Received: 14 August 2024 | Revised: 27 January 2025 | Accepted: 18 March 2025

DOI: 10.1002/ecs2.70288

ECOSPHERE
AN ESA OPEN ACCESS JOURNAL

ARTICLE
Animal Ecology

DNA metabarcoding provides new insight into the diet of invasive chital deer (*Axis axis*) in a tropical savanna landscape

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Funding information
Australasian Wildlife Management Society, Postgraduate Student Research Award; Ecological Society of Australia, Holsworth Wildlife Research Endowment; Sporting Shooters' Association of Australia; Australian Research Council, Grant/Award Number: LP180100267; James Cook University

Handling Editor: Guoliang Li

Abstract

The introduction of non-native species into new environments can cause significant ecological harm and is considered a major conservation threat. As populations of invasive species continue to establish and increase across the globe, novel methods can provide new insights into their biology and potentially aid in management. In this study, we examined the diet of non-native chital deer (*Axis axis*) in a tropical savanna environment in northern Australia. Using DNA metabarcoding of fecal samples, we described the dietary items consumed by 149 individuals over a two-year sampling period and associated each item with individual body condition. The DNA metabarcoding method detected significantly more dietary items consumed by individual chital deer at each of the taxonomic levels (family, genus, and species) when compared with previous analyses. We observed marked differences in diet composition across multiple seasons and sites. Significantly more sequences from the genera *Terminalia*, *Diospyros*, *Jasminum*, and *Hakea* were detected in samples collected from individuals in poor condition during the dry season, suggesting that a different suite of food resources is being consumed by a subset of individuals during periods when forage quantity and quality is low. Most notably, our results indicated that chital are consuming a browse-dominated diet throughout the year, differing from previous macroscopy analyses which suggested chital are predominantly grazers during the wet season in northern Australia. Our findings give support for the use of DNA metabarcoding to qualitatively assess diet composition compared to macroscopic analysis and suggest that the restricted availability of food during the dry season may result in the consumption of poor quality and detrimental dietary items.

KEYWORDS
Axis axis, body condition, chital, deer, diet, drought, fecal DNA, invasive, macroscopy, metabarcoding, savanna, ungulate

INTRODUCTION

Invasions by non-native species are a leading cause of species extinctions and a major contributor to ecosystem change (David et al., 2017; Gurevitch & Padilla, 2004). Impacts of invasive species include predation, competition, parasitism, and disease, which can increase mortality and alter population sizes of one or more species, thereby modifying complex ecological processes (Doody et al., 2009; Sakai et al., 2001; Vitousek, 1990). Many non-native ungulate species have been introduced around the world and have since established naturalized populations in a variety of environments (Forsyth & Duncan, 2001; Griffith et al., 1989; Moriarty, 2004; Spear & Chown, 2009). Herbivory by invading ungulate species can reduce vegetation biomass and alter nutrient cycles, which can negatively impact other co-existing native herbivores and ecosystem interactions (Motta et al., 2020; Rooney & Waller, 2003).

In Australia, non-native deer have colonized environmental and agricultural areas through natural dispersal, farm escapes, and human-assisted liberations (Davis et al., 2023; Moriarty, 2004). Chital deer (*Axis axis*), also referred to as spotted or axis deer in their native range, were first introduced to the tropical savanna ecosystems of northern Australia in 1886 (Roff, 1960). The initial founding population size was only four individuals, but the species has since dramatically increased in both population size and distribution (Forsyth et al., 2019; Moriarty, 2004). The attitudes of landowners toward chital are mostly negative due to the damage they cause to native vegetation and sensitive ecosystems, competition for food with cattle, their potential to act as vectors for disease, and the subsequent costs associated with management (Cripps et al., 2019; Davis et al., 2016; Figgins & Holland, 2012). Given that their population size and distribution are expanding, exploring and implementing effective methods to understand and restrict further range expansion is a high priority for land managers (Forsyth et al., 2019; Kelly et al., 2021; Simberloff, 2003).

All animals require a continuous supply of energy to grow, maintain homeostasis, and reproduce (Hughes et al., 2018; McCue, 2010). When sources of energy are limited or unavailable, animals must rely on energy reserves to survive, thereby increasing mortality risk (Bender et al., 2008). Body condition, which is assumed to be related to an animal's fitness, is directly impacted by the composition and availability of dietary items (Dunn et al., 2018; Fløjgaard et al., 2017). To maintain healthy body condition, many herbivores select habitat based on food availability. Identifying the dietary components that significantly influence body condition is therefore essential for understanding how animals respond and adapt to new environments (McNaughton, 1988; Rahman et al., 2017).

Animals of the suborder Ruminantia, which includes deer, are generally classified into one of three main dietary groups: browsers, intermediate feeders, and grazers (Hofmann & Stewart, 1972). The diet of browsers is dominated by woody and nonwoody items, including tree foliage, fruits, and shrubs, while grazers feed mostly on grasses and grass-like plants (Gordon, 2003). Somewhere in the middle of this range, intermediate feeders consume a mixture of browse and graze material, mostly in response to seasonal variation in forage availability (Hofmann & Stewart, 1972). In northern Australia, chital deer have been classified as predominantly grazers during the wet season, through the use of macroscopic analysis of rumen samples (Watter, Baxter, Brennan, et al., 2020). Although useful, macroscopic techniques are often influenced by different digestion rates of various plants, which can result in species richness being underestimated (Kessler et al., 1981; McCaffery et al., 1974; Wheeler et al., 2004). Recent advances in DNA analysis, in the form of metabarcoding, have enabled rapid, efficient, and accurate identification of species composition from a variety of sample types (Lopes et al., 2015; Nichols et al., 2016). Items consumed by a species of interest can be reliably identified from degraded fecal samples, provided that appropriate molecular markers are selected and access to adequate sequence reference databases is available (Kartzinel et al., 2015). Numerous studies advocate DNA metabarcoding over traditional dietary analysis techniques such as macroscopic analyses, citing advantages including higher sensitivity, increased taxonomic detail, and cost efficiency (Milla et al., 2021; Nichols et al., 2016). However, the quantitative ability of metabarcoding to reliably infer the amounts of consumed plant biomass from sequence read abundance still lacks support; thus, quantitative interpretation should be met with a degree of caution, especially in non-controlled settings.

In this study, we used DNA metabarcoding methods to analyze chital deer fecal samples and assess the dietary composition of the species in an invaded tropical savanna landscape. Our overall goal was to detail the spatial and temporal variation in the diet of the species, while also providing a point of comparison with previous dietary research. We compared the plant taxonomic resolution provided by DNA metabarcoding with previous macroscopic analysis of rumen samples performed by Watter, Baxter, Brennan, et al. (2020), assessing differences in the number of dietary items detected at several taxonomic levels. We categorized each dietary item detected using the DNA metabarcoding technique as one of five different plant growth forms (climber, forb, grass, shrub, or tree), and used sequence read depth and occurrence data to document changes in the diet of chital over multiple seasons. We examined differences in dietary composition of chital between seasons, sites, and sexes, to identify

drivers of dietary variation. Associations between the plant families and genera consumed by chital individuals and their subsequent body condition were investigated to determine if and what dietary items may have influenced body condition. Given the species invasive status, we expected that associations among significant dietary items and body condition would be important information for predicting and managing further expansion of the species into new environments.

METHODS

Study area

This study was conducted across two cattle stations in the Einasleigh Uplands bioregion of northern Australia, approximately 110 km north of Charters Towers (20°4′35.1″ S, 146°15′24.6″ E; Figure 1). Spyglass (19°29′26.4″ S, 145°41′28.7″ E) and Niall (19°25′10.6″ S, 145°18′21.8″ E) stations are similar in size (38,200 and 43,200 ha, respectively) and carrying capacity of adult cattle during periods of average rainfall (O'Reagain et al., 2009; Watter et al., 2019). Chital deer densities differed significantly across the two sites, with 40 deer per km^2 estimated at Niall and 10 deer per km^2 at Spyglass (Pople et al., 2023). Both stations are within 30 km of the original chital deer release location at Maryvale station in 1886.

The broader study area is an open tropical savanna, with areas cleared to improve grazing conditions for cattle. Spyglass is dominated by narrow-leaved ironbark (*Eucalyptus crebra*) and yellowjacket (*Eucalyptus similis*), in combination with native (*Dactyloctenium radulans*, *Chrysopogon fallax*) and non-native (*Heteropogon contortus* and *Echinochloa mosambicenis*) graminoids. The predominant tree species at Niall are ironbark (*Eucalyptus crebra*), box (*Eucalyptus persistens*), and black gidyea (*Acacia argyrodendron*), and both sites support similar blends of introduced pasture grasses (*Bothriochloa pertusa* and *Cenchrus ciliaris*). A reduction in the total vegetation cover of grasses and forbs, but not shrubs, is observed during the dry season, resulting in a nutritional bottleneck as herbaceous plants senesce (Watter, Baxter, Brennan, et al., 2020). The forage quality of grasses and forbs is low during this time, particularly in terms of digestibility and protein content (Burrows et al., 1990; Poppi & McLennan, 1995). The mean temperature in the region ranges from a low of 11.6°C in June to a high of 34.7°C in December. The timing and amount of rainfall vary significantly across seasons, and an average of 644.6 mm is captured annually, ~75% of which falls between November and March (Australian Bureau of Meteorology, 2023).

Fecal sample collection

Fecal samples were collected from Niall and Spyglass during sampling programs in the October 2014 and 2015 dry seasons, and the March 2015 and 2016 wet seasons. Annual rainfall was below average for the entire sampling period, resulting in the local government areas where Spyglass and Niall are positioned being drought-declared

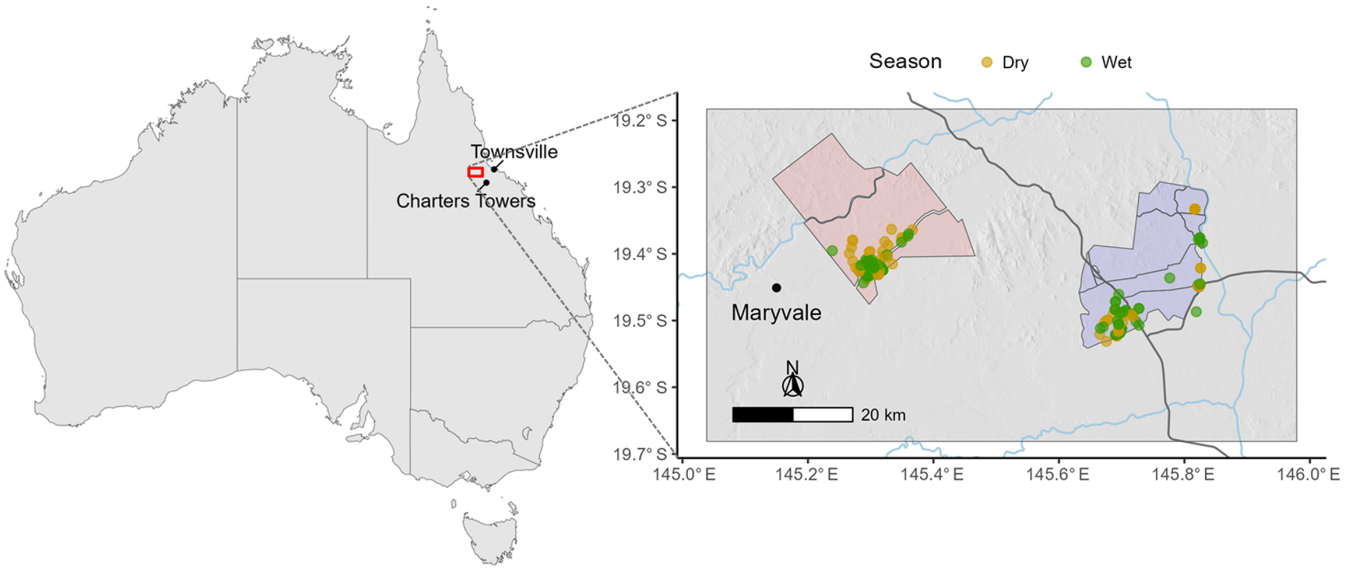

FIGURE 1 Study area in the Einasleigh Uplands bioregion of northern Australia, showing the collection locations for 152 chital deer fecal samples across two successive wet and dry seasons. Niall station appears in red, and Spyglass station appears in blue. The original release location of chital deer, Maryvale, is also shown.

(Pople et al., 2023). Chital culls were carried out under the Queensland Department of Agriculture and Fisheries Animal Ethics approval (AEC SA2014/07/475). A total of 67 males and 85 females were necropsied across each of the four sampling events (Table 1). For each of the sampled deer, fecal samples were removed from the rectum, placed in separate field collection bags, and frozen at −4°C. Samples were removed directly from culled individuals, thereby reducing the likelihood of contamination by pollen, seeds, or plant material not consumed by deer. Morphological information, including sex, body mass, body length, and kidney fat index (KFI), was recorded for each deer (Watter et al., 2019).

DNA extraction and PCR amplification

Fecal samples for the DNA extraction protocol were prepared by homogenizing the collected pellets from each individual deer within their separate field collection bags. Then, 200 mg from each homogenized fecal sample was transferred into a 2-mL microcentrifuge tube, and DNA extractions were performed using a QIAmp Fast DNA Stool Mini Kit (Qiagen), according to the manufacturer's instructions. All extractions were undertaken in a polymerase chain reaction (PCR) free room, treated with 15 μL of Proteinase K, and eluted in a final volume of 200 μL of Buffer ATE. Throughout the process, six extractions containing no fecal material were performed to monitor potential contamination and serve as negative controls. DNA extraction concentrations were quantified with a NanoDrop Spectrophotometer and stored at 4°C.

The g and *h* primers were used to amplify segments of the chloroplast *trnL* intron P6 loop gene region (Taberlet et al., 2007). The associated sequences for the g and h primers are 5′-GGGCAATCCTGAGCCAA-3′ and 5′-CCATTGAGTCTCTGCACCTATC-3′, respectively. An Illumina nextera adapter sequence followed by the *trnL* gene specific primer sequence was used in the first round of PCR. Each PCR mixture comprised 15.5 μL of nuclease-free H_2O, 0.5 μL of MyTaq HS DNA Polymerase (Bioline), 5 μL of 5× MyTaq Reaction Buffer (containing dNTPs, $MgCl_2$, and enhancers at optimal concentrations), 0.5 μL of each forward and reverse primer (10 μM), and 3 μL of DNA template for a 25-μL reaction. The PCR conditions contained an initial denaturation step at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 10 s, followed by a final extension step at 72°C for 2 min. In cases where samples failed to amplify, DNA samples were diluted at 1/10 and 1/100 rates with nuclease-free H_2O until amplification was successful. PCRs included both negative extraction and nuclease-free H_2O controls to test for contamination. Totally 162 PCR products were sent to the Australian Genome Research Facility (AGRF), where amplicons underwent final purification, indexing, and normalization steps to ensure equal DNA concentrations across all samples. Sequencing of amplicons was performed using a MiSeq reagent V3 kit (150 cycles) on an Illumina Miseq High-Throughput sequencer.

TABLE 1 The number of male and female chital deer sampled across the Niall and Spyglass sites, in dry and wet seasons.

	Niall		Spyglass	
Sex	**Dry**	**Wet**	**Dry**	**Wet**
Female	16	20	26	23
Male	17	20	15	15
Total	33	40	41	38

Bioinformatics and data filtering

Sequenced amplicons were de-multiplexed based on unique identifiers incorporated in the Illumina MiSeq protocol and were downloaded as fastq files for bioinformatic analysis. We inspected the Illumina reads and removed all occurrences of the *trnL* intron P6 loop PCR primers in the forward, forward complement, reverse, and reverse complement directions, using the software *CUTADAPT* (v. 4.0; Martin, 2011). We identified amplicon sequence variants (ASVs) using the *DADA2* package (v. 1.16.0; Callahan et al., 2016) in R (v. 4.2.2; R Core Team, 2022). ASVs are a commonly used clustering approach and can distinguish sequence variants with single-nucleotide differences. Using *DADA2*, we filtered low-quality sequences by specifying a maximum expected error value of 2, a truncation value of 2, and a minimum length of 10, as this matched the minimum possible length of sequences using the *trnL* primer pair. Sequences were error corrected using a default parametric error learning algorithm implemented in *DADA2*, and forward and reverse sequences were then merged using a minimum overlap of 12 bases. We filtered out any chimeras from the dataset using the bimera algorithm and ran a final filtering step to remove any remaining sequences that were not within the expected 10-143 bp range. At the completion of the *DADA2* pipeline, we constructed a table containing the sequence read depth for each inferred ASV across all samples.

Although taxonomy can be assigned to the ASVs generated by *DADA2*, the *trnL* sequences amplified from fecal samples are shorter than the minimum length

required for taxonomic assignment using the naïve Bayesian classifier implemented within the package. Instead, we extracted the ASV sequences in fasta file format using the *seqRFLP* package (v. 1.0.1; Ding & Zhang, 2012). We uploaded the fasta file to the National Centre for Biotechnology Information (NCBI) GenBank nucleotide database, and aligned the ASVs using default blastn parameters in BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990). To limit the results to records for our specific gene region of interest, we included the following Entrez query: (trnL OR tRNA-Leu OR trn-L OR trn L) AND (chloroplast[Filter] OR plastid [Filter]). We imported the output from BLAST into MEGAN (v.6; Huson et al., 2016) for taxonomic analysis using the weighted lowest common ancestor (wLCA) algorithm. We used default settings, except for a specified minimum score of 50, a maximum expected value of 0.01, a top percent value of 5, and a minimum support percentage of 0.01.

The Atlas of Living Australia (www.ala.org.au) database was consulted to ensure that all taxonomic classifications inferred by MEGAN software had previously been recorded within the study area and broader bioregion. We used the package *AusTraits* (v. 2.1.2; Falster et al., 2021) to obtain plant trait data for all ASVs with taxonomic classifications in the dataset. The *phyloseq* package (v. 1.42.0; McMurdie & Holmes, 2013) was used to merge and filter the ASV table, taxonomic classifications, and sample data for further analysis in R. We used the *decontam* package (v. 1.20.0; Davis et al., 2018) to remove potential contaminant sequences that were identified in the DNA extraction and PCR controls, using the "prevalence" method with a threshold of 0.5. Next, we filtered the entire dataset based on a minimum taxonomic classification of "Order," and hence any ASV that was not determined to at least this rank was removed. We filtered out any ASV where the sum of that taxon across all samples was less than 0.0001% of all ASVs. This value was selected to maximize the amount of dietary information retained, and preserve rare dietary items within the dataset. Our comparison of this threshold value with other regularly used values in dietary metabarcoding studies (i.e., 0.001%, 0.01%, and 0.1%), showed no differences among the most frequently occurring taxa or read depth across samples, and therefore we continued our analysis with this threshold (Appendix S1: Figures S1–S4). All remaining ASVs were merged at their lowest level of taxonomic identification using the "tax_glom" function in *phyloseq*, allowing all unique items to remain in the dataset at varying levels of order, family, genus, and species. We subsequently use the terms taxon or taxa to refer to these ASVs merged at different levels.

Statistical analysis

To determine whether the diet of chital deer had been sampled sufficiently, the cumulative richness of all detected taxa with an increasing number of samples was explored using a custom function in R. This function performed 999 randomized permutations of the data to estimate the mean and standard error of detected taxa with an increasing sample size. The metabarcoding dataset and previous macroscopy analysis performed by Watter, Baxter, Brennan, et al. (2020) on samples from the same deer individuals were explored in several ways. We compared the mean number of items detected using our metabarcoding techniques to the previous macroscopy analysis at four different taxonomic levels using a Wilcoxon signed-rank test, as the macroscopy data were not normally distributed (Shapiro–Wilk: $W = 0.831$, $p < 0.01$; Kolmogorov–Smirnov: $p < 0.01$). We used a Mantel test to explore the relationship among pairs of samples analyzed using the macroscopy and metabarcoding methods, using a Jaccard pairwise dissimilarity matrix for the two techniques. A positive correlation for this test would suggest that both techniques showed similarities among pairs of samples in terms of dietary composition.

Using the DNA metabarcoding dataset, we calculated the relative read abundance (RRA) of each taxon by dividing the sequence read depth (i.e., total reads per sample) for each taxon by the total sequence read depth for all samples. In dietary studies, the RRA of each taxon has been interpreted as a measure of relative biomass consumed, however, further work is needed for this to be broadly accepted (Lamb et al., 2019; Stapleton et al., 2022). We also calculated the frequency of occurrence (FOO) by dividing the number of samples in which an individual taxon was detected by the total number of samples, to indicate how common a dietary item is among samples. We explored several methods to determine the diet of chital deer and describe any seasonal variability. Each ASV was classified into one of five different growth form categories based on the plant trait data obtained from *AusTraits*. These categories were: "climber, forb, grass, shrub, and tree". First, we used the sequence read depth to provide a proxy measure of overall plant biomass from each of the five plant growth forms consumed by chital. Second, we calculated the number of observations for each growth form and compared changes in the proportion of these items between wet and dry seasons using a chi-square test, because the use of sequence read depth to indicate the relative amounts of individual items contained within a sample is still debated. Biological factors, such as differences in the DNA quantity per unit mass of tissue (e.g., in leaves, stems, and roots), and technical factors such as inefficient

DNA extraction protocols or primer biases can result in discrepancies between the amount of an item consumed, and the amount of DNA recovered (Alberdi et al., 2018; Ando et al., 2020; Pompanon et al., 2012). Therefore, we explored abundance and frequency measures to respond to these concerns. Lastly, we compared the number of detected items for each growth form between wet and dry seasons using Wilcoxon signed-rank tests, to assess the overall seasonal richness of the growth forms.

We compared the number of taxa detected within each sample between seasons, sites, and sexes using Wilcoxon signed-rank tests. This nonparametric test was selected because the richness of taxa within the metabarcoding dataset was not normally distributed (Shapiro–Wilk: $W = 0.981$, $p = 0.03$; Kolmogorov–Smirnov: $p < 0.01$). To explore the plant species composition of fecal samples among seasons, sites, and chital sexes, we used nonmetric multidimensional scaling (NMDS), generated using Bray–Curtis distance measures on abundance data in the *vegan* package (v. 2.6.4; Oksanen et al., 2022). Stress values were used to evaluate the goodness-of-fit of the NMDS ordination, with values >0.3 signifying unsatisfactory representation of the data (Clarke & Warwick, 2001). Differences in the composition of fecal samples between seasons, sites, and chital sexes were explored using a permutational multivariate analysis of variance (PERMANOVA) analysis in *vegan*, using the "adonis" function with 9999 permutations. Specifically, the PERMANOVA analysis was performed using two different data types; a Bray–Curtis distance matrix generated from abundance data, and a Jaccard distance matrix generated from presence/absence data. These matrices compared diet among all pairs of fecal samples, with values close to 1 suggesting complete similarity in dietary composition, and values close to 0 suggesting little to no similarity. We performed analyses for both distance measures to account for differences that could arise from sequence read abundance (Bray–Curtis) and sequence presence/absence (Jaccard). We then performed an analysis of multivariate homogeneity of group dispersions using the "betadisper" function in *vegan*, to explore patterns of dispersion among groups (seasons, sites, and chital sexes).

To explore associations between the presence of dietary items and body condition, we performed differential abundance analysis using the package *DESeq2* (v. 1.38.3; Love et al., 2014). We report log2 fold changes for significantly associated dietary items, which indicates effect size estimates. Chital deer samples were grouped into two categories of body condition depending on their KFI (method available in Watter et al., 2019). We considered that any animal depleting their bone marrow fat reserves were in poor condition for the analysis, which is the last point of fat depletion, and is a clear threshold reflecting long-term nutritional status. Watter et al. (2019) reported this occurring at KFI values below ~30% in the chital deer population from which the fecal samples were collected (i.e., the mass of the fat on the kidneys was 30% of the total lean mass of the kidneys). Individuals were classified as being in "poor condition" if their KFI was less than or equal to 30%, and "good condition" if their KFI was greater than 30%. We performed differential abundance analyses for each season, but included individuals from both sexes and all age classes in the same analyses, as Watter et al. (2019) reported no significant differences in body condition among different sexes or ages of chital deer. Subsequent tests for significance were performed using a Wald test.

RESULTS

Of the 152 samples sequenced in this study, 149 passed quality-filtering steps and were retained in the final analyses. We obtained a total of 8,621,751 filtered reads across the entire dataset, which comprised 359 unique dietary ASVs. These ASVs represented 30 unique plant orders, 68 families, and 92 genera. A total of 50 ASVs were confidently identified to species level (Appendix S1: Table S1).

Comparison to macroscopy

The rate of new taxa detections with additional fecal samples decreased substantially at a sample size of approximately 130 fecal samples, suggesting that a representative number of fecal samples had been obtained for adequately assessing the diet of chital deer (Figure 2).

The Bray–Curtis dissimilarities among pairs of samples in the metabarcoding matrix were positively correlated with the dissimilarities among the same pairs of samples in the macroscopy matrix. This suggested that the metabarcoding and macroscopy techniques both detected similar patterns of dietary similarity among pairs of samples (Mantel test; $r = 0.1717$, $p < 0.01$). The metabarcoding method detected significantly more dietary items at the levels of family (Wilcoxon signed-rank test; $W = 21{,}429$, $p < 0.01$), genus (Wilcoxon signed-rank test; $W = 21{,}065$, $p < 0.01$), species (Wilcoxon signed-rank test; $W = 18{,}786$, $p < 0.01$), and taxon (Wilcoxon signed-rank test; $W = 21{,}441$, $p < 0.01$; Figure 3a). Between the two methods, a total of 70 different families, 110 genera, and 76 species were detected (Figure 3b). While metabarcoding identified ~97% of the families (71.4% uniquely) in the combined dataset, this technique detected relatively fewer unique plant items at lower taxonomic levels (69.1% of unique plant items at genus and 60.8% of unique plant items at species level).

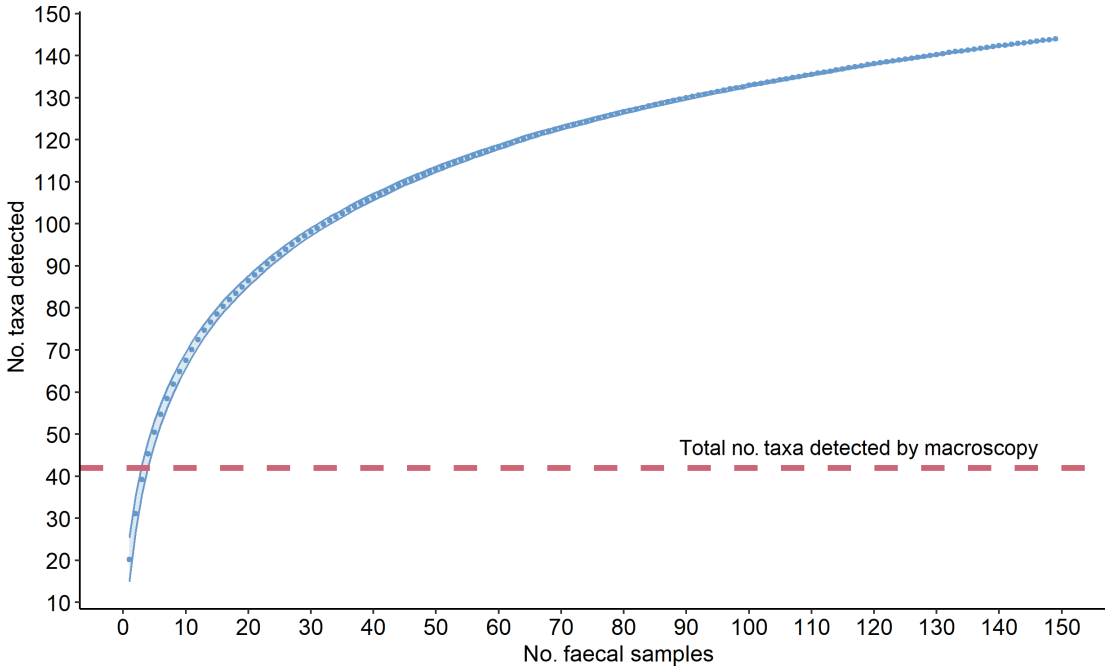

FIGURE 2 Cumulative observed richness of chital deer dietary items at the taxon level. The shaded blue area represents the SE of the estimated mean. The red dashed line corresponds to the total number of taxa observed using macroscopy (Watter, Baxter, Brennan, et al., 2020).

Metabarcoding dietary composition

Using the metabarcoding dataset, the most frequently occurring plant families detected in the diet of chital deer were the Fabaceae (legumes; observed in 99.3% of samples), Poaceae (grasses; 97.9% of samples), Myrtaceae (myrtles; 96.6% of samples), Apocynaceae (dogbanes; 95.3% of samples), and Malvaceae (mallows; 85.2% of samples). The relationship between read abundance and occurrence of dietary items was not linear, as some taxa, such as the Poaceae and Malvaceae, were observed in most samples but only represented <10% of the sequence reads (Figure 4).

Sequence read abundance data indicated that trees were the major dietary component during the dry and wet seasons, followed by climbers (31.3% and 16.8% of the sequence reads in the dry and wet seasons respectively; Figure 5a). Using frequency data, the proportions of growth forms in the diet between dry and wet seasons differed significantly (chi-squared; $X^2 = 171.6$, df $= 4$, $p < 0.01$). Specifically, the proportions of grasses (z-test; $X^2 = 28.9$, df $= 1$, $p < 0.01$) and forbs (z-test; $X^2 = 91.1$, df $= 1$, $p < 0.01$) increased in the wet season, while the proportion of trees (z-test; $X^2 = 80.6$, df $= 1$, $p < 0.01$) declined. While most of the sequence read depth was attributed to trees across both seasons, comparisons of richness for each of the five growth forms revealed that shrub dietary items were the most diverse among samples (Figure 5b). Comparing richness of growth forms between seasons, there were larger numbers of grasses (Wilcoxon signed-rank test; $W = 1100$, $p < 0.01$) and forbs (Wilcoxon signed-rank test; $W = 1115$, $p < 0.01$) detected in samples in the wet season than in the dry season, while the opposite was true for tree dietary items (Wilcoxon signed-rank test; $W = 4342$, $p < 0.01$).

Community level characteristics

The number of dietary items detected in the chital deer fecal samples differed significantly between seasons (Wilcoxon signed-rank test; $W = 1951$, $p < 0.01$) and sites (Wilcoxon signed-rank test; $W = 2248$, $p = 0.04$), but not sexes (Wilcoxon signed-rank test; $W = 2999$, $p = 0.31$). Samples clearly differed between season and site in the ordination generated using NMDS (Figure 6). Season (Bray–Curtis; $F_{1,148} = 12.249$, $r^2 = 0.077$, $p < 0.01$; Jaccard; $F_{1,148} = 12.749$, $r^2 = 0.057$, $p < 0.01$) and site (Bray–Curtis; $F_{1,148} = 32.31$, $r^2 = 0.181$, $p < 0.01$; Jaccard; $F_{1,148} = 22.803$, $r^2 = 0.134$, $p < 0.01$) were the most important factors in explaining the dietary variation among samples in the PERMANOVA analysis. The composition of plant species in male and female fecal samples was not significantly different (Bray–Curtis; $F_{1,148} = 1.628$, $r^2 = 0.011$, $p = 0.15$; Jaccard; $F_{1,148} = 1.733$, $r^2 = 0.016$, $p = 0.07$). The results of the permutational dispersion tests were significant for season (Beta-dispersion; $F_{1,148} = 20.761$,

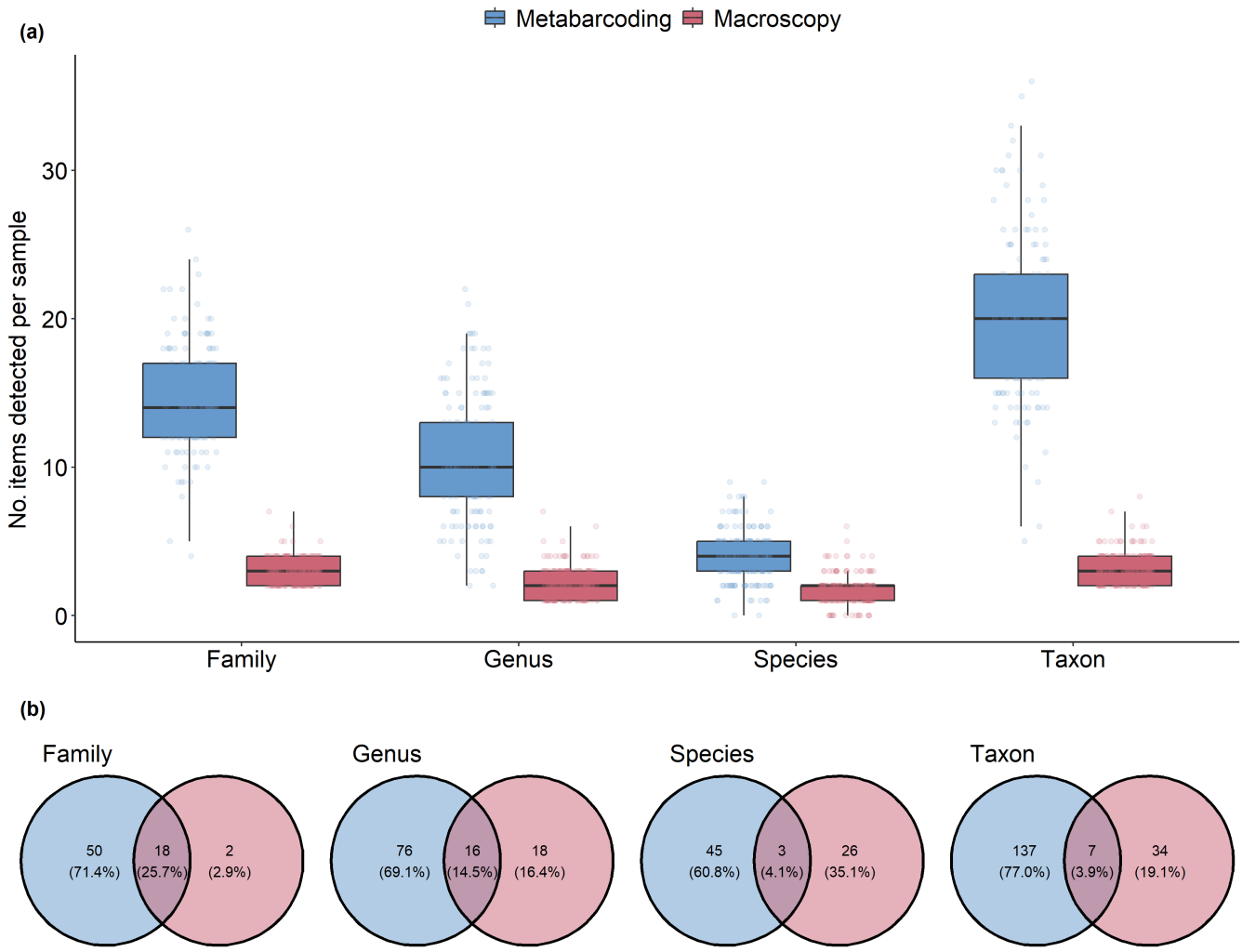

FIGURE 3 (a) Comparison of dietary items detected per sample by metabarcoding (blue) and macroscopy (red) at different taxonomic levels. (b) Overlap among the number of unique items at the family, genus, species, and taxon levels detected in samples analyzed with metabarcoding (blue) and macroscopy (red) methods. For the box plots, the midline represents the median, the box hinges represent the upper and lower quartiles, and the whiskers indicate the minimum and maximum data values (excluding outliers). Individual points represent the raw data summarized by the box plot.

$p < 0.01$) and site (Beta-dispersion; $F_{1,148} = 28.512$, $p < 0.01$), suggesting that the variance within the groups tested was different and may have inflated the patterns observed in the PERMANOVA analyses. However, inspection of the ordination (Figure 6) showed very few outliers and similar patterns of sample distances relative to each of the group centroids. This suggests that variance among groups, and not within, was likely driving the differences among season and site, corroborating the results of the PERMANOVA.

Associations between diet and body condition

While most plant families consumed during the wet season had little impact on chital deer body condition, some plant families were associated with individuals in poor condition during the dry season (Figure 7). Combretaceae (bushwillows; log2 fold change = 23.66, $p < 0.01$), Anacardiaceae (cashews; log2 fold change = 22.77, $p < 0.01$), Oleaceae (olives; log2 fold change = 22.31, $p < 0.01$), Casuarinaceae (sheoaks; log2 fold change = 9.96, $p = 0.02$), Meliaceae (mahoganies; log2 fold change = 7.81, $p = 0.05$), and Ebenaceae (ebonies; log2 fold change = 7.17, $p = 0.05$) all exhibited large, positive log2 fold changes in sequence read depth, indicating an increased abundance of these items in the diets of individuals in poorer condition during the dry season. More specifically, in the dry season, the genera *Terminalia* (log2 fold change = 24.04, $p < 0.01$), *Jasminum* (log2 fold change = 23.31, $p < 0.01$), *Diospyros* (log2 fold change = 22.87, $p < 0.01$), and *Hakea* (log2 fold

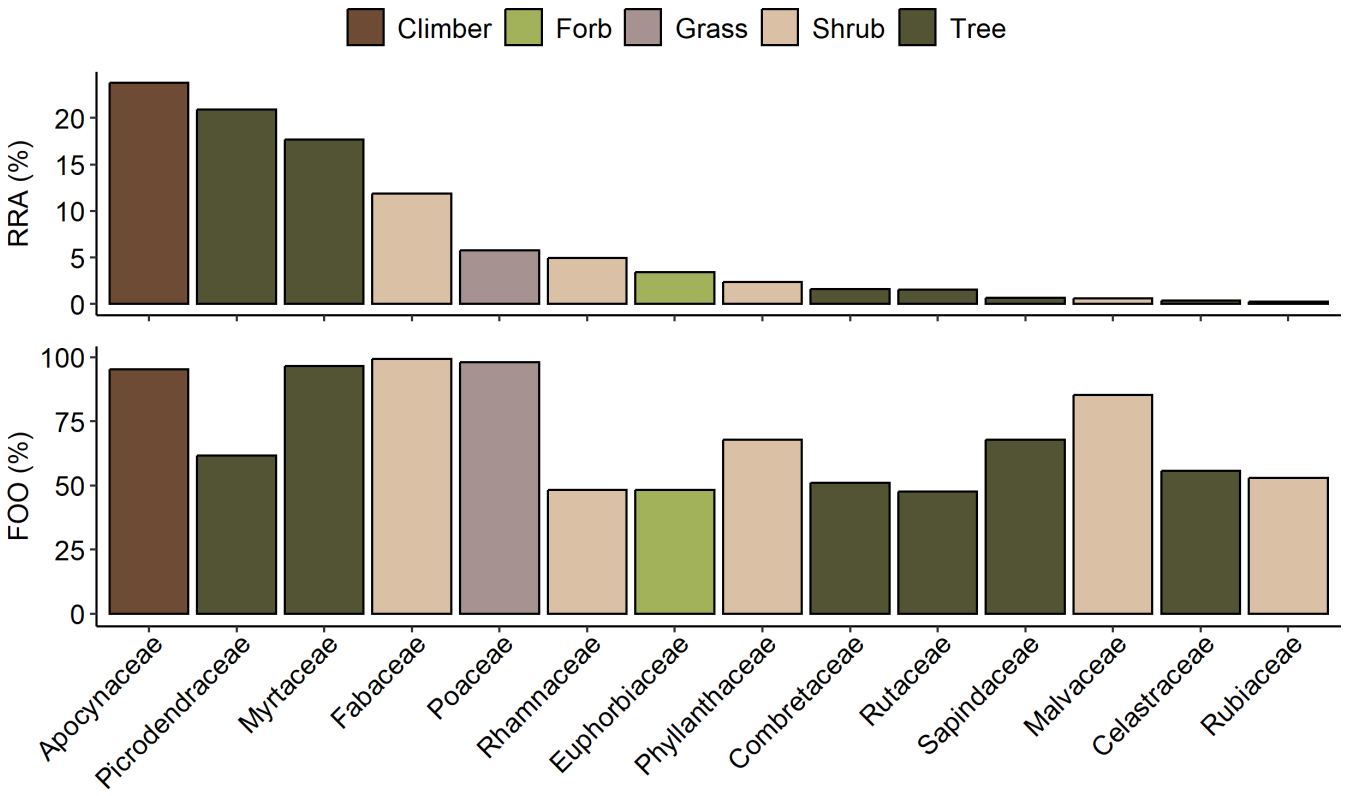

FIGURE 4 Relative read abundance (RRA) and frequency of occurrence (FOO) for the plant families contributing the most sequence reads to the analysis. RRA is calculated as the number of DNA sequences for an item divided by the total number of sequences for that item across all samples. FOO is calculated as the number of samples in which an item is detected, divided by the total number of samples. Colors represent the most common plant growth form identified for each plant family in this study.

change = 22.57, $p < 0.01$) were associated with individuals in poor condition. During the wet season, several plant families displayed moderate log2 fold changes in abundance in the diets of individuals in poor body condition, although none were statistically significant. Only the family Cleomaceae (spider-flowers; log2 fold change = −20.71, $p < 0.01$), which comprises the genus *Cleome* (log2 fold change = −23.98, $p < 0.01$) was significantly associated with good body condition during the wet season.

DISCUSSION

DNA metabarcoding of fecal samples proved to be a valuable tool for extracting new dietary information from chital individuals previously analyzed by conventional methods. Compared with traditional, yet invasive, rumen sample analyses, the metabarcoding method detected significantly more dietary items consumed by individual chital deer at each of the taxonomic levels of family, genus, and species, and detected unique items not previously observed with macroscopy. Importantly, the metabarcoding technique led to the conclusion that chital deer diet in the tropical savanna landscapes of northern Australia is dominated by browse dietary items, in contrast to previous macroscopy results using rumen samples from the same individuals (Watter, Baxter, Brennan, et al., 2020).

Dietary composition and methodological considerations

Although most cervid species are considered browsers or intermediate feeders, research into the diet of chital deer has produced contrasting conclusions depending on the environment they inhabit. The early work of Hofmann (1985) categorized chital as intermediate feeders, and a more recent study of the morphological characteristics of the gastrointestinal tract of free-ranging chital has corroborated this classification (Pérez et al., 2015). In contrast, Henke et al. (1988) observed a high contribution of grasses in the diet of chital deer in the Edwards Plateau ecoregion in the United States, and chital utilize grasslands when forage quality is high in their native range (Moe & Wegge, 1994). While environment likely plays a major role in the diverse diet of chital across the globe, it does not explain the discrepancy observed between the results of this study and the previous work of Watter,

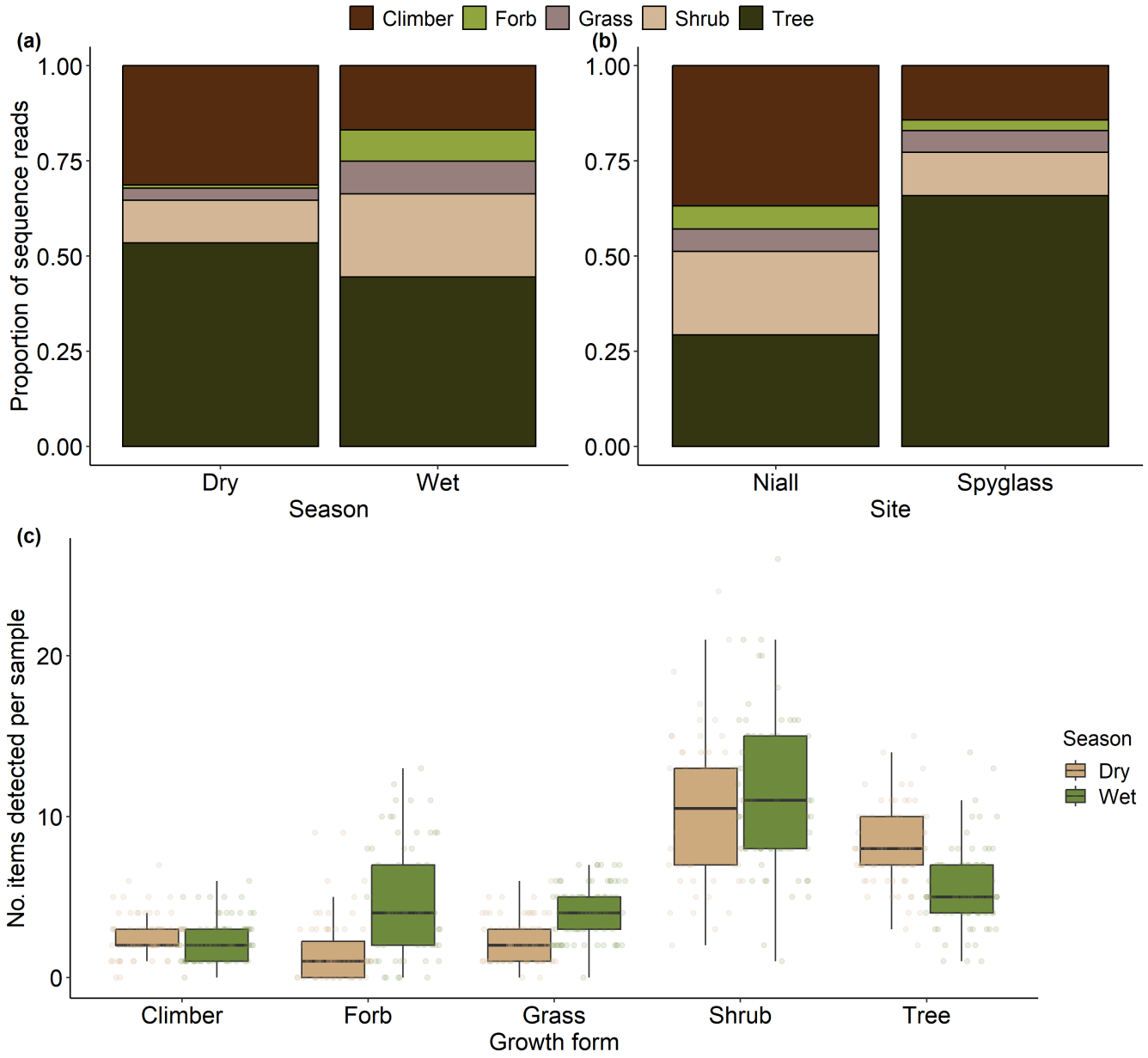

FIGURE 5 The proportion of sequence reads classified as climbers, forbs, grasses, shrubs, and trees, using all samples, and categorized by (a) season and (b) site. (c) Comparison of the richness of each growth form per sample. For the box plots, the midline represents the median, the box hinges represent the upper and lower quartiles, and the whiskers indicate the minimum and maximum data values (excluding outliers). Individual points represent the raw data summarized by the box plot.

Baxter, Brennan, et al. (2020), which used samples derived from the same individuals.

Compared to macroscopy, a key advantage of DNA metabarcