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Minimal Sample, Maximum Insight: DNA Metabarcoding Uncovers the Diet of Endangered Dholes in Central India

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ABSTRACT

Understanding the dietary ecology of endangered carnivores is critical for their conservation and management, as it elucidates their interaction with prey, other co-occurring carnivores, and humans. We used DNA metabarcoding to examine the seasonal and spatial variation in the diet of dholes (*Cuon alpinus*) in Tadoba Andhari Tiger Reserve, a key stronghold for the species in central India. We evaluated a simpler non-invasive fecal sample collection method and optimized a novel blocking oligonucleotide to suppress host DNA amplification. Our results revealed that pinches of scat in lysis buffer, coupled with N-spacer-tagged primers for amplifying diet DNA, significantly improved prey detection, sequencing efficiency, and reduced host contamination compared to traditional whole-scat-in-silica methods. Across 169 genetically confirmed dhole scats collected over two different seasons, we identified 15 vertebrate prey species. Dhole diet was dominated by wild ungulates, especially chital (*Axis axis*) and sambar (*Rusa unicolor*), with negligible consumption of domestic species. However, we observed strong seasonal and spatial variation; monsoon diet was diverse with greater variability, while winter diet was more consistent and dominated by large-bodied prey. Core zone exhibited wider dietary profiles than buffer areas that included mixed use landscapes. These findings suggest that wild prey availability, habitat quality, and seasonal foraging constraints shape dhole dietary patterns. Our study underscores the value of improved sampling and sequencing strategies for carnivore dietary studies and highlights the importance of sustaining wild prey populations to ensure the long-term persistence of dholes in multi-use landscapes.

1 | Introduction

A comprehensive understanding of the dietary requirements and trophic interactions of a species and the factors that influence them is a fundamental aspect of species ecology (Estes et al. 2011; Shehzad et al. 2012). Carnivores are among the species most affected by the ongoing biodiversity crisis and habitat loss due to their large home ranges, low population densities, and slow growth rates, conflict with humans further compounding these threats (Fernández-Sepúlveda and Martín 2022; Ripple et al. 2014; Torres-Romero et al. 2025). Their global decline and local extinction underscore the need to quantify their dietary requirements due to their ecological importance (Estes et al. 2011; Hacker et al. 2024). Information on dietary composition and variation can help identify key prey species (Ghaskadbi et al. 2022; Ghosh-Harihar et al. 2024), highlight the requirements to maintain connectivity and habitat use, assess coexistence and niche differentiation with other co-occurring species (Foster et al. 2013), and evaluate the potential for human–carnivore interactions and

conflict (Hacker et al. 2024; Srivathsa, Sharma, and Oli 2020). Therefore, insights from dietary analyses can significantly inform landscape-level conservation and management of carnivores (Clements et al. 2014).

Non-invasive fecal samples have become increasingly important for studying the diet of elusive and endangered species. Fecal DNA-based approaches have allowed finer taxonomic resolution and detailed dietary breadth compared to traditional methods like micro-histological analysis, which rely on the identification of undigested remains such as hairs or bones (Deagle et al. 2013; Ghaskadbi et al. 2022; Pompanon et al. 2012; Roffler et al. 2021). Although widely used, such conventional techniques are labor-intensive, prone to observer bias, and often limited by incomplete reference libraries, leading to misidentification, especially for soft-bodied prey or closely related taxa (da Silva et al. 2019; Massey et al. 2021; Spaulding et al. 2000).

DNA metabarcoding provides a scalable and non-invasive approach that enables rapid, high-throughput dietary profiling from fecal samples (Deagle et al. 2013). This method allows processing of large sample size and provides fine-scale taxonomic resolution of consumed prey. However, fecal DNA is often degraded and dominated by host DNA, which can limit the detection of prey species (Pompanon et al. 2012; Taberlet et al. 2012). These challenges necessitate the development of improved metabarcoding strategies, including careful sample collection, marker selection, curated reference databases, and suppression of host DNA amplification using blocking oligonucleotides.

Most recent metabarcoding studies have used methods involving the collection of whole scats in ziplock bags or centrifuge tubes with silica beads (Massey et al. 2021; Roffler et al. 2021; Shao et al. 2021; Mishrikotkar et al. 2026) or ethanol (Caspi et al. 2025; Henger et al. 2022). These methods, while commonly used, are challenging for large-scale studies due to issues related to sample storage, processing time, and increased risk of contamination during DNA extraction. Thus, improving sampling protocols can further enhance the scalability and robustness of fecal DNA-based diet studies. In addition, most published studies that rely on a single amplicon require a 20% PhiX spike during Illumina sequencing to increase nucleotide diversity, which reduces the effective yield of dietary reads (Ghosh-Harihar et al. 2021; Hawke et al. 2022). Optimizing amplicon strategies could thus improve sequencing efficiency and data output.

While these refinements are crucial, understanding how carnivore diets vary across space and seasons is equally critical for addressing ecological and conservation questions. Unfortunately, very few studies have attempted to explore this variation in depth for large carnivores (but see Hacker et al. 2024; Kunz et al. 2025; Roffler et al. 2021; Shi et al. 2021). A better understanding of such variation can help address key knowledge gaps in carnivore ecology. For example, in monsoon, waterlogged terrain may limit the accessibility of large prey species, potentially shifting diet to include more opportunistic prey species. In contrast, reduced prey availability in degraded and human-dominated landscapes may force predators to depredate on domestic species. Such insights are pivotal for designing data informed conservation and management strategies to mitigate human–carnivore conflict and promote coexistence (Hacker et al. 2024).

Dholes (*Cuon alpinus*), also known as Asiatic wild dogs, are among the most threatened carnivores globally and are currently listed as Endangered by the IUCN (Kamler et al. 2015; Wolf and Ripple 2017). Endemic to South and Southeast Asia, dholes have suffered a substantial range contraction of over 80% from their historical distribution (Kamler et al. 2015; Wolf and Ripple 2017). In India alone, dholes have lost nearly 60% of their range over the past century. Nevertheless, India still supports the largest remaining dhole population in the world (Srivathsa, Majgaonkar, et al. 2020). Within India, three primary metapopulations persist in the Western Ghats, Central India, and Northeast India, typically structured as source populations within protected reserves, surrounded by unprotected or modified habitats that may function as demographic sinks (Srivathsa, Sharma, and Oli 2020). The Central Indian forests and the Western Ghats harbor relatively high dhole densities, largely due to the extent of protected habitat and the availability of wild prey (Karanth et al. 2009). Dholes, being social carnivores, play a crucial role in maintaining ecosystem structure and function by regulating prey populations. Ensuring sufficient prey availability is critical for dhole conservation, as their hypercarnivorous diet imposes high energetic demands (Kamler et al. 2015; Karanth et al. 2004).

In this study, we develop and evaluate an improved fecal DNA metabarcoding and sample collection strategy that requires less sample, is easier to implement, and minimizes data loss during sequencing. We test whether this method performs better than conventional approaches. Further, we implement these methods to assess spatial and seasonal variation in dhole diet within Tadoba Andhari Tiger Reserve. Specifically, we expected to observe (a) differences in diet composition across seasons, and (b) a restricted diet in buffer areas where regulated human activities are permitted, compared with core areas, which have the highest level of protection and do not allow human activities. By combining methodological advancements with ecological insights, our study refines non-invasive dietary assessments and informs conservation management of dholes in human-dominated landscapes.

