 µL reactions using 10 µL GoTaq® Colorless Master Mix, 0.6 µL (10 mM) of each primer, and 6 µL of DNA template and 2 µL of water. Thermocycling followed a program of initial denaturing at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a 2-min final extension at 72°C.

The integrity of DNA amplified by each primer was assessed by gel electrophoresis. Specifically, 3 µL of each DNA extract was analyzed in a 2% agarose gel and was visualized by UV illumination. If positive, the PCR products were cleaned with the Qiagen PCR Purification Kit (Valencia, CA). For the two-step PCR, appropriate Illumina barcodes were appended to each sample as an index tag for each unique sample (Ushio et al., 2017). The index PCR was performed as a 20 µL reaction: 10 µL of Amplitaq Gold360 Mastermix reactions (with 2.5 mM MgCl₂, 200 IM each dNTP, 0.1 mg/ml BSA, 4% DMSO), 1 µL (of each primer), 3 µL of purified DNA amplicons, and 6 µL of H₂O. Cycling conditions used initial denaturing at 95°C for 10 min, followed by 12 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30s, and extension at 72°C for 10 s.

The second PCR products were quantified using a Qubit fluorometer (ThermoFisher Scientific) with the dsDNA High Sensitivity Assay Kit (Life Technologies Corp.), normalized to equimolar concentrations, and

pooled together before purification using QIAquick PCR Purification Kit (Qiagen). Sequencing was performed on a MiSeq platform using the MiSeq Reagent Kit v3 for 2 x 300 bp paired-end reads (Illumina) and run at the Stanford University PAN Facility. PhiX DNA spike-in control (18%) was added to improve the data quality.

2.7 | Plant ITS2 bioinformatics

We used exact sequence variants (ESVs), which are considered as unique DNA read sequences with variation down to single-nucleotide resolution having biological meaning (Hajibabaei et al., 2019). This method offers several advantages for community analyses, for example, better characterization of intraspecific genetic diversity revealing community patterns, computational tractability, reproducibility of analysis, and the ability to perform meta-analyses across different laboratories (Callahan et al., 2017; Elbrecht et al., 2018; Glassman & Martiny, 2018; Mueller et al., 2016; Porter & Hajibabaei, 2020; Tapolczai et al., 2021).

In this study, sequencing success and read quality were verified using FastQC v0.11.8, and reads were demultiplexed by using Illumina-utils v2.6 (iu-demultiplex) using default settings. Sequences of each sample were then merged using the -fastq_mergepairs option in Usearch v11.0.667 (Edgar, 2010). The forward primer (5'-TGTGAATTGCARRATYCMG-3') and reverse primer (5'-CCCGHYTGAYYTGRGGTDC-3') for plant ITS2 sequences were removed using Cutadapt v1.18 (Martin, 2011). Cutadapt was also used to discard sequences with lengths below 187 bp and above 387 bp for ITS2 sequences. Expected error filtering as implemented in Usearch was then used to discard low-quality reads (max_ee = 0.5) (Edgar & Flyvbjerg, 2015). Instead of OTU clustering, reads affected by sequencing and PCR errors were then removed using the UNOISE3 algorithm with an alpha value of 5 (Edgar, 2016). This denoising was applied to each sample, and ESVs were compiled including sequences and read counts for each sample.

Taxonomy was assigned to each ESV by mapping them against GenBank reference data (Benson et al., 2005) and Jonah Ventures (jonahventures.com) voucher sequence records, using usearch_global with -maxaccepts 0 and -maxrejects 0 to ensure mapping accuracy. Consensus taxonomy was generated from the hit tables, by first considering 100% matches, and then decreasing in 1% steps until hits were present for each ESV. In the respective 1% bracket, we used a cutoff for taxonomy present in at least 90% of the hits, or reported an NA, if several taxa matched the ESV. To reduce errors caused by misidentified taxa, the bracket was increased to 2% if matches of 97% or higher were present, and no family level taxonomy was returned. ESVs only identified to Phylum or Order were removed from the analysis. All ESVs with <10 ITS2 reads were excluded from analysis for diet. Non-target ESVs were removed because the ITS2 primer is known to amplify fungi and bacteria (Moorhouse-Gann et al., 2018). Finally, all sequences with >1% of the total reads in the negative controls were removed from our samples (Meyer et al., 2020).

The ESVs are assigned to taxonomy based on the reference databases, which are often incomplete, especially in remote and poorly sampled areas such as the DRC (Elbrecht et al., 2017). Thus, we chose to analyze the data as taxonomy-free post-filtration of non-target ESVs, for example, fungi and bacteria, to retain poorly identified ESVs (Tapolczai et al., 2021). This method allows information on the ecology of sequences belonging to the same species and of unassigned sequences to be kept (Tapolczai et al., 2021) and for more diverse patterns to be identified.

To describe the composition in the diet at the taxonomic level (not for the taxonomic-free analyses), we joined matched ESVs to the same taxa using a match criteria threshold of >90% base similarity to reference sequences (Dunn et al., 2018; Hatzenbuehler et al., 2017; Leempoel et al., 2020), making a single molecular unit (da Silva et al., 2020). We then labeled them as operational taxonomic units "OTUs" in the study for convenience as this term is more commonly used in the metabarcoding literature (Minerovic et al., 2020). For example, multiple ESVs were identified to the *Echinochloa* genus, and the reads would be combined to create one unique OTU for this genus.

2.8 | Diet composition and biodiversity analysis

All further data processing and statistical analyses were done in R (version 3.5.2). We converted the filtered sequence read data into read abundance and occurrence data to examine the utility of both data types for food web construction. As suggested by Deagle et al., (2019), we quantify diet composition using frequency of occurrence (FOO), which is calculated as the number of occurrences divided by the total number of samples and we normalized all data by transforming sequence counts into relative read abundances (RRA) defined as the proportion of unique Illumina sequence reads in a sample divided by the final (i.e., after quality control) number of sequence reads in that sample post-filtering (Kartzinel et al., 2015).

OTUs were categorized into plant functional trait groups. Plants were identified as Grasses, Herbs, or Woody vegetation (trees and shrubs) through the TRY database (Kattge et al., 2020). When the OTUs could not be identified to a single group, they were labeled as Unknown. The mean percent of RRA was used to compare diet type (e.g., Grass vs. Woody vegetation) among the focal species (Pansu et al., 2019).

We determined individual niche breadth by calculating average ESVs or OTUs richness per sample and the diversity of diet using the Shannon diversity index (H). Shannon diversity accounts for both abundance and evenness of the species present in the diet and was calculated for each species' diet using the *vegan* package (Oksanen et al., 2019). We subsequently computed niche overlap between elephant sexes and among species using Pianka's niche overlap index (Pianka, 1973).

We used EcoSim null models (version 1; <http://grayentsminger.com/ecosim.htm>) to test whether the extent of niche overlap is greater than expected by chance (Gotelli et al., 2015). We generated 1,000 simulated matrices of randomized OTU diet composition,

using the randomization algorithm 3, where the niche utilization values are reshuffled within each row of the matrix to detect non-random niche overlap patterns (Winemiller & Pianka, 1990). The observed niche overlap (O_{jk}) was then compared with the simulated niche overlap values (Gotelli et al., 2015). For all pairwise comparisons, we deemed niche overlap to be significant when the observed value was greater than at least 99% of the simulated values.

To determine whether the number of collected fecal samples captured enough of the diet richness for this study, we used the function *specaccum* in the R package *vegan* to determine the OTUs accumulation curve (without replacement; Gotelli & Colwell, 2001). Occurrence data of OTUs were used without singletons; this allowed for the assessment of plants making up most of the diet.

The relationship of sampling sites, individual elephants (sex and age), and different species were visualized using a nonmetric multidimensional scaling (nMDS) based on a Bray–Curtis dissimilarity matrix using the RRA data (square root transformed for normality, which is best suited for dDNA metabarcoding data (Laporte et al., 2021)) of ESVs and OTUs (permutations = 999, $k = 3$; Casey et al., 2019; Kartzinel et al., 2015). Permutational multivariate analyses of variance (perMANOVA) were performed with 999 permutations using the *adonis2* function (Anderson, 2017) in the *vegan* package in R to determine any compositional difference in plant taxa identified between habitats, sex, age, and focal species. An analysis of multivariate homogeneity of group dispersions (betadisper), to evaluate the pattern of dispersion of samples within each category (Anderson et al., 2006). The very conservative Bonferroni correction of the p -values was applied to ascertain the contribution of differences seen with the *pairwise.adonis* function. We tested for differences among species, habitat types, and between sexes in RRA of ESVs and OTUs in our samples using ANOVAs followed by Tukey honest significant difference (Tukey HSD).

3 | RESULTS

3.1 | Description of raw data

We collected a total of 84 fecal samples from three megaherbivores (forest elephant *L. cyclotis* ($n = 58$), buffalo *S. c. caffer* ($n = 13$), and giraffe *G. c. antiquorum* ($n = 13$), during a rapid collection period in the early rainy season of 2019 across three habitat types, Grasslands, Woodlands, and a Human-Altered Landscape (HAL) (Figure 1). Elephant was the only species found in the Woodlands and HAL, as expected. Giraffe inhabit the southern and central savanna grasslands of Garamba NP only (De Meroode et al., 2000; D'haen et al., 2019), while buffalo *S. c. caffer* are not found in the denser woodlands (African Parks, pers. comm 2019).

The average number of GPS locations per elephant throughout 2019 was 6,624 (min = 2,063; max = 8,737). The movement data revealed that elephants spend on average $50 \pm 4\%$ of their time in the forests, $45 \pm 4\%$ in the grasslands, and $4 \pm 0.5\%$ in agricultural areas. Elephants are spending around 34%–62% of their time in the

grasslands each month, thus highlighting it as a constant resource for this population of forest elephants (Figure 1). Because there were only three males collared, we were not able to assess sex difference between habitat use.

Plant diet DNA (dDNA) was successfully sequenced in 80 samples from 57 elephants across the three habitats, and 12 buffalo and 11 giraffe samples in the grasslands (Figure 1, Table 1). dDNA metabarcoding of ITS2 revealed a highly diverse taxonomic composition of plants across all focal species. From the 80 samples sequenced, a total of 1,202,602 raw reads were generated and 2762 Exact Sequence Variants (ESVs), with 1,110,405 reads and 2215 ESVs remaining after conservative filtration and the removal of one elephant sample for poor quality and inability to identify sex (392 ESVs <10 reads; Table S1). The average number of reads per sample was $13,857 \pm 462$ SE (min = 1180, max = 23,009). The total number of ESVs per sample ranged from 11 to 164, with giraffe showing the lowest richness, averaging 51.64 ± 11.02 SE. A total of 188 unique DNA groupings of plant operational taxonomic units (OTUs) were identified among all fecal samples, encompassing 22 orders, 42 families, 122 genera, and 130 species (Table S2). There was only

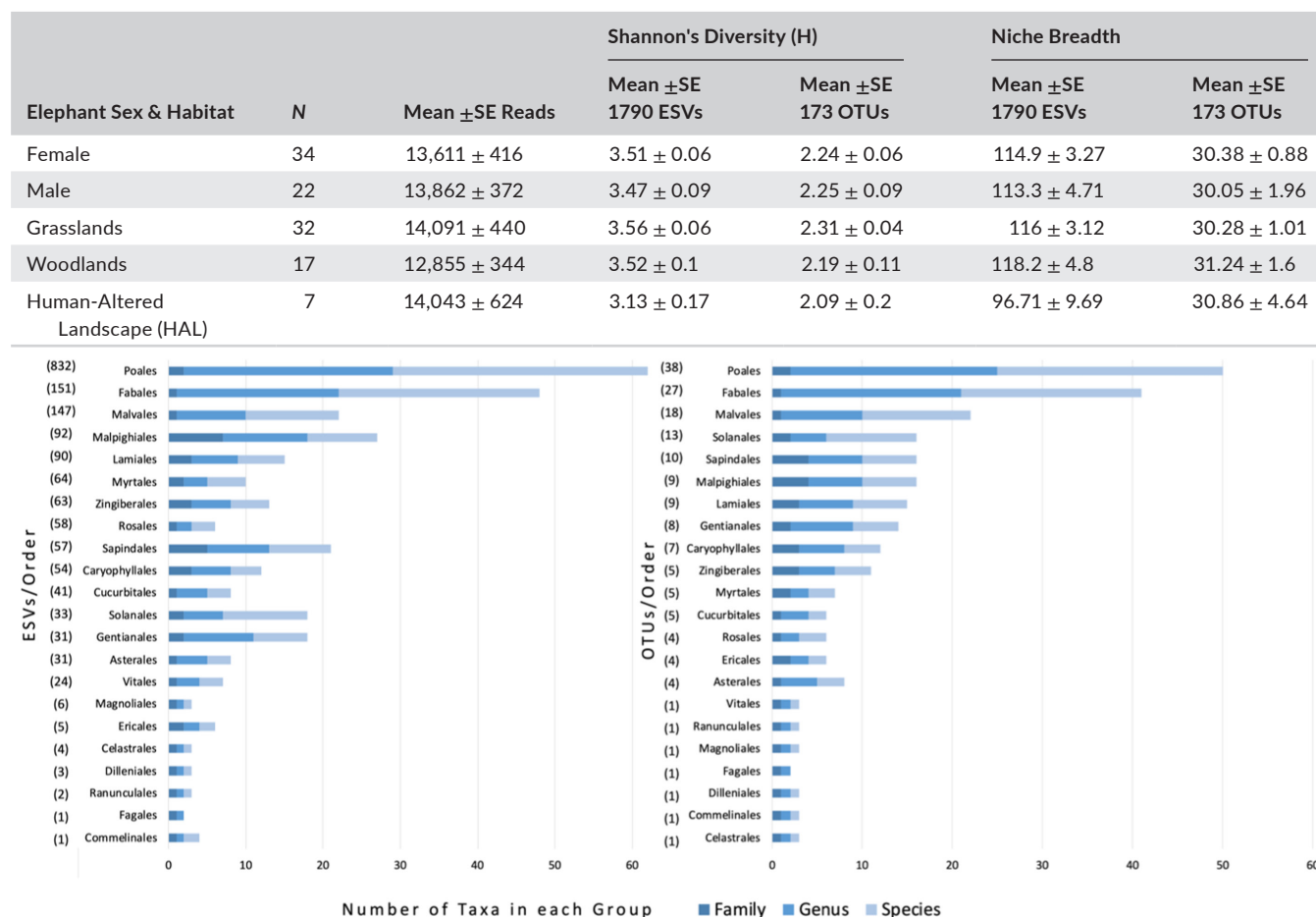
one negative control with reads (45) containing Brassica; no other samples had this ESV, therefore is being removed. According to the species accumulation curves, the estimated asymptote was reached for each of the species when focusing on the OTUs greater than one occurrence (Figure S2).

We recognized a clustering difference in the nMDS plots between the older (± 3 days) versus fresh giraffe samples that were collected (see Figure S3). Further investigation revealed Poaceae (grasses) present in the older samples of these browsers, likely representing wind-blown pollen deposition on the outside of the pellets over time (Kraaijeveld et al., 2015; Meyer et al., 2020). After removing Poaceae from the analysis, the giraffe samples clustered together and are represented in Table 1 (Table. S1 and S2).

3.2 | Elephant diet composition and diversity by habitat, sex, and age

We identified a total of 1790 unique plant ESVs, averaging $13,707 \pm 290$ (SE) reads per sample, in the diet of male ($n = 22$) and

TABLE 2 GNP forest elephant sample size between sexes and among habitat types, with the mean number of reads, Shannon's diversity and richness \pm SE and ESVs per sample for the 1790 taxonomic-free ESVs and 173 unique OTUs (no sex difference for diversity and richness ($p > 0.05$); Habitat difference (ANOVA: $p < 0.05$) for each, diets in the grasslands and woodlands are richer and more diverse than HAL (Tukey's HSD, $p < 0.05$). Bar graph of how each order is represented in the elephant diet with the number of families, genus, and species recorded in each, along with the number of ESVs or OTUs/order listed to the right of the figure



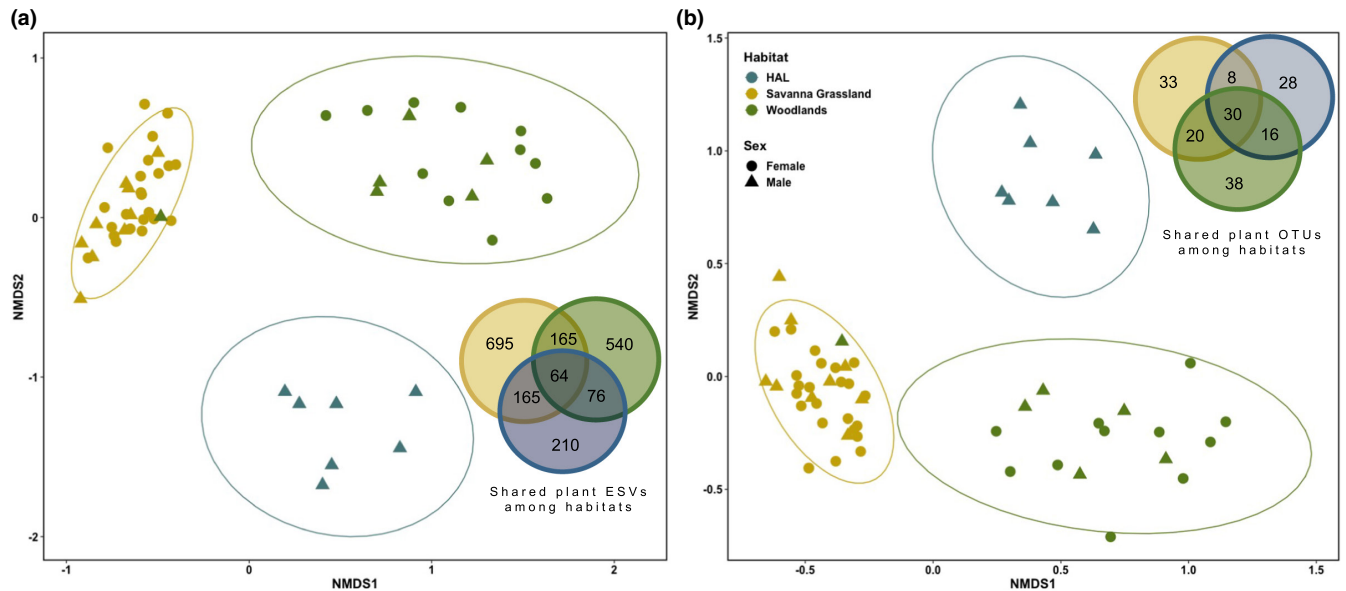


FIGURE 2 Diet divergence between habitats. Non-metric multidimensional scaling plots (nMDS) using a Bray–Curtis dissimilarity of elephant dietary relative read abundance, showing differences in habitat type where samples were collected. Points represent individual samples with 95% confidence ellipses for HAL ($n = 7$, 7 male), savanna grasslands ($n = 32$, 23 female, 9 male), and woodlands ($n = 17$, 11 female, 6 male). Venn diagram represents the number of shared plant ESVs and OTUs among habitat types. Each sample collected confidently provides a representation of the diet of that individual within each habitat for (a) 1790 ESVs (Stress = 0.10, PerMANOVA: $pseudo-F_{2,53} = 10.83$, $R^2 = 0.29$, $p = 0.001$, pairwise $P_{adj} < 0.01$) and for (b) 173 OTUs (Stress = 0.11, $Pseudo-F_{2,53} = 17.36$, $R^2 = 0.40$, $p = 0.001$, pairwise $P_{adj} < 0.01$). There was a sex difference in diet (a) $pseudo-F_{1,54} = 2.06$, $R^2 = 0.04$, $p = 0.02$, pairwise $P_{adj} < 0.01$; (b) $pseudo-F_{1,54} = 2.50$, $R^2 = 0.04$, $p = 0.02$; however, was driven by habitat type: Habitat:Sex (a) $pseudo-F_{1,51} = 1.34$, $R^2 = 0.02$, $p = 0.13$ (b) $pseudo-F_{1,51} = 0.1$, $R^2 = 0.00$, $p = 0.39$

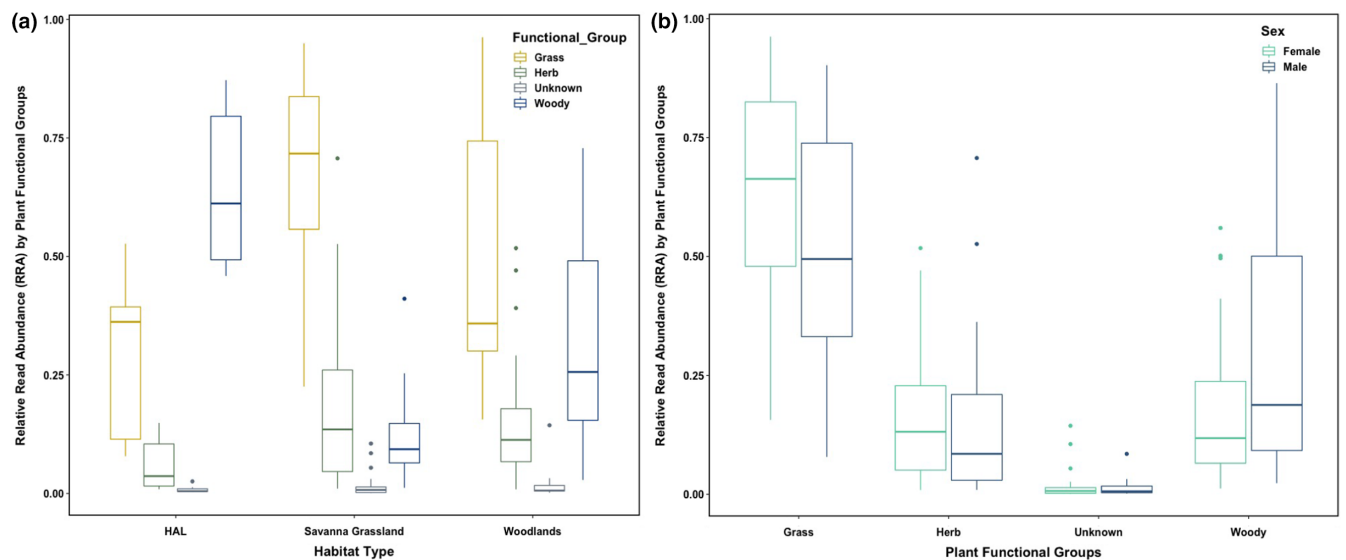


FIGURE 3 Plant functional groups consumed within each habitat and by sex. Box-plot of the mean proportion of plant functional groups (grass, herb, woody (tree/shrub), and unknown) consumed per elephant fecal sample collected a) within different habitat types (Functional Groups:Habitat $F_{6,208} = 20.93$, $p < 0.0001$; More woody vegetation (TukeyHSD $p < 0.05$) was consumed in HAL habitat and more grasses in the Savanna Grasslands and Woodlands ($p > 0.05$) and b) consumed by the different sexes (FG: $F_{3,208} = 92.74$, $p < 0.0001$; FG:Sex $F_{3,208} = 5.46$, $p < 0.001$); males and females consumed significantly more grass and males consumed more woody vegetation compared with females (TukeyHSD $p < 0.05$)

female ($n = 34$) elephants (Table 2). To taxonomically assign ESVs, we included data with a $\geq 90\%$ match to the database, leaving 1527 ESVs and 698,829 reads. For ecological analysis, we combined all the ESVs

with the same taxonomic ID to analyze the plant functional groups and plant diet composition. After combining ESVs, there were a total of 173 plant OTUs representing 22 orders, 39 families, 110 genera,

and 117 species (Table 2; Table S3). Within the elephant diets, there was no difference in the total reads per sample among habitat or between the sexes ($p > 0.05$) (Table 2).

First approaching the diet data through a taxonomic-free lens, we assessed variance among habitat type and elephant sex and age through the 1,790 ESVs identified. The fecal samples collected from each habitat significantly cluster together in the Bray–Curtis dissimilarity nMDS plot (Figure 2a). Although elephants travel between habitats, in our study, we only found one male elephant sample collected in the woodlands that clustered with the savanna grassland habitat, suggesting possible rapid movement between the habitats for that individual. There were no female elephant fecal samples collected in the human-altered landscape, as expected from rangers reporting only male elephant sightings within this area (personal comms.).

Male and female diets significantly differed across the GNPC; however, when accounting for within habitat type, they were consuming similar diets (Figure 2a). Interestingly, we observe similar patterns when focusing on the identified plant OTUs (Figure 2b), showing no difference between taxonomically free ESVs and concentrated OTUs. We found juveniles ($n = 10$) to be consuming similar diets to the adult females ($n = 29$) within the same habitat (Age:Habitat $Pseudo-F_{1,36} = 1.45$, $R^2 = 0.31$, $p = 0.13$).

Dietary diversity and the individual niche breadth were assessed across habitat types and between the sexes. The diet in the grasslands and woodlands were richer and more diverse than in the human-altered landscape, with no sex differences in the woodlands and grasslands (niche breadth: $F_{2,52} = 3.36$ $p = 0.04$; Sex:Habitat $F_{1,51} = 1.26$, $p = 0.27$; Shannon's Diversity $F_{2,52} = 4.09$ $p = 0.02$; Sex:Habitat $F_{1,51} = 0.20$, $p = 0.65$) (TukeyHSD = $p < 0.05$) (full list per habitat: Table S4–S6).

We identified each of the 173 plant OTUs by their functional groups where possible. Some OTUs were only identified to the family or genus level, encompassing multiple functional groups, and were therefore labeled as unknown. Elephants mostly consumed grasses across all habitats, for both males (51%) and females (64%), although males consumed significantly more woody vegetation than female elephants (33% vs 17%; Figure 3b). Herbaceous vegetation comprised the smallest proportion of the diet (16% for both). The proportion of the diet from each plant functional group is approximately aligned with the plant structure of each habitat type (Figure 3a). For example, most of the savanna grasslands consist of grasses and herbaceous plants with few trees (<5% tree cover); the Woodlands are comprised of both grass and woody vegetation, while the HAL habitat is a combination of non-native and native trees and riparian habitat. There was no difference between the sexes in their selection of plant functional groups within each habitat; however, males were only found in the human-altered landscape consuming a significant amount of woody vegetation, driving the overall difference between the sexes in consumed woody vegetation ($p < 0.05$). Elephants consumed more grasses in the savanna grasslands and more woody vegetation in the woodlands compared with the grasslands, as expected ($p < 0.05$) (Figure 3a). Key species

were identified in each habitat type (Table 3), and the full list can be found in Tables S4–S6.

Male and female elephant diet niches do significantly overlap across habitats (Ojk = 0.84, $P(\text{Obs} > \text{null}) = 99\%$); however, we do see some differences in specific plant selection (Figure 4). Males consumed plants from ten different family groups that females did not, and females only had four different family groups. We compared the RRA of plant families that were consumed between the sexes and made up >0.1% of the diet (Figure 4a). While both sexes consumed 58% (101 OTUs) of the same plant taxa, males ate 28 different OTUs while females ate 44 unique OTUs (Figure 4c), noting the differences in sample size for males and females. Of those differences, females consumed more different taxa of woody vegetation whereas males chose a varied diet of grasses vegetation (females 22 woody plant taxa compared to 7 for males, 2 vs 7 of grasses, 18 vs 12 of herbs, and 2 vs 2 of unknown vegetation).

Our nMDS model demonstrated that families chose a more similar diet to each other, compared with other family groups within the same habitat, with Bray–Curtis dissimilarity scores significantly clustering for each group ($pseudo-F_{5,41} = 6.3$, $R^2 = 0.47$, $p > 0.001$; pairwise $P_{adj} > 0.05$ for savanna family groups) (Figure 5). Within the woodlands, all families are different from one another ($p > 0.01$), however, not when adjusting the p-value as a result of small sample size.

We wanted to know if elephants were consuming pioneer woody species while in the grasslands. These genera include *Combretum*, *Mimosa*, *Piliostigma*, *Vitex*, *Terminalia*, and *Grewia*. Across all habitat types, these species made up 8% of the RRA of the elephant's diet, with the majority being consumed in the HAL habitat (61%), followed by the grasslands (24%). These species were found frequently in the diet, with the mean number of pioneer ESVs per sample in the Grasslands at 2.9 ± 0.2 (SE), Woodlands 2.1 ± 0.4 , and HAL 5.6 ± 0.3 . *Piliostigma reticulatum* was in 100% of the fecal samples collected within the grasslands and *Vitex* in 60% of the samples.

3.3 | Megaherbivore niche partitioning

We assessed the diets of two additional megaherbivores (>1000 kg), the buffalo and giraffe, in the GNPC to compare the dietary niche of elephants across habitats and within the grasslands. Elephant and buffalo consumed a richer and more diverse diet than the giraffe when assessing ecological patterns with ESVs, while the elephant overall consumed a greater diversity and on average more plant OTUs (Table 1).

We used nMDS to visualize patterns in dietary dissimilarity within and among megaherbivores (Figure 6). The diets differed strongly among species, with a minor overlap of samples, both across all habitats (Figure 6a, c) and when within the same grassland habitat (Figure 6b, d). To elucidate within-guild niche segregation, we ran perMANOVA and found that species consume a diet that is significantly more similar to conspecifics and less similar to other species for both ESVs and OTUs (Figure 6).

TABLE 3 Top ten plant taxa in the forest elephant diet within the grasslands, woodlands, and human-altered landscape (HAL) and the megaherbivore diet within the grasslands using frequency of occurrence (FOO) and the mean relative read abundance (RRA) across all samples

Elephant Top 10 Taxa in Grasslands								
Family	Grassland OTUs	Plant Form	FOO	Family	OTUs	Plant Form	Mean RRA (N = 32)	±SE
Poaceae	Poaceae	Grass	1.00	Poaceae	Poaceae	Grass	16.55%	1.96%
Poaceae	<i>Setaria parviflora</i>	Grass	1.00	Poaceae	<i>Setaria parviflora</i>	Grass	12.97%	2.03%
Poaceae	<i>Hyparrhenia</i>	Grass	1.00	Poaceae	<i>Hyparrhenia</i>	Grass	7.75%	1.63%
Poaceae	<i>Digitaria</i>	Grass	1.00	Poaceae	<i>Digitaria</i>	Grass	7.59%	1.58%
Poaceae	<i>Megathyrsus maximus</i>	Grass	1.00	Poaceae	<i>Urochloa mutica</i>	Grass	4.87%	1.85%
Ograceae	<i>Ludwigia abyssinica</i>	Herb	1.00	Poaceae	<i>Leersia oryzoides</i>	Grass	3.18%	1.18%
Fabaceae	<i>Piliostigma reticulatum</i>	Woody	1.00	Poaceae	<i>Megathyrsus maximus</i>	Grass	3.14%	0.60%
Poaceae	<i>Brachiaria ramosa</i>	Grass	1.00	Poaceae	<i>Panicum repens</i>	Grass	3.14%	0.90%
Poaceae	<i>Paspalum</i>	Grass	0.94	Poaceae	<i>Paspalum</i>	Grass	3.00%	0.79%
Poaceae	<i>Schizachyrium</i>	Grass	0.94	Ograceae	<i>Ludwigia abyssinica</i>	Herb	2.92%	0.59%
Elephant Top 10 Taxa in Woodlands								
Family	Woodland OTUs	Plant Form	FOO	Family	OTUs	Plant Form	Mean RRA (N = 17)	±SE
Poaceae	Poaceae	Grass	1.00	Poaceae	Poaceae	Grass	18.00%	3.92%
Poaceae	<i>Brachiaria ramosa</i>	Grass	1.00	Poaceae	<i>Setaria parviflora</i>	Grass	10.57%	4.24%
Poaceae	<i>Paspalum</i>	Grass	0.94	Lamiaceae	<i>Clerodendrum silvanum</i>	Woody	8.65%	3.10%
Poaceae	<i>Setaria parviflora</i>	Grass	0.88	Moraceae	<i>Ficus drupacea</i>	Woody	7.67%	1.92%
Moraceae	<i>Ficus drupacea</i>	Woody	0.88	Poaceae	<i>Paspalum</i>	Grass	6.34%	2.14%
Phyllanthaceae	<i>Bridelia micrantha</i>	Woody	0.88	Costaceae	<i>Costus dubius</i>	Herb	5.15%	2.58%
Fabaceae	<i>Desmodium velutinum</i>	Woody	0.88	Poaceae	<i>Setaria plicata</i>	Grass	4.97%	1.98%
Costaceae	<i>Costus dubius</i>	Herb	0.82	Poaceae	<i>Cenchrus purpureus</i>	Grass	3.48%	2.97%
Poaceae	<i>Setaria plicata</i>	Grass	0.82	Marantaceae	<i>Hypselodelphys hirsuta</i>	Herb	3.35%	4.23%
Poaceae	<i>Schizachyrium</i>	Grass	0.82	Fabaceae	<i>Dalbergia</i>	Woody	2.84%	1.05%
Elephant Top 10 Taxa in HAL								
Family	Human-Altered Landscap OTUs	Plant Form	FOO	Family	OTUs	Plant Form	Mean RRA (N = 7)	±SE
Malvaceae	<i>Grewia</i>	Woody	1.00	Malvaceae	<i>Grewia</i>	Woody	29.22%	6.70%
Poaceae	<i>Hyparrhenia</i>	Grass	1.00	Poaceae	<i>Hyparrhenia</i>	Grass	14.44%	6.44%
Malvaceae	<i>Grewia trichocarpa</i>	Woody	1.00	Moraceae	<i>Ficus drupacea</i>	Woody	12.51%	8.08%
Fabaceae	<i>Piliostigma reticulatum</i>	Woody	1.00	Poaceae	<i>Cenchrus</i>	Grass	6.26%	2.25%
Fabaceae	<i>Piliostigma thonningii</i>	Woody	1.00	Malvaceae	<i>Grewia trichocarpa</i>	Woody	5.83%	1.45%
Poaceae	Poaceae	Grass	1.00	Fabaceae	<i>Piliostigma reticulatum</i>	Woody	2.78%	1.05%
Fabaceae	Fabaceae	Woody	1.00	Asteraceae	<i>Vernonia cumingian</i>	Woody	1.90%	
Moraceae	<i>Ficus drupacea</i>	Woody	0.86	Poaceae	<i>Paspalum</i>	Woody	1.87%	1.91%

TABLE 3 (Continued)

Elephant Top 10 Taxa in HAL								
Family	Human-Altered Landscap OTUs	Plant Form	FOO	Family	OTUs	Plant Form	Mean RRA (N = 7)	±SE
Poaceae	<i>Cenchrus</i>	Grass	0.86	Anacardiaceae	<i>Mangifera zeylanica</i>	Woody	1.79%	0.52%
Anacardiaceae	<i>Mangifera zeylanica</i>	Woody	0.86	Anacardiaceae	<i>Mangifera</i>	Woody	1.77%	0.50%
Megaherbivore Top 10 Taxa in Grasslands								
Family	Grassland OTUs	Plant Form	FOO	Family	OTUs	Plant Form	Mean RRA (N = 55)	±SE
Poaceae	Poaceae	Grass	0.80	Poaceae	Poaceae	Grass	17.97%	2.16%
Poaceae	<i>Hyparrhenia</i>	Grass	0.80	Lamiaceae	<i>Vitex triflora</i>	Woody	10.04%	3.30%
Poaceae	<i>Megathyrus maximus</i>	Grass	0.80	Poaceae	<i>Hyparrhenia</i>	Grass	5.36%	1.10%
Onagraceae	<i>Ludwigia abyssinica</i>	Herb	0.80	Poaceae	<i>Setaria parviflora</i>	Grass	7.66%	1.53%
Lamiaceae	<i>Vitex triflora</i>	Woody	0.76	Poaceae	<i>Digitaria</i>	Grass	4.64%	1.12%
Poaceae	<i>Digitaria</i>	Grass	0.76	Poaceae	<i>Megathyrus maximus</i>	Grass	3.59%	0.97%
Poaceae	<i>Schizachyrium</i>	Grass	0.76	Annonaceae	<i>Annona squamosa</i>	Woody	6.69%	1.33%
Poaceae	<i>Brachiaria ramosa</i>	Grass	0.76	Poaceae	<i>Schizachyrium</i>	Grass	3.49%	1.27%
Poaceae	<i>Setaria parviflora</i>	Grass	0.71	Poaceae	<i>Urochloa mutica</i>	Grass	2.96%	1.29%
Fabaceae	<i>Piliostigma reticulatum</i>	Woody	0.69	Malvaceae	<i>Sida</i>	NA	2.89%	1.69%

When using Pianka's niche overlap, we find the elephant and buffalo diet overlapped the most in the grasslands when assessed using OTUs ($O_{jk} = 0.72$, $P(\text{Obs} > \text{null}) = 99\%$) and to a lesser extent when assessed with ESVs ($O_{jk} = 0.59$, $P(\text{Obs} > \text{null}) = 99\%$). The majority of their diets consisted of grasses in the grasslands (Figure 7); however, the diversity of those grasses differed greatly both across habitats and even within the grasslands (Figure 7). Although buffalo and elephants partially overlapped in their diet of grasses (Poaceae), buffalo generally consumed more herbs (Lamiaceae), while elephants preferred more tree and legume taxa (Fabaceae). Elephants ate 107 unique OTUs not found in the diets of other species across habitats and 33 different plants within the grasslands (Figure 7). While the giraffe almost exclusively consumed woody vegetation, the trees consumed by the giraffe were different from those consumed by elephants.

4 | DISCUSSION

4.1 | Forest elephants in the grasslands

The recently separated forest elephant *L. cyclotis* is the lesser-known and rarer of the two African elephant species, in part because they are found almost exclusively in the remote central African rainforest and other forested areas of West Africa. In a transition zone between these forests and the grasslands, we find a unique and rather isolated population of forest elephants that spend much of their time out on the savanna grasslands of the greater Garamba National

Park Complex (GNPC; De Merode et al., 2000; Hillman-Smith et al., 1995). These elephants seem to be exhibiting behavioral plasticity—shifting to consume a large proportion of grasses and possibly playing a functional role in maintaining the grassland system, in contrast to their typical frugivorous diet and role of “gardening” forests (Blake et al., 2009; Campos-Arceiz & Blake, 2011; Cardoso et al., 2019; Theuerkauf et al., 2000).

Metabarcoding of fecal diet DNA (dDNA) samples provided valuable insights into the plant biodiversity of each habitat type. We found individual elephant diet composition to significantly cluster within the broader habitat type in which it was found (Figure 2), therefore confidently providing a representation of the diet of that individual within each habitat. This is valuable to know for elephants, as a fecal sample could be deposited far away from where food was consumed since it takes approximately 40 h for foodstuff to pass through the elephant gut (Beirne et al., 2019) and elephants can travel large distances in short periods. We did have one male sample that might represent this movement between habitats. Detailed corroboration of the frequency, relative abundance, and identities of plant species in each sampling area is thus a valuable way to assign plants to the area from which dung was collected (Table 3).

In the early wet season of GNPC, the largest population of forest elephants in the Congo consumed mostly grasses in both the grassland (69%) and woodland (45%) habitats, however, composed of different species compositions (Figures 4, 7; Table 3). Elephants consumed an abundance of the dominant grass genera, *Hyparrhenia* in the grasslands and human-altered landscape (HAL), and *Setaria*

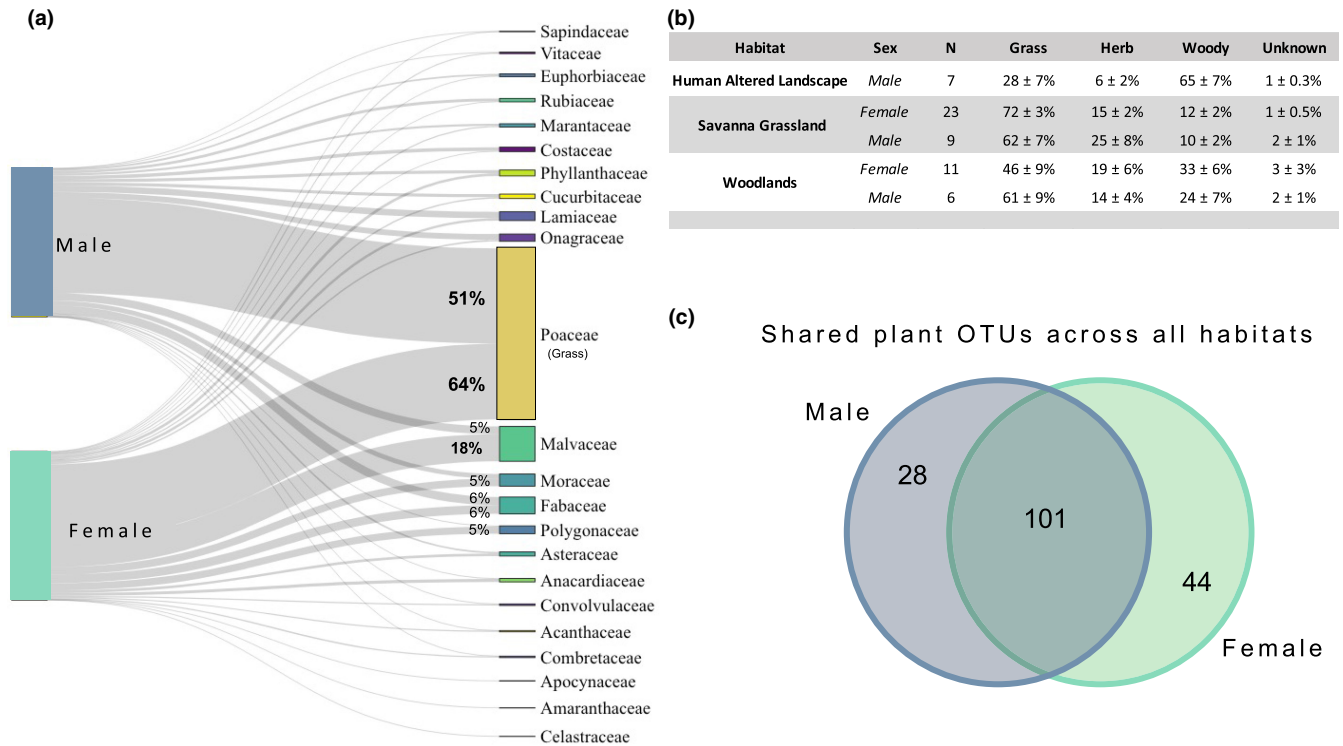


FIGURE 4 Male and female forest elephant diet composition. (a) The relative read abundance (RRA) per plant family ($\geq 0.1\%$) consumed by male ($n = 22$) and female ($n = 34$) elephants (percentages indicate the most abundant plant family in each diet). (b) Sex preference for plant functional groups while in the same habitat. Mean RRA \pm Standard Error of plant functional groups (grass, herb, woody, and unknown) consumed in each habitat type by elephant sex. Male and females consumed a similar abundance of plant groups within each habitat ($p > 0.05$). (c) The Venn diagram represents the number of shared plant OTUs between the sexes across all habitats, in which diet niches significantly overlap ($O_{jk} = 0.84$, $P(\text{Obs} > \text{null}) = 99\%$)

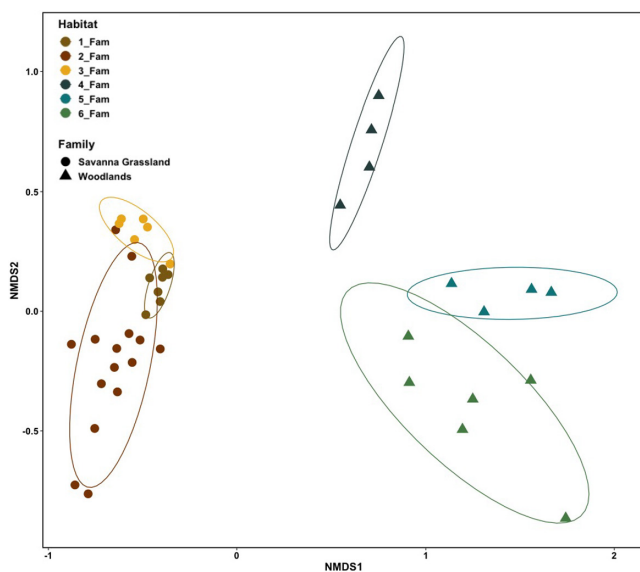


FIGURE 5 Diet divergence between forest elephant families. nMDS plot showing how predicted family groups are significantly clustering (stress = 0.08; $F_{5,41} = 6.3$, $R^2 = 0.47$, $p > 0.001$; pairwise comparison shows all families within the grasslands are different from one another ($P_{adj} < 0.05$), and in the woodlands, however, not when adjusting P -value)

parviflora in the woodlands and grasslands, while other species were specific to habitats (e.g., *Cenchrus purpureus* in the woodlands and *Urochloa mutica* in the grasslands). The grass abundance in the diet is similar to the savanna elephant diet in the wet season (Cerling et al., 2006; Shannon et al., 2013) and contrary to other studies of forest elephant diet where they found almost no consumed grasses (Beirne et al., 2020; Blake et al., 2009; Campos-Arceiz & Blake, 2011; Danquah & Oppong, 2006; Short, 1981; Theuerkauf et al., 2000; White et al., 1993). In a region where forest elephants spend a large amount of time along a forest edge, they consumed fruit and browse and not grasses (Cardoso et al., 2019). However, this and all previous studies on forest elephant diet have used morphological analysis, and not dDNA metabarcoding, which is more sensitive to the detection of different plant species and genotypes (Bush et al., 2019; Elbrecht et al., 2017; Meyer et al., 2020). Interestingly, a recent study found a higher C_4 isotopic signature indicative of more grasses and shrubs in West African forest elephant ivory from the 1600s, grouping closer with the isotopic signature of savanna elephant diets (de Flamingh et al., 2021). Thus, the presence of grass may have been missed in their diet to a degree that there are regional variations in their diet selection and/or their foraging behavior has shifted over time.

Although this study only captured a snapshot of the wet season diet, the annual collar data reveal elephants using the grasslands

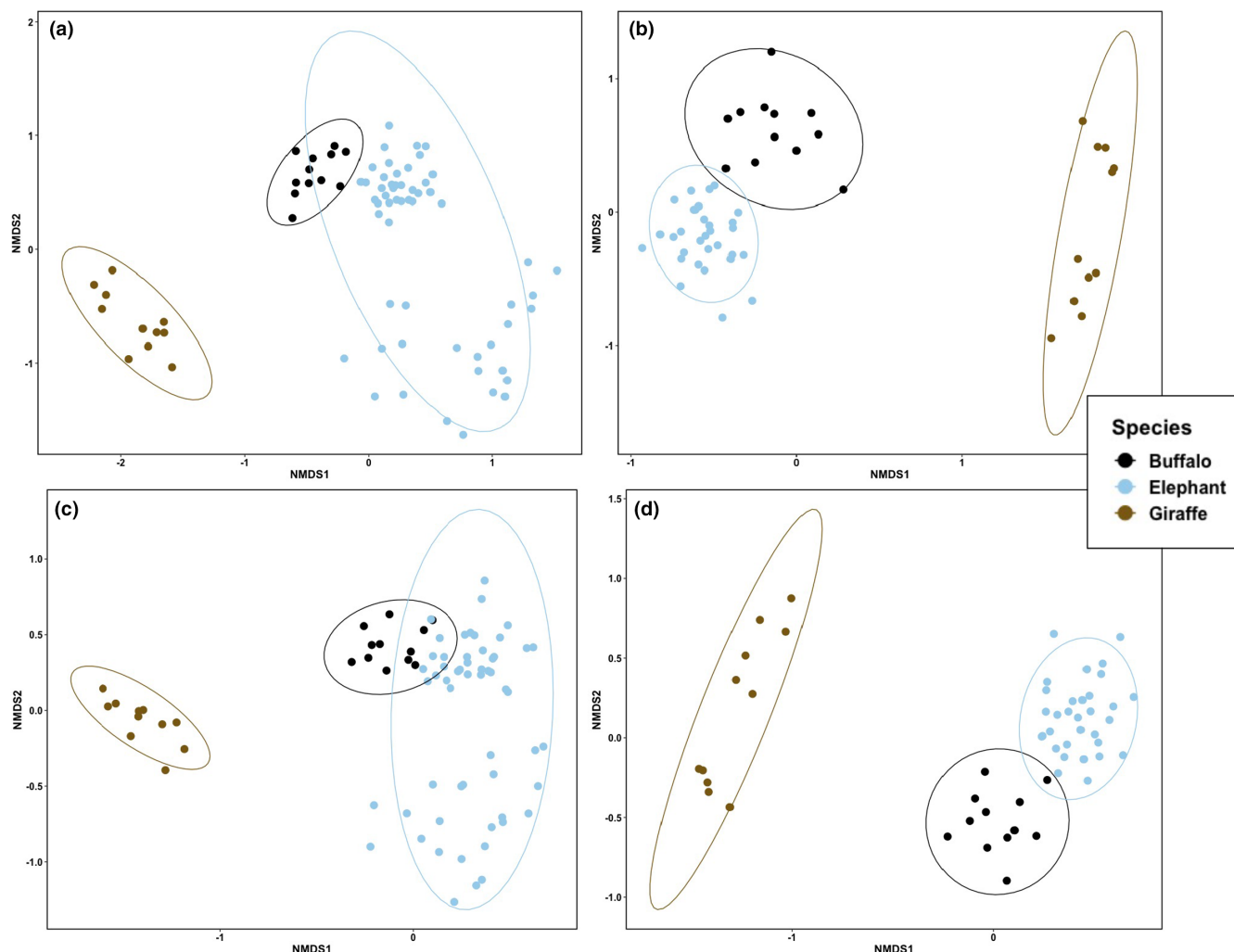


FIGURE 6 Niche partitioning as a function of habitat type and ESVs vs OTUs. Points represent individual samples with 95% confidence ellipses for elephant (a, c) $n = 56$ and (b, d) $n = 32$, buffalo ($n = 12$), and giraffe ($n = 11$). NMDS plots using a Bray–Curtis dissimilarity of mammal species dietary relative read abundance (a) of 2215 ESVs across habitats (stress = 0.10; perMANOVA: $pseudo-F_{2,78} = 10.1$, $R^2 = 0.21$, $p > 0.001$) and (b) of 1416 ESVs within the savanna grasslands only (stress = 0.10; $pseudo-F_{2,54} = 13.6$, $R^2 = 0.34$, $p > 0.001$). NMDS representing (c) the 188 of uniquely identified OTUs across all habitats (stress = 0.10; $pseudo-F_{2,78} = 16.1$, $R^2 = 0.30$, $p > 0.001$) and (d) within the savanna grasslands only (114 OTUs) (stress = 0.09; $pseudo-F_{2,54} = 24.1$, $R^2 = 0.48$, $p > 0.001$). Diet compositions of large mammal species were all significantly different between each other after pairwise Bonferroni correction for multiple comparisons ($P_{adj} = 0.003$)

throughout the year (average of 45% each month), irrelevant of the season (Figure 3). One study conducted in another grassland-forest mosaic landscape found forest female elephants to be spending 68% of their time in forests and 30% in grasslands driven by season (Mills et al., 2018), quite different than this population. There could be other factors that are driving the use of the grasslands outside of the seasons, possibly fear of poaching from the northern section of the park (De Merode et al., 2000).

These unique behaviors may be assisting in fulfilling the role of the locally extinct savanna elephant. Genetic evidence of their existence remains in the forest-savanna hybrid signature in GNPC populations (Ishida et al., 2011a, 2011b; Roca et al., 2005); however, this study suggests that the hybridization occurred sometime in the distant past. Savanna elephants were most likely the first to

be hunted out for their ivory by Europeans since their tusks are twice as long as forest elephants (Grubb et al., 2000) and they are more easily found in the open savannas, as suggested in other hybrid zones (Mondol et al., 2015). The extent of hybridization within this population and the potential behavioral differences of hybrid individuals have not been addressed, and we will be assessing this in future research.

Without the high numbers of elephants and other megaherbivores favoring woody vegetation (Daskin et al., 2016; Pringle et al., 2016; Shannon et al., 2008), this unique savanna grassland may transition into forest (Wolf et al., 2015; Young et al., 2014, 2016). Aerial photographs and remote sensing show that the northern grasslands of GNPC are being reclaimed at a rapid rate by the surrounding woodlands, an area where elephants rarely venture due to

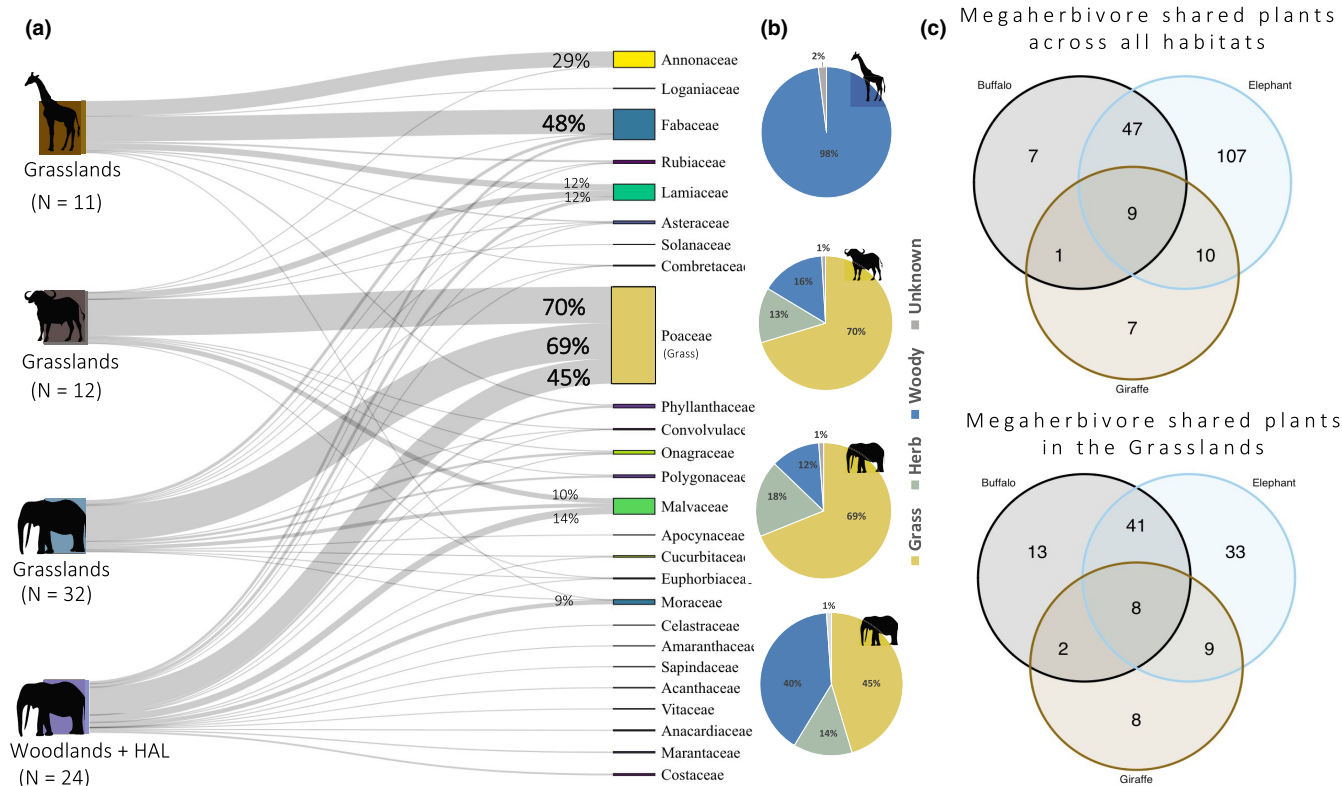


FIGURE 7 Large mammal diet composition. (a) Bipartite networks depicting diet composition using relative read abundance (RRA) per plant family ($\geq 0.1\%$ of diet) and for each focal species in the grasslands and additionally for elephants the two wooded habitats (woodlands + HAL) combined to represent woodlands (left), where percentages indicate the RRA of the most abundant plant families in the diet for each species. (b) The proportion of diet comprised of grasses, herbaceous, and woody vegetation for each focal species (unknown represents OTUs where plant functional group could not be determined). (c) Overlap of plant taxa in the diets of megaherbivores across all habitats and within the grasslands, note difference in sample size

human threats (De Merode et al., 2000; Hillman Smith et al., 2014). Although other factors, such as fire and climate change, cannot be discounted, extensive studies in Africa have shown that megafauna, especially elephants, can reduce woody species cover by up to 95% (Asner et al., 2016; Bakker et al., 2016; Keesing & Young, 2014).

In this study, the elephant diet frequently contained known pioneer woody species, such as *Combretum* and *Terminalia*, which were represented in all samples while foraging in the grasslands. The most abundant and frequently consumed species *Piliostigma reticulatum* is an important shrub that once established promotes the growth of other woody species (Hernandez et al., 2015). Also, *Piliostigma*'s evolutionary strategy for fire is to rapidly rebuild aboveground structures (Midgley et al., 2010), thus important for it to be controlled for the maintenance of the grasslands. Therefore, forest elephant gardening could be responsible for reducing encroaching woody species in the south of the park where elephants are concentrated; however, currently their low numbers may not allow for a significant impact in the north. We also did not find any *Mangifera* (mango) or other gallery forest tree taxa in the diet of elephants in the savanna, suggesting that even if they are being dispersed from the HAL and woodlands, they are not seeding in these gardened grasslands of the south.

4.2 | Intrinsic factors affecting diet

Sexual differences in diet and habitat use have been recorded in many sexually dimorphic species, including savanna elephants (Shannon et al., 2013; Stokke & Du Toit, 2000; Woolley et al., 2009); however, to date no studies have investigated this in forest elephants. We found that male and female forest elephants seem to have similar diet preferences in the same habitat, both mostly selecting kinds of grass (51% and 63% respectively). Males, however, did consume a greater abundance of woody vegetation within the HAL, a habitat only they entered (Figure 2). Minor differences between sexes emerged when we investigated the 42% of plant OTUs unique to males or females. Females ate more woody plants (however in low abundance), while males ate more grass OTUs (Figure 4). The non-significant difference could be driven by the abundance of new grass during the growing season that the study was conducted in; however, we found that these forest elephants spend an equal proportion of their time in these habitats throughout the year anyway. The generalizability of the results is, however, limited by reduced male sample size within the woodlands and grasslands. This was also the case for GPS-collared individuals. Further studies with a greater number of male samples and across seasons would be advised.

Typically, males from sexually dimorphic species are more willing to tolerate the risk of predation to seek more abundant, high-quality forage, whereas females may sacrifice forage quality to minimize risk (Hay et al., 2008; Main et al., 1996; Pellman et al., 2017). In the HAL, where non-indigenous fruits are plentiful and enticing, only male dung samples were found, the diet of which showed an abundance of figs and ripe non-native mangos. Females have never been seen within this area of GNPC (AP unpublished data), supporting the hypothesis that male elephants are more willing to take risks for an energetic pay-off. This is also evident in savanna elephants, where males were willing to take a higher risk than females to crop-raid for nutrient-rich resources (Chiyo & Cochrane, 2005; Chiyo et al., 2011; Songhurst et al., 2016).

Habitat configuration shapes the social structure and behavior of animals (He et al., 2019), and it is known that the forest elephant's small social structure is very different from the large savanna elephant family groups as a result of habitat (Goldenberg et al., 2020). We identified possible family groups (using GPS collar data as described above) within the grasslands and woodlands and discovered individuals from each "family" group consumed more similar diets than other family groups within the grasslands (Figure 5). Diet selection is thought to be learned by juveniles eating as their mothers do, as we recorded juveniles eating the same as adult females in this study, but our data suggest that these family choices may persist even in the same proximate environment. In other words: elephants are choosy, even when offered the same buffet. These groups out on the grasslands are also seen in large numbers (Hillman Smith et al., 2014; pers. obs.), similar to savanna elephants; thus, the annual use of grasslands might also be changing the size of their social groups. Although beyond the scope of this study, it would be beneficial to identify true family groups and to better understand the social dynamics and possible effects of these "larger" groups, differing diets, and habitat use compared with previous forest elephant studies. Indeed, the study of forest elephant ecology in these different habitats and over longer periods of time is greatly needed.

4.3 | Fulfilling a niche in the grasslands

Although diets vary seasonally, our data focused on the early wet season when resources are abundant, and species had equal access across the open grasslands and woodlands. Elephants were, however, the only species we found samples for in the woodlands and HAL. Kordofan giraffe *G. c. antiquorum*, which are typically more suited to savanna woodland habitat, were more abundant and restricted their movements in the southern and central savanna grasslands of Garamba NP only (De Merode et al., 2000; D'haen et al., 2019). Buffalo *S. c. caffer* are found in the central and southern sector of the park, but not in the denser woodlands where elephants were found (African Parks, pers. comm 2019).

Dietary niche partitioning occurred among the three megaherbivores (forest elephant, Kordofan giraffe, and buffalo) in the grasslands, with each species consuming significantly different

diets (Figure 6), as expected. The giraffe diet was comprised mostly of trees in the Fabaceae (Legumes), Lamiaceae (genus *Vitex*), and Annonaceae (Custard apples) families. This coincides with previous findings of giraffe in GNPC eating *Vitex doniana* and *Piliostigma thonningii* (Fabaceae) (D'haen, 2017). It is interesting to note that in West Africa, giraffe (*Giraffa camelopardalis peralta*) have been recorded avoiding the genera *Annona* (Caister et al., 2003), whereas we found it in the diet of all giraffes sampled. For both the buffalo and elephant, Poaceae (grasses) made up the greatest proportion of their diets (~70%); however, the diversity of the grasses differed (Figure 7). The diversity of the buffalo diet (64 OTUs, 503 ESVs) far exceeded expectations compared with morphological diet studies (21 to 39 plant species (Erena et al., 2019) (Landman & Kerley, 2001)). However, our results were similar to a dDNA metabarcoding study, which also found buffalo to be taxonomic generalists (142 taxa), having the most diverse diet of a large spectrum of herbivores (Kartzinel & Pringle, 2020). African buffalo are known to add browse to their diet during the dry season (Erena et al., 2019); however, in this study woody taxa made up 16% of the diet in the wet season, most likely consuming small saplings while grazing and contributing to the grassland maintenance. Our findings are consistent with models of other large mammal herbivores niche partitioning within similar habitats (Cromsigt & Olff, 2012; Kartzinel et al., 2015).

Metabarcoding identified frequently consumed and the relative abundance of key plant taxa in each of the habitats (Table 3; Table S4–S6). Elephants are targeting fruiting trees such as *Ficus* and the non-native *Mangifera* in the woodlands and HAL, while also consuming an abundance of the genera *Dalbergia* and *Grewia* that are both often found in savanna elephant diets and only the *Grewia* in forest elephants diets (Theuerkauf et al., 2000). *Desmodium velutinum* is an important legume in the woodlands, with high nutritive quality of edible material and low amounts of tannins. The central species in the megaherbivore grassland food web are grasses, the herbaceous genus *Ludwigia* and woody genera *Vitex* and *Piliostigma*. Camel's thorn *Piliostigma reticulatum* is key in the grasslands for elephants and other megaherbivores, which again is a pioneer species that is heavily encroaching in the northern section of the grasslands. Monitoring these essential plant species in the greater system will enable management to determine the impacts of interventions and understand the food web trophic perturbations (Jordán, 2009).

4.4 | Taxonomic-free assessment in remote areas

Metabarcoding is a relatively new approach in assessing the diets (Pompanon et al., 2012), biodiversity (Leempoel et al., 2020), food webs (Meyer et al., 2020), and ecosystems (Bush et al., 2019). Much is to be learned, as the field is growing and a major limitation is the databases used for identifying species (Elbrecht et al., 2017; Hestetun et al., 2020), especially in remote and lesser-studied regions. Comparing taxonomically free ESVs and combined taxa ESVs (labeled as OTUs in paper), both captured the same ecological patterns, therefore can apply metabarcoding in remote areas even

where the database may be lacking, question specific. The large number of ESVs creates more variance among individuals and identified a finer scale of diversity to be assessed that might otherwise be missed within an ecosystem. Reviewing these data, we see over 800 ESVs were identified in the Order Poales, which combined to just 38 individual OTUs, suggesting much DNA variance in the grasses or few species in the database. While the taxonomy-free approach allows for ecological pattern identification, it does not allow for key species identification within ecosystems, an important aspect to understand when exploring food webs and interspecies interactions in dynamic and recovering landscapes.

It must be noted that the variance in dDNA recovery among digested plant taxa could be limiting. For example, grasses and woody tree material are less digested than fruits and, therefore, might bias the abundance of grasses that are being identified versus the fruits. Therefore, it is important to not only look at the RRA but also the frequency of plant taxa within the diet, to possibly identify any discrepancies (Table 3).

4.5 | Conservation implications and conclusion

Our dDNA metabarcoding approach revealed a detailed insight into the diet selection of the critically endangered forest elephant and Kordofan giraffe and the Cape buffalo. Each of these megaherbivores had its niche function within the savanna grasslands, assisting in sustaining or rehabilitating these unique grassland habitats that are diminishing globally (Hillman-Smith et al., 2014; Sala & Maestre, 2014). Additionally, the data created from these analyses provided an effective biodiversity monitoring mechanism across the different habitats.

Forest elephants are, for the most part, thought to spend most of their time in the forests and consuming fruits and browse, spending a small proportion of time, if any, on grasslands. This limits the areas in which protection is prioritized for forest elephants, when in fact, as this study shows, they can utilize grasslands throughout the year. For example, the IUCN Red List of Threatened Species has developed methods for the estimation of the Area of Habitat (AOH) that is habitable by a particular species and using this to guide conservation for the species (Brooks et al., 2019). The two habitats labeled of "Major Importance" for forest elephants are Forest-Subtropical/Tropical Moist Lowland and Forest-Subtropical/Tropical Swamp, with Savanna listed as "Marginal," while grasslands are not included. Broadening the scope of habitable landscapes and realizing their potential of maintaining both forests and grasslands is fundamental for both the elephants and these habitats.

The forest elephants seem to be playing a critical role in the maintenance of the southern grasslands of Garamba National Park, as they are spending half of their time in the grasslands throughout the year, and as this study captured in a snapshot of time, consuming large quantities of grasses and selecting pioneer woody species while they are in the grasslands. This leads us to reconsider their purely "frugivorous" status and highlights regional variations in their

ecology. As African Parks continue to secure the park the elephant population will continue to recover and move back into the northern area of the park to restore the grasslands where woody encroachment is the highest (Hillman-Smith et al., 2014). Our future research will aim to use fecal eDNA to identify the proportion of hybrids in this population and determine whether the savanna elephant genetic signature is possibly driving this unique elephant ecology. Future studies should also monitor the group dynamics and fecundity of this population to determine other behavioral changes related to this abundant and novel resource. It is clear though that we need to protect the current megaherbivores of this area to maintain this essential ecosystem within the Congo Basin.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

JM designed the study, performed the field and laboratory work, analyzed the data, and wrote the manuscript. NC supported the design of the study and sample collection. EH supervised the research and contributed to editing the manuscript. All authors contributed to the article and approved the submitted version.

DATA AVAILABILITY

The dataset presented in this study is available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.sxksn035d>

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