2 | Materials and Methods

2.1 | Study Site

This study was conducted in Tadoba Andhari Tiger Reserve (TATR), a 1727 km^2 protected area, separated into core and buffer zones, located in the Chandrapur district of Maharashtra state, India (Habib et al. 2019). TATR falls within the Central Indian Landscape (CIL) and serves as a major source population for multiple wild species in the region. It is a tropical dry deciduous forest interspersed with teak-mixed bamboo forests, grasslands, and riparian forests (Champion and Seth 1968; Paliwal and Mathur 2014). Mixed

bamboo forest is the dominant vegetation type, covering more than 75% of the reserve (Paliwal and Mathur 2014). The region experiences three seasons: summer, monsoon, and winter. Winters are short and mild, summers are hot and prolonged, while monsoons are moderate and peak in August to mid-September. TATR also supports carnivore species other than the dhole (*C. alpinus*), such as the tiger (*Panthera tigris*) and leopard (*Panthera pardus*). It hosts several herbivore species, including sambar (*Rusa unicolor*), chital (*Axis axis*), gaur (*Bos gaurus*), nilgai (*Boselaphus tragocamelus*), and wild boar (*Sus scrofa*). The estimated prey density in the reserve is 18.67 per km^2 (Habib et al. 2019).

2.2 | Sample Collection

Since dhole packs are known to defecate along forest roads, we conducted sweep sampling on roads and trails in the core and buffer regions during two seasons: monsoon (Aug–Sep 2022) and winter (Jan–Feb 2023). For comparing collection methods, sampling was restricted to winter (Jan–Feb 2023). In the field, dhole scats were identified by morphology and odor, and secondary signs like pugmarks found nearby were examined to confirm species identification.

To compare the efficacy of collection methods, the same samples were collected using two different methods. In the old or whole scat method, a small chunk of the scat was placed in a ziplock bag containing a packet of silica beads. While stored in the field, the silica beads were replaced multiple times after reaching maximum absorption capacity. Additionally, we employed a new collection method in this study. The scat bolus was broken open, and 4–5 small pinches were collected from different interior portions using sterilized forceps (Figure 1). These were placed in 2 mL microcentrifuge tubes containing 1 mL of Longmire buffer (Longmire et al. 1997). Two replicates were collected per sample in separate microcentrifuge tubes. A fresh pair of gloves and sterilized forceps was used for each sample to prevent cross-contamination. The tube and forceps method was used for collecting all other scats for dietary analysis. Only fresh scats (approximately < 3 days old) were collected, identified based on physical attributes such as appearance (e.g., shiny outer surface), moisture content, smell, and insect activity.

We also accounted for rainfall events during fieldwork and, following rainy days, collected only very fresh samples (mostly same-day samples). Any samples for which there was uncertainty regarding age were not collected. For each sample, we recorded the GPS location, date and time of collection, probable species (based on morphology), and estimated sample age. All the samples were stored in the field for a month (during the sampling period) and were then transported to the lab and stored at −20°C until further analysis. To avoid sampling the same individuals and overrepresenting a single feeding event, each road was sampled only once. After samples were collected from a given trail or road, it was not surveyed again. We collected soil samples as field negative controls to account for potential environmental DNA contamination from nearby substrate into a 2 mL vial containing Longmire buffer (Longmire et al. 1997).

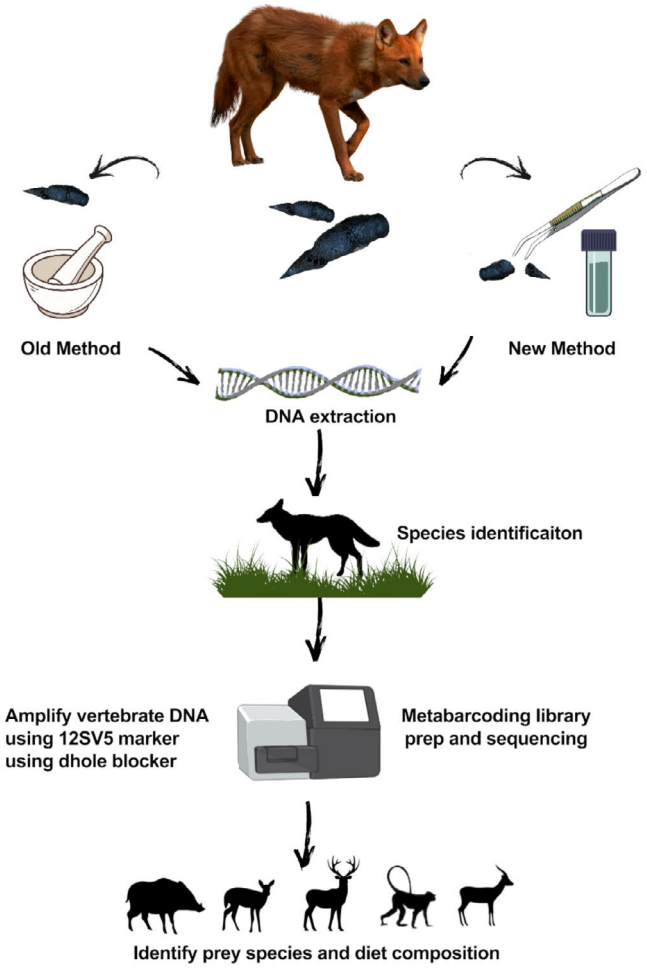

FIGURE 1 | Schematic representation of the two sampling methods compared and the downstream workflow. The old method involves collecting a whole scat chunk in silica beads followed by DNA extraction via pulverization using a mortar and pestle, whereas the new method involves collecting four to five pinches from the interior of the scat using forceps and placing them directly into lysis buffer.

2.3 | DNA Extraction and Species Identification

For samples collected using the whole scat method, liquid nitrogen was added to the scat chunk, which was then pulverized using a mortar and pestle. The resulting powdered scat was thoroughly mixed to ensure homogenization and used for DNA extraction. For both methods, DNA extraction was performed using the QIAamp Fast DNA Stool Mini Kit (QIAGEN, Germany). Extractions were conducted in a dedicated room designed for handling DNA from non-invasive sources to minimize contamination, while adhering to standard safety and contamination control protocols. Extractions were carried out in batches of 23 samples, with one extraction control included per batch. Field controls were processed using the same protocol as the samples to ensure consistency.

After DNA extraction, molecular species identification was performed using dhole-specific primers (Modi et al. 2018) to confirm the genetic identity of the collected samples. Only samples that showed positive amplification were considered conclusively identified and were processed further for dietary analysis.

2.4 | Design and Standardization of Dhole Blocking Oligonucleotide

2.4.1 | Design of Blocking Oligonucleotide

For restricting dhole DNA amplification from fecal DNA using the universal vertebrate 12S rRNA marker (12SV5; Riaz et al. 2011) used to study the dietary composition of dhole, we designed a dhole-specific blocking oligonucleotide (DhlBlk: 5′-CTATGCTTAGCCCTAAACATAGATAATTCTACAACA AAATAATTCGCC [SpcC3]-3′), adapted from the sequence described by Woo et al. (2022). DhlBlk is a 48 bp-long oligonucleotide whose first six bases overlap with the 3′ end of the forward primer of the 12SV5 marker (Riaz et al. 2011). Additionally, DhlBlk has a 3-carbon spacer at its 3′ end and is designed to bind specifically to dhole DNA, thereby selectively blocking its amplification during PCR using the 12SV5 marker. Details of all primers used in this study are provided in Table S2.

2.4.2 | Standardization of Blocking Oligonucleotide

To amplify vertebrate prey DNA, we followed the PCR conditions described by Shao et al. (2021), with modifications to the annealing temperature and the number of reaction cycles. Gradient PCR (ranges from 55°C to 65°C) was performed using dhole scat and tissue samples to determine the annealing temperature at which the blocking oligonucleotide was most effective. PCR was also repeated using various concentrations (from 20 to 50 μM) to identify the optimal blocking concentration. Blocking efficiency was assessed based on the absence of amplification in tissue-derived DNA while maintaining amplification in fecal samples. An annealing temperature of 63°C and a concentration of 40 μM yielded the highest blocking efficiency for dhole DNA, and these conditions were used for subsequent amplifications.

2.5 | DNA Metabarcoding

2.5.1 | Amplification Using N-Spacer Primers

To overcome issues related to low base diversity during single amplicon sequencing on Illumina platforms, we used a pool of 12SV5 primers with random "N" spacers (0–10 bp) added to the 5′ end (Figure S1), an approach adapted and tested for 16S sequencing (Naik et al. 2023). This strategy introduces base complexity across sequencing reads, removing the need for PhiX spike-in and improving sequencing quality and throughput (Naik et al. 2023).

2.5.2 | Library Preparation and Sequencing

We used a 10 μM pool of 11 sets of 12SV5 primers with random "N" spacers as described in the previous section that targets all vertebrates to identify the dietary composition of dholes. We amplified all the samples using this primer pool and followed the library preparation method used in a previous study with slight modifications (Ghosh-Harihar et al. 2021).

The PCR reactions were carried out in 20 μL reaction volume consisted of 10 μL of 2X Qiagen Multiplex Master Mix (Qiagen), 1 μL each from a 10 μL pool of reverse and forward 12SV5 primers, 3 μL of dhole blocking oligonucleotide (40 μM concentration), 4 μL of DNA and 1 μL of PCR grade water to make up the volume. The PCR conditions had an initial denaturation step of 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 90 s at 63°C, and no elongation (Henger et al. 2022; Shao et al. 2021). Amplifications were confirmed by electrophoresis using 1.2% agarose gel. All the samples which showed amplification were taken forward to library preparation. The PCR products were purified using a volume of 1.5X of AMPure XP Beads (Beckman Coulter, Fullerton, CA, USA). The purified products were taken for indexing using the indexes used in a previous study (Tyagi et al. 2024). The indexing PCR was carried out using 25 μL of 2X Qiagen Multiplex Master Mix, 5 μL of i5 and i7 indexes each, 5 μL of purified amplicon, and 10 μL of PCR grade water. The PCR conditions included an initial denaturation step at 95°C for 15 min, followed by 8 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, followed by a final extension step for 5 min at 72°C.

The indexed products were visualized using a TapeStation (Agilent Technologies, Santa Clara, USA). The libraries were then pooled in equal volume (5 μL each) and then subjected to purification using 0.9X volume of AMPure XP Beads (Beckman Coulter, Fullerton, CA, USA) to remove unbound primers. The purified pooled library was then loaded to a V2 300 cycle-kit for 150X2 paired-end sequencing on an Illumina MiSeq platform.

2.6 | Data Analysis and Filtering

We demultiplexed raw reads based on the index information and then used *MetReTrim* (Naik et al. 2023) to remove the "N" spacers from the 5′ end of all the reads. Post trimming, we used *OBITools* (Boyer et al. 2016) for analysis, sequence filtering, and taxonomic identification. Filtering parameters followed previously published metabarcoding workflows (Ghosh-Harihar et al. 2021; Shao et al. 2021). These thresholds were chosen to remove low-quality reads, sequencing artifacts, and low-abundance variants while retaining biologically meaningful sequences. The forward and reverse reads were first merged using the *illuminapairedend* tool, after which low-quality reads (quality score below 40) were filtered out with *obigrep*. Using the *ngsfilter* command (Shehzad et al. 2012), sequences were retained if they had exact tag matches and no more than two mismatches in primer regions. Redundant reads were consolidated into unique sequences through *obiuniq*. Further filtering with *obigrep* excluded sequences shorter than 80 bp and sequences with fewer than 10 reads across the entire dataset. To eliminate potential PCR and sequencing artifacts, sequences differing by only one nucleotide and occurring at less than 50% of the dominant sequence's frequency were identified and removed using *obiclean*. We also discarded all singleton sequences based on the *obiclean* assignment to further minimize the influence of PCR and sequencing errors. A reference database was then built by retrieving vertebrate 12S gene sequences targeted by the 12SV5 primers from EMBL release 143 using the *ecoPCR* tool (Ficetola et al. 2010).

Taxonomic assignment of sequences was carried out using a combination of similarity-based, abundance-based, and phylogenetic approaches to delineate molecular operational taxonomic units (MOTUs). Initially, sequences were assigned to taxa using the *ecotag* command based on similarity to the curated reference database. To improve accuracy, sequences matching human or the focal carnivore species, as well as low-frequency variants (< 5% relative abundance and/or counts below those observed in extraction, PCR blanks, and field controls), were excluded to reduce the influence of potential contamination and sequencing artifacts. Sequences were then clustered into MOTUs based on a 2% sequence divergence threshold and their relative abundance. For divergent sequences (> 2%), phylogenetic tree topologies were used to further resolve taxonomic identities. BLAST searches against the NCBI GenBank database were conducted with stringent parameters (E-value ≤ 1e−10; ≥ 98% query coverage and identity) to validate species-level matches. Assignments were refined using biogeographic data and known local species distributions: sequences matching a single local species with ≥ 98% identity were assigned to that species; if multiple local matches were found, the assignment was made at the lowest common taxonomic level; sequences with poor matches (< 98% identity) or no local correspondence were considered unidentifiable and excluded from downstream dietary analysis. This threshold is commonly used in metabarcoding studies for reliable species-level identification (Ghosh-Harihar et al. 2021, 2024; Shao et al. 2021).

2.7 | Method Comparison

To evaluate whether our sampling was sufficient for comparing diet diversity and the effectiveness of the two sample collection methods, we performed rarefaction analysis using the R package *iNEXT* (Hsieh et al. 2016). We then assessed differences in the composition of species detection between the methods by calculating Bray–Curtis dissimilarity matrices and visualizing the patterns using non-metric multidimensional scaling (NMDS). The NMDS ordination was performed using the R package *vegan* (Oksanen 2010).

2.8 | Diet Diversity Analyses

To characterize diet composition from fecal metabarcoding data, we used two commonly employed metrics: frequency of occurrence (FOO) and relative read abundance (RRA). FOO indicates the presence or absence of a species in each sample and was calculated as the number of samples in which a diet item appeared divided by the total number of samples. RRA reflects the proportion of sequence reads assigned to each taxon relative to the total number of reads. While both metrics are subject to specific biases—FOO may overemphasize rare or contaminant taxa, and RRA does not always correspond to actual biomass consumed—they are complementary and widely used for qualitative comparisons of diet (Ando et al. 2020; Caspi et al. 2025; Deagle et al. 2019; Mallott et al. 2018; Massey et al. 2021; Walker et al. 2023). We used FOO for summarizing diet richness and broad patterns at the population level and RRA to assess differences in dietary composition among seasons and areas. To evaluate whether our sample size was sufficient for comparisons, we generated dietary rarefaction curves using the R package *iNEXT* (Hsieh et al. 2016). To assess variation across seasons and areas, we calculated Jaccard dissimilarity matrices for FOO and Bray–Curtis matrices for RRA using the *vegdist* function in the R package *vegan*. We visualized these differences using non-metric multidimensional scaling (NMDS) and tested for statistical significance using permutation-based multivariate analysis of variance (PERMANOVA) with 999 permutations implemented using the *adonis* function in *vegan*. We conducted all statistical analyses and generated figures in R version 4.1.2 (R Development Core Team, 2021).

3 | Results

3.1 | Data Summary and Sample Information

We collected a total of 99 and 84 putative dhole scats in the monsoon and winter seasons, respectively, based on field identification using morphology and secondary signs. Additionally, during winter, we collected 48 more samples using two different methods: whole scat preserved in silica and scat pinches stored in Longmire buffer (Longmire et al. 1997). After genetic species identification, 76, 49, and 44 samples from the monsoon, winter, and method comparison sets, respectively, were confirmed to be of dhole origin. Sampling was conducted across extensive spatial coverage (Figure 2) to assess potential variation in dhole diet across different areas of the reserve. All confirmed samples, along with field, extraction, PCR, and library preparation controls, were processed for DNA metabarcoding library preparation and sequencing. For dietary analysis, we used the 76 monsoon and 49 winter samples, as well as the 44 additional winter samples collected using our standardized forceps-and-tube sampling method.

3.2 | Efficiency of N-Spacer Primers

The use of N-spacer primers enabled a single-primer metabarcoding run on the MiSeq platform without the need for a PhiX spike-in, allowing us to save approximately 20% of the sequencing output. Our sequencing run generated over 34 million reads with a mean quality score above Q30, and ≥ 97% of the reads were retained after adapter and N-spacer trimming.

3.3 | Method Comparison

To assess the efficacy of sample collection methods, we compared 44 genetically confirmed dhole scats collected using both approaches. Metrics evaluated included total reads retained after filtering, host DNA amplification despite the use of a blocking oligonucleotide, and the number of dietary taxa recovered per sample. The new method of scat pinches collected with forceps performed significantly better than the traditional whole-scat-in-silica approach in terms of obtaining the number of reads post-filtering (Figure 3A; Table S3; Wilcoxon rank-sum test: $W = 1413$, $p = 0.00015$).

Despite the use of a dhole-specific blocking oligonucleotide (DhlBlk) with the 12SV5 primer, amplification of host DNA

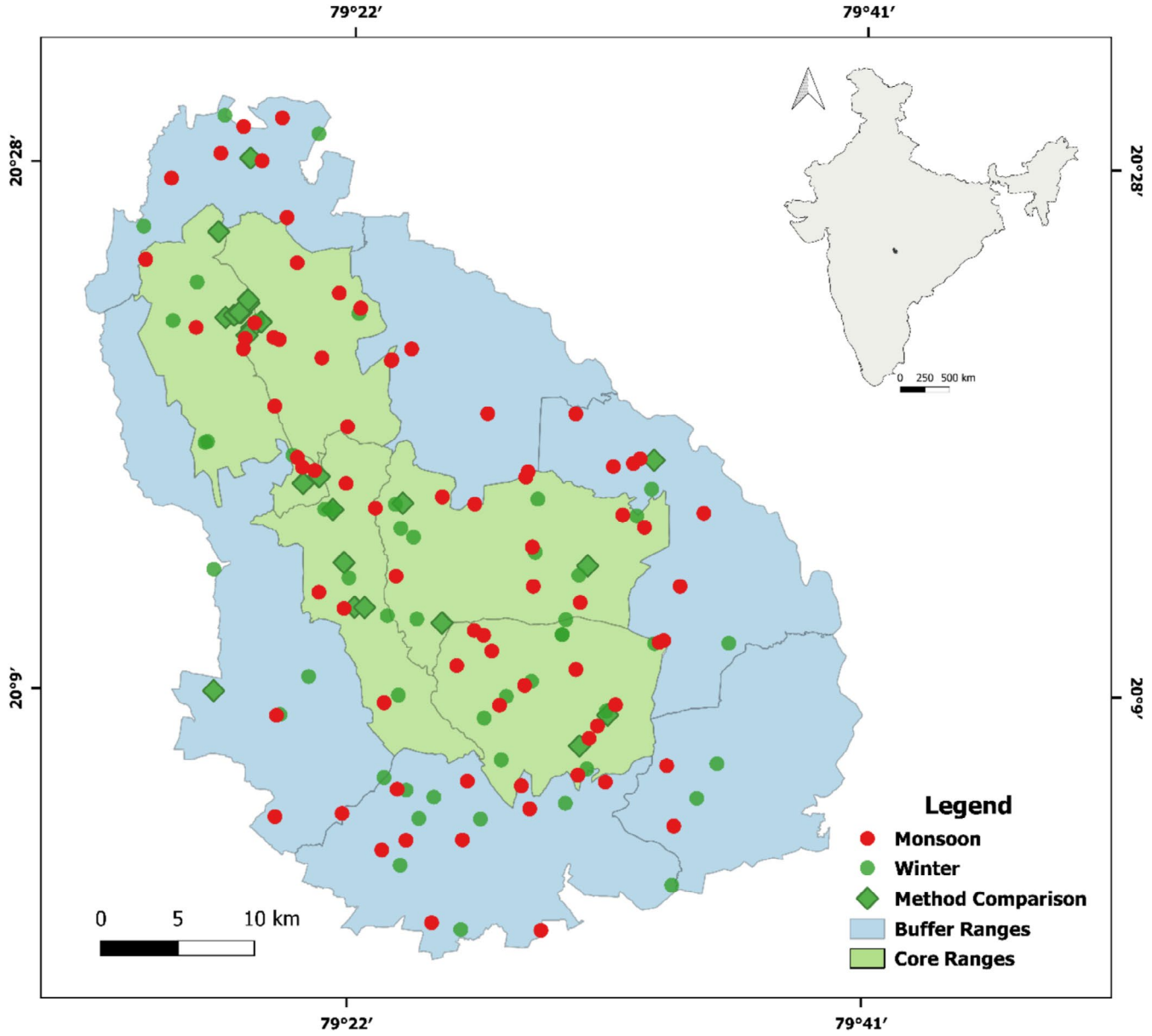

FIGURE 2 | Study area and sample locations. Map of Tadoba Andhari Tiger Reserve showing the locations of all genetically identified dhole samples from monsoon (red dots; $N = 76$) and winter (green dots; $N = 49$, green diamonds; $N = 44$) seasons. The core and buffer zones of the reserve are also depicted in different colors. Green diamonds represent the samples collected using two different methods during the winter season for method comparison.

was observed in samples collected using both methods. Of the 44 samples collected using the older method, 41 exhibited dhole DNA amplification, compared to 35 from the new method. The proportion of dhole reads relative to total reads was also significantly lower in the new method (Figure 3B; Table S3; $W = 528.5$, $p = 0.00023$), indicating higher blocking efficiency.

The new method significantly yielded better dietary resolution, recovering a higher number of dietary taxa per sample (Figure 3C; $W = 1418$, $p = 6.82\text{e-}05$). All samples yielded at least one dietary species. However, three samples collected using the whole-scat method yielded only dhole sequences and no dietary data (Figure 3C). Non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity revealed tighter clustering among samples collected using the new method (stress = 0.08; Figure 3D), indicating greater consistency in species composition. In contrast, samples from the older method exhibited higher dispersion, highlighting greater variability and stochasticity across samples. Rarefaction analysis confirmed that the sample size was sufficient to robustly compare the observed differences in species composition due to sample collection method (Figure 3E).

3.4 | Dietary Composition

We determined the overall diet of dholes using 169 samples collected across two seasons. In total, we recovered 15 unique vertebrate prey species. Based on frequency of occurrence

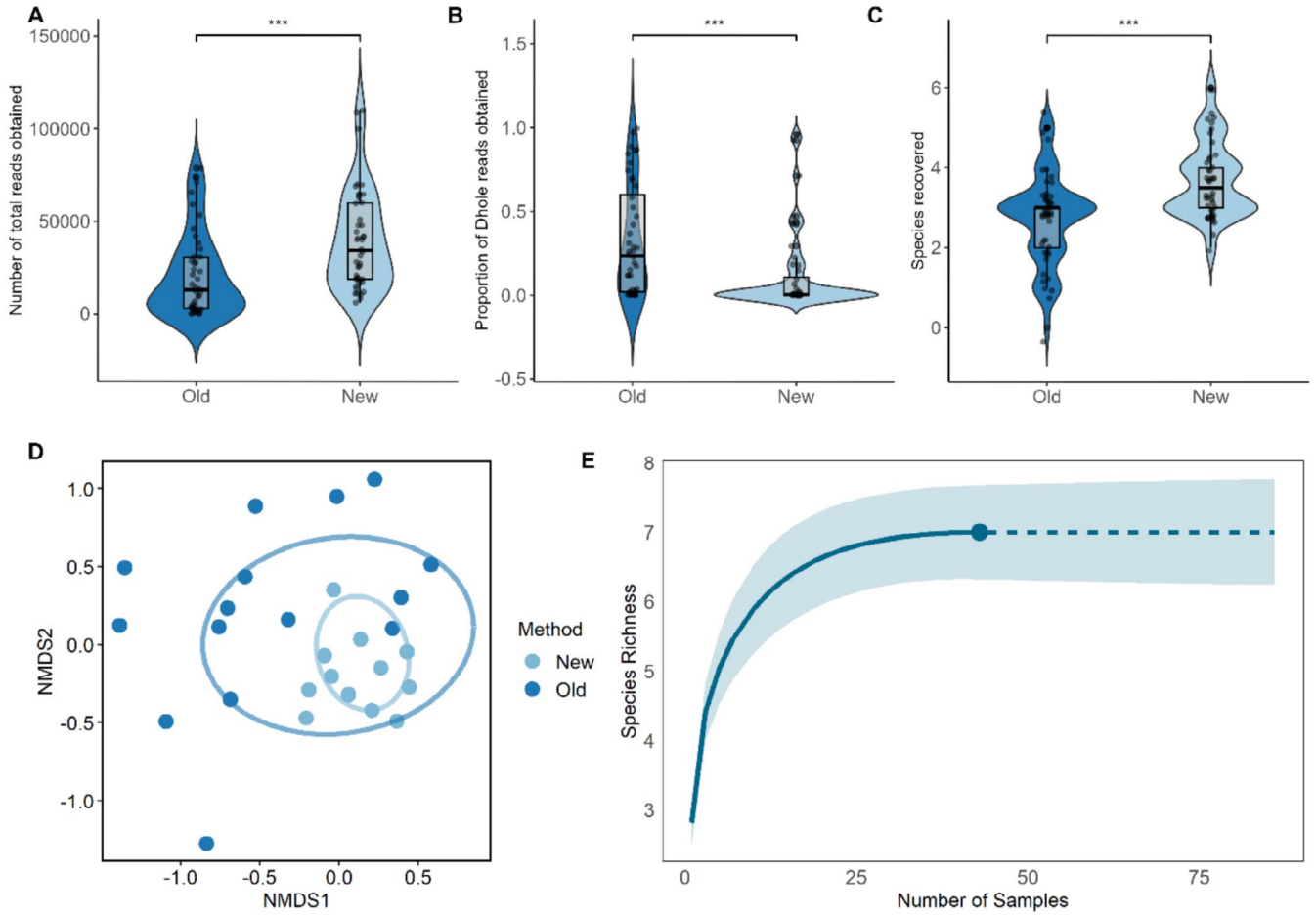

FIGURE 3 | Comparison of sample collection methods. (A) Number of reads retained after quality filtering. (B) Proportion of host (dhole) reads relative to total reads. (C) Number of dietary taxa recovered per sample. Statistical significance (Wilcoxon rank-sum test) is indicated by bars above the plots (***$p < 0.001$). (D) Non-metric multidimensional scaling (NMDS) plot based on Bray–Curtis dissimilarity where points represent individual samples and ellipses indicate the 95% confidence regions for samples grouped by sampling method. (E) Rarefaction curve: Solid line represents the rarefied species accumulation curve, the dashed line represents extrapolated species richness beyond the observed sample size, and the shaded area indicates 95% confidence intervals.

(FOO), the most commonly detected species was chital (*Axis axis*; FOO = 0.704), followed by sambar (*Rusa unicolor*; 0.669), barking deer (*Muntiacus muntjak*; 0.556), black-naped hare (*Lepus nigricollis*; 0.308), wild boar (*Sus scrofa*; 0.266), and four-horned antelope (*Tetracerus quadricornis*; 0.112). The remaining nine species had FOO values below 10% (Figure 4, Table S1). Relative read abundance (RRA) values followed a broadly similar pattern, with chital (0.331), sambar (0.259), black-naped hare (0.145), wild boar (0.108), barking deer (0.051), and four-horned antelope (0.051) comprising the majority of dietary reads. RRA values for all other taxa were below 1% (Figure 4, Table S1). We found very low proportions of domestic prey species in the dhole diet based on both FOO (cattle: 0.065; buffalo: 0.077) and RRA (cattle: 0.008; buffalo: 0.003). Some unexpected species were also identified, including Asian palm civet (*Paradoxurus hermaphroditus*), small Indian civet (*Viverricula indica*), and rusty spotted cat (*Prionailurus rubiginosus*).

3.5 | Dietary Diversity and Spatio-Temporal Variation

We observed significant seasonal and spatial variation in dhole diet composition among core and buffer ranges of the Tadoba Andhari Tiger Reserve. Compositional differences were assessed using permutational multivariate analysis of variance (PERMANOVA) and visualized with non-metric multidimensional scaling (NMDS). Diet composition in the monsoon season was more variable than in winter, with all 15 identified prey species detected during the monsoon, compared to only 11 in winter. The winter diet was primarily dominated by large wild ungulates based on RRA (Figure 5A, Figure S3). PERMANOVA results confirmed significant seasonal differences (FOO: $F = 16.54$, $R^2 = 0.08$, $p < 0.001$; RRA: $F = 2.02$, $R^2 = 0.012$, $p < 0.05$).

For presence–absence data, we used Jaccard distances to generate NMDS plots, while Bray–Curtis distances were used for

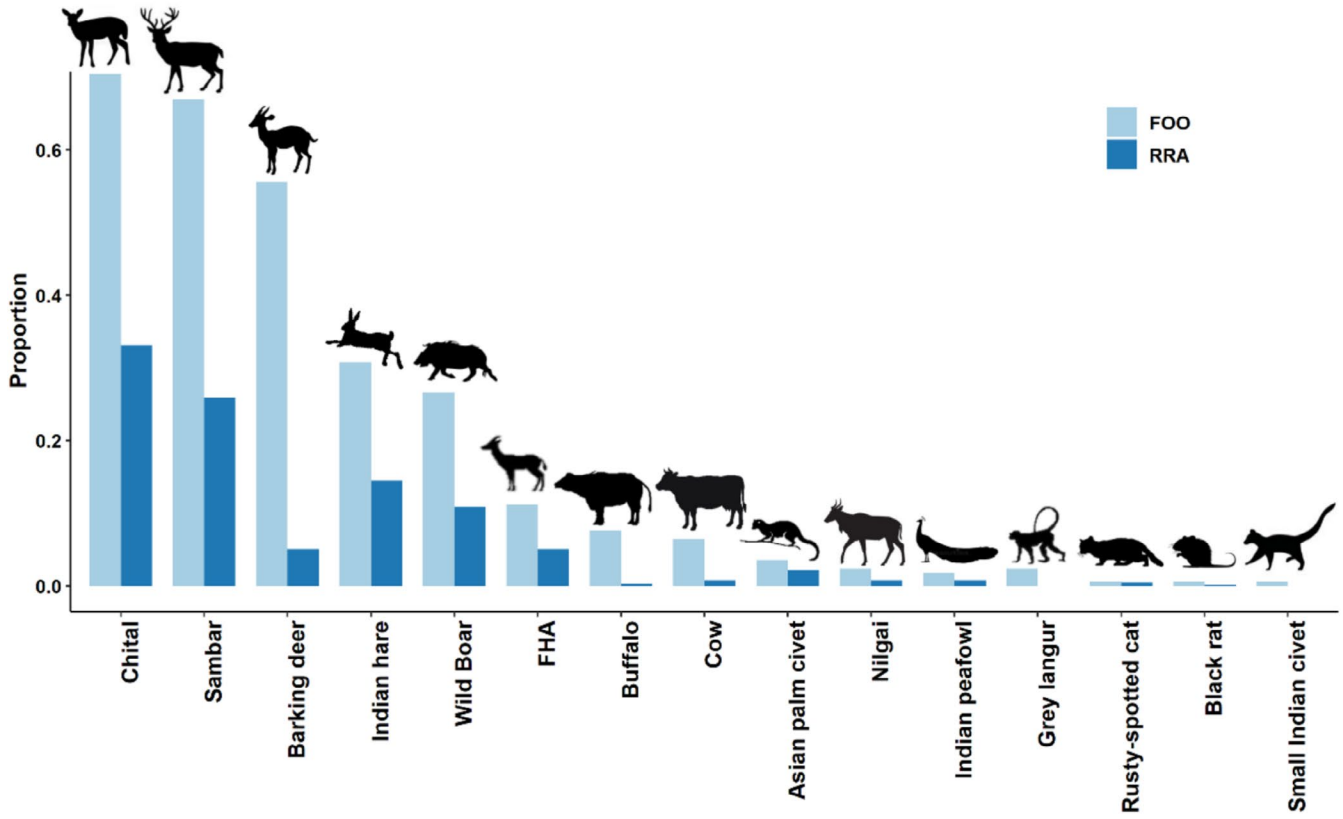

FIGURE 4 | Bar plots showing overall dietary composition of dholes based on Frequency of Occurrence (FOO) and Relative Read Abundance (RRA).

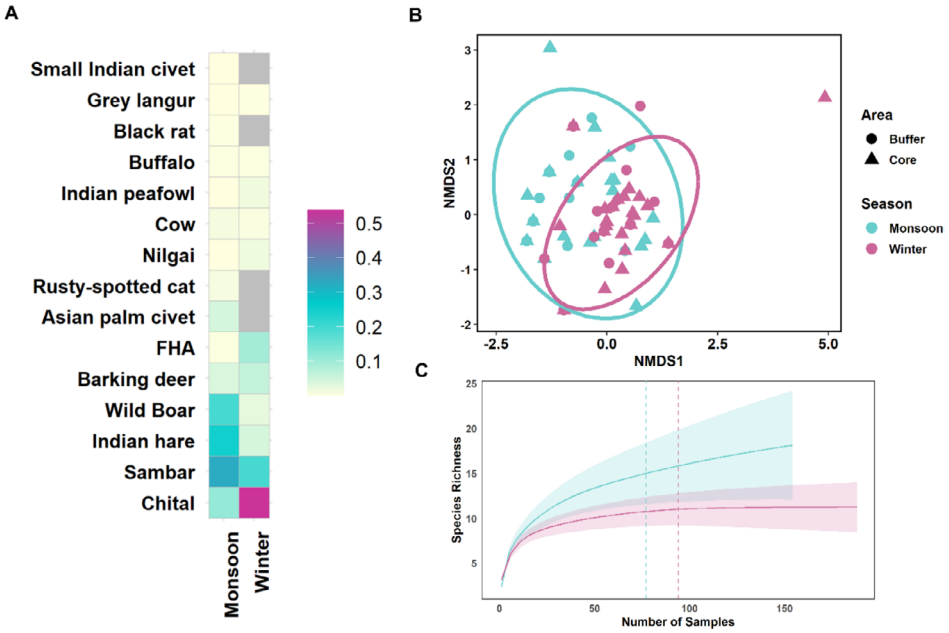

FIGURE 5 | Seasonal variation in dhole diet. (A) Heatmap based on relative read abundance (RRA) illustrating differences in prey species consumed/detected during the monsoon and winter seasons. Gray cells indicate absence of a given species in that season. (B) Non-metric multidimensional scaling (NMDS) plot based on frequency of occurrence (FOO) showing variation in diet composition between seasons. (C) Rarefaction curves comparing dietary species richness between monsoon and winter samples. The vertical dashed line represents the season-wise sample size.

RRA-based analyses. The NMDS ordination revealed tighter clustering of winter samples, suggesting greater consistency in diet during that season, whereas monsoon samples were more dispersed, indicating higher dietary variability (NMDS stress = 0.082; Figure 5B). One winter sample appeared as an outlier in the NMDS ordination because it contained only

Indian peafowl (*Pavo cristatus*) reads, leading to high dissimilarity from other samples based on Bray–Curtis distances.

Spatially, core areas exhibited more diverse and variable diet than buffer zones (PERMANOVA: FOO: $F = 19.12$, $R^2 = 0.094$, $p < 0.001$; RRA: $F = 3.58$, $R^2 = 0.02$, $p < 0.01$; Figure S2). Among all factors tested, season emerged as the strongest driver of dietary variation, followed by spatial zone (core vs. buffer), and then their interaction. Rarefaction analysis further confirmed that the lower dietary richness observed in winter was not due to limited sample size (Figure 5C). Overall, dholes exhibited pronounced seasonal and spatial variation in diet composition, with relative prey abundance shifting more strongly by area and interacting with season. Interestingly, we also detected sequences corresponding to *Panthera* species in dhole samples, with higher occurrence in the monsoon season (14 samples) compared to winter (6 samples), suggesting increased trophic or spatial interactions among co-occurring carnivores during the monsoon.

4 | Discussion

Our study provides an assessment of dhole (*C. alpinus*) dietary ecology in the Tadoba Andhari Tiger Reserve (TATR) using methodological advancements in sample collection and metabarcoding procedures. Our results demonstrate the efficiency of the sample collection method we tested, and our metabarcoding approach eliminated the need to spike non-target libraries to maintain diversity during Illumina sequencing. Importantly, our findings show that dhole diet varies across habitat types and seasons; however, the species remains heavily dependent on wild prey, with minimal reliance on domestic species. We recovered 15 prey species, the most complete dietary profile described for dholes to date, highlighting the species' broad but wild-prey focused diet. Overall, our findings not only underscore the value of improved methodological practices in wildlife diet studies but also provide new insights into dhole ecology in this critical conservation landscape.

4.1 | Methodological Advancements in Non-Invasive Diet Analysis

Fecal samples have been extensively used in dietary studies on a wide range of taxa, including carnivores such as tiger, leopard, snow leopard, cheetah, coyote, wolf, various meso-carnivores, as well as herbivores and several bird species (Andheria et al. 2007; Ghosh-Harihar et al. 2024; Hacker et al. 2024; Henger et al. 2022; Ingvaldsen et al. 2024; Lu et al. 2021; Shao et al. 2021; Thuo et al. 2019). Molecular dietary analysis has revolutionized the study of food webs and trophic interactions by enabling highly resolved, accurate, and reproducible reconstruction of animal diets (Pringle and Hutchinson 2020; Symondson 2002). While there is substantial evidence that DNA metabarcoding is a robust method for studying diet (Mishrikotkar et al. 2026), our results highlight that sampling methods can significantly impact the efficiency of DNA metabarcoding outcomes.

We show that collecting scat pinches directly into lysis buffer significantly improves DNA yield, PCR efficiency, reduces host DNA contamination, and enhances dietary resolution compared to the commonly used whole-scat-in-silica method. Fecal DNA is enriched in PCR inhibitors (Schrader et al. 2012; Tyagi et al. 2022) like bile pigments, which negatively impact data generation through metabarcoding, as successful amplification of dietary DNA is the first critical step during library preparation. Taking small pinches reduces the amount of PCR inhibitors present in the extracted DNA, resulting in higher PCR efficiency and, therefore, better data generation post-Illumina sequencing (Figure 3A). Additionally, our minimalist sampling approach that leaves most of the scat at its original location in situ preserves the territorial and olfactory signals critical for social and spatial communication for wild species (Wooldridge et al. 2019). Conventional approaches that remove entire scats risk disrupting these cues, with possible implications for species behavior, territory maintenance, and broader ecological dynamics.

We also developed a novel and effective blocking oligonucleotide for dholes targeting the 12S rRNA gene. The use of blocking oligonucleotide is essential as host DNA is typically much more abundant in fecal samples than the DNA of consumed species (Mannise et al. 2024; Woo et al. 2022). Despite using the blocking oligonucleotide, we still observed some host DNA amplification; however, the amplification with the new method was significantly reduced compared to the whole-scat method (Figure 3B). This improvement could be attributed to collecting pinches from the interior of the scat, which reduces host epithelial cells that are more concentrated on the outer surface. Our sampling strategy effectively minimizes host DNA contamination during collection itself, thereby enhancing the blocking oligonucleotide's performance. This aligns with previous studies emphasizing the importance of early-stage preservation and reduced sample complexity to maximize prey detectability (De Barba et al. 2014; Pompanon et al. 2012).

These improvements led to a greater number of dietary taxa being detected and improved consistency in species composition across samples using the new method (Figure 3C,D). Additionally, the method is easier to implement in the field, sample storage and transportation are simplified, and the time required for DNA extraction is also reduced since no pulverization step is needed. This also effectively eliminates the risk of cross-sample contamination during DNA extraction.

Furthermore, the use of N-spacer-tagged primers eliminated the need for PhiX spike-in during sequencing, thereby increasing data output efficiency by ~20%. Although such primer designs are still underutilized in vertebrate diet studies, our findings support recent recommendations advocating their broader application in single amplicon sequencing workflows (Naik et al. 2023).

4.2 | Dietary Composition

Several studies across the dhole range have examined their dietary ecology, including in India (Ghaskadbi et al. 2022; Srivathsa et al. 2023; Srivathsa, Sharma, and Oli 2020), Nepal (Aryal et al. 2015), Bhutan (Thinley et al. 2011), Laos (Kamler et al. 2020), and Thailand (Grassman et al. 2005). Most of these have relied on traditional approaches such as direct observations or micro-histological analyses. In contrast, our study applies

DNA metabarcoding to investigate dhole diet and describes the widest dietary breadth recorded for the species to date, comprising 15 prey species. This represents an important methodological advancement in understanding the trophic ecology of dholes.

Our results confirm that sambar and chital are the two primary and most frequently consumed prey species by dholes in TATR, which is consistent with previous findings from this landscape (Borah et al. 2009; Ghaskadbi et al. 2022). In addition to these large ungulates, we also detected frequent consumption of smaller prey species such as four-horned antelope, barking deer, wild boar, and Indian hare, although their composition varied across seasons and areas within the reserve (Figure 5A, Figures S2 and S3). These species have been previously reported in dhole diet studies, but our results provide the first evidence of their occurrence shifting in response to spatial and seasonal factors (Ghaskadbi et al. 2022; Karanth and Sunquist 2000).

Interestingly, we did not detect significant consumption of other large-bodied herbivores such as gaur (*B. gaurus*) and nilgai (*B. tragocamelus*), despite their known presence in the landscape and earlier reports of their occurrence in dhole diet (Ghaskadbi et al. 2022; Srivathsa, Sharma, and Oli 2020). This pattern may reflect prey preferences or constraints linked to prey vulnerability or pack hunting strategies. Overall, our findings indicate that the dhole population in TATR relies predominantly on six wild prey species: chital, sambar, barking deer, Indian hare, wild boar, and four-horned antelope. This highlights a strong dependence on medium to large-bodied wild ungulates. The marker we used cannot distinguish between wild boar and domestic pig, as both belong to the same species (*S. scrofa*); however, given the absence of domestic pigs in the core areas, sequences identified as *S. scrofa* most likely represent wild boar, although their presence in buffer areas cannot be entirely excluded. Our results are consistent with Srivathsa, Sharma, and Oli (2020), which showed that wild prey availability influences dhole-livestock interactions and human attitudes towards dholes. In our study, livestock consumption was negligible, further supporting the idea that high wild prey densities can reduce the likelihood of negative human–carnivore interactions. These findings emphasize the importance of conserving prey-rich habitats beyond protected areas to support dhole persistence in multiple-use landscapes, where prey depletion and competition with sympatric carnivores such as leopards may otherwise constrain dhole distribution and abundance (Habib et al. 2019).

Diet estimates derived from DNA metabarcoding can be influenced by how sequence data are summarized, we interpreted our results using both Frequency of Occurrence (FOO) and Relative Read Abundance (RRA). Each metric has inherent limitations: FOO may inflate the importance of prey species that occur frequently but contribute relatively little biomass, particularly when prey species differ substantially in body size. Whereas RRA may better approximate the relative biomass consumed but can be influenced by PCR amplification biases, differential digestion, and variation in DNA recovery among taxa (Deagle et al. 2019). Previous work suggests that occurrence-based metrics often overestimate rare prey items, while RRA can provide a more realistic approximation of population-level diet composition despite moderate recovery biases (Deagle et al. 2019). Our use of both metrics complimentarily, therefore allows a more balanced interpretation of dhole diet composition while accounting for potential biases associated with each approach.

4.3 | Spatial and Seasonal Variation in Diet

Our results highlight that dhole dietary composition varies across both area and season, and the spatio-temporal variation we observed could be attributed to differences in prey densities, prey availability, co-predator densities, and varying levels of anthropogenic disturbance in and around TATR (Habib et al. 2019). Seasonally, we found that the monsoon exhibits greater dietary variability (all 15 species detected) compared to winter (11 species). During winter, chital and sambar were the dominant prey species, whereas in the monsoon, the diet was more diverse and no single prey species dominated (Figure 5A). Along with large ungulates, species like wild boar and Indian hare formed a substantial portion of the diet (Figure 5A, Figures S2 and S3, Table S1). This could reflect the increased availability of smaller-bodied prey or the difficulty of hunting large hoofed prey such as sambar and chital during the monsoon, when dense vegetation and waterlogged terrain may hamper dhole hunting efficiency. Similar patterns have been observed in African wild dogs, where feeding behavior shifts occur in response to prey densities, with smaller prey becoming dominant with decreasing large prey availability (Woodroffe et al. 2007).

Interestingly, we found that dhole diet was more variable (i.e., more prey species detected) in the core compared to the buffer zones (Figure S2). This pattern may be driven by lower wild prey densities in buffer areas (Habib et al. 2019) combined with dholes' apparent avoidance of domestic livestock. In buffer areas, we did observe higher occurrences of four-horned antelope and nilgai, this likely reflects prey availability rather than a preference for these species. Our findings also revealed occasional predation on other taxa such as civets, rodents, langurs, and birds. Domestic species such as cattle, buffalo, and goats were rarely detected in dhole diet, unlike in other co-occurring large carnivores such as tigers and leopards (Ramesh et al. 2020; Srivathsa, Sharma, and Oli 2020). However, a study conducted in Bhutan documented cattle presence in dhole diet but only during wet season (Thinley et al. 2011).

We did detect a small number of occurrences of domestic species like cattle and buffalo in dhole scats. This is likely due to scavenging behavior, as dholes have been observed feeding on kills made by tigers or leopards (Ghaskadbi et al. 2022). Kleptoparasitism by dholes has been documented in TATR, where dholes feed on kills made by other carnivores, and vice versa (Ghaskadbi et al. 2022). Our observations of domestic species in the diet are consistent with previous studies from the same landscape and likely reflect scavenging of tiger kills or feeding on improperly disposed livestock carcasses in buffer zones, especially given that forest department records do not report dhole attacks on livestock in this area (Ghaskadbi et al. 2022). Another layer of evidence comes from the detection of *Panthera* species DNA in dhole scats collected during the monsoon, indicating increased interactions among co-occurring carnivores during this season. This underscores the potential for future studies to investigate

trophic and spatial interactions between sympatric carnivores in this landscape.

This study provides an important foundation for understanding the dietary ecology of dholes using a modern molecular approach. However, several important questions remain. First, incorporating biomass-based estimates would allow more ecologically meaningful interpretations of diet by accounting for the relative size and contribution of each prey species. Second, pairing dietary data with independent prey abundance estimates would enable assessments of prey selection and preference, rather than only prey use. Third, a simultaneous dietary assessment of sympatric carnivores—such as leopard and tiger using the same metabarcoding framework—could reveal important insights into interspecific competition and niche partitioning, especially in shared landscapes with overlapping prey bases, as demonstrated by several studies in different landscapes (Hacker et al. 2024; Shao et al. 2021). Together, such integrative approaches would be important for developing a more nuanced understanding of trophic dynamics and for informing conservation strategies that maintain functional predator guilds in increasingly human-dominated ecosystems.

Our study also has a few limitations and caveats that are worth mentioning. We only collected samples during the monsoon and winter seasons, and dietary variation during the summer could not be assessed due to delays in sample collection and technical constraints. Future studies incorporating year-round sampling would provide a more comprehensive understanding of seasonal variation in dhole diet. In addition, technical PCR replicates were not included in the metabarcoding workflow due to several limitations during data generation. Instead, we prioritized analyzing a larger number of samples to better capture population-level dietary patterns. Increasing sample size can improve diet estimates by capturing greater variability among individuals (Thuo et al. 2019), although incorporating technical replicates in future studies would further improve detection reliability and reduce stochastic amplification effects.

4.4 | Conservation and Management Implications

Wild species, especially carnivores, are globally facing numerous threats to their survival, including habitat loss and fragmentation (Ripple et al. 2014), depletion of prey populations (Creel et al. 2025; Wolf and Ripple 2017), loss of connectivity (Crooks et al. 2011; Thatte et al. 2020, 2021; Tyagi et al. 2024), and increased interaction and competition for resources with both wild and domestic counterparts (Creel et al. 2025; Tyagi et al. 2023). There is a pressing need to apply genomics and advanced genetic tools to better understand the impacts of these stressors on wild populations, particularly in human-dominated and rapidly transforming landscapes such as those in South and Southeast Asia (Khan and Tyagi 2021). Here, we present a simplified and replicable DNA metabarcoding approach to assess the diet of an endangered carnivore. Such insights are important for strengthening carnivore conservation and management strategies, particularly for species like the dhole, for whom prey depletion remains a significant threat across their range. Our study, the first to use DNA metabarcoding to assess spatial and seasonal variation in dhole diet from a high-density dhole landscape in India, provides a detailed understanding of prey consumption and highlights the species' strong reliance on medium to large-bodied wild ungulates.

The near absence of domestic livestock in the dhole diet in our study area underscores the role of healthy wild prey populations in mitigating potential human–carnivore conflict. This is particularly important in multiple-use landscapes where dholes coexist with other large carnivores and people. Our findings suggest that sustained availability of key herbivore species is essential for supporting dhole populations and minimizing resource competition with sympatric carnivores such as leopards and tigers. Importantly, decisions regarding herbivore reintroduction and prey population management, whether within protected areas or in buffer and territorial forests, can benefit from such dietary assessments. Understanding key species can help prioritize ungulate species for recovery and restoring efforts, especially in prey-depleted regions. Looking beyond dholes, many carnivores in India and across tropical Asia persist in shared spaces where high human densities, livestock presence, and competition with conspecifics pose significant management challenges. Our approach offers a scalable and cost-effective framework that can be applied to other species to better understand predator–prey dynamics in such landscapes. Standardizing diet assessments using metabarcoding can thus play an important role in shaping broader carnivore management, guiding targeted prey monitoring, and informing conflict mitigation strategies across the subcontinent.

Author Contributions

Conceptualization: A.T. and U.R. Data collection: A.T., and A.M., P.H. Laboratory work: A.T. and A.M. Data analyses: A.T. Project administration: A.T., N.K., J.R., and U.R. Data curation: A.T., A.M., and P.H. Supervision: A.T. and U.R. Funding acquisition: A.T., N.K., J.R., and U.R. Writing – original draft: A.T. Writing – review and editing: A.T., A.M., P.H., N.K., J.R., and U.R.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive (SRA) and are available under BioProject accession number PRJNA1290056.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Schematic representation of 12sV5 primers with "N"-spacers and Illumina adapter overhangs. The pool of these 11 pair of primers was used to amplify vertebrate DNA from dhole diet along with dhole blocking oligo to block the amplification of host DNA. **Figure S2:** Faceted bar plot showing the frequency of occurrence (FOO) of dietary taxa in dhole scats across different areas (core vs. buffer) and seasons (monsoon vs. winter). Each bar represents the proportion of samples in which a given taxon was detected. **Figure S3:** Faceted bar plot showing the relative read abundance (RRA) of dietary taxa in dhole scats across different areas (core vs. buffer) and seasons (monsoon vs. winter). Each bar represents the proportion of samples in which a given taxon was detected. **Table S1:** Dietary profile of the dhole population in Tadoba Andhari Tiger Reserve. Prey species are reported with frequency of occurrence (FOO) and relative read abundance (RRA) [*all presented values are rounded to three decimal places]. **Table S2:** Information on all primer sets used in this study. DhlBlk is the dhole blocking oligo designed in this study. The DholespID primer pair was used to confirm the species identity of all dhole fecal samples collected, and the 12SV5 primer pair was used to amplify diet taxa from dhole fecal samples. **Table S3:** Information on the total number of reads obtained and the proportion of dhole reads recovered from the method comparison dataset. The corresponding plots are shown in Figure 3 for better visualization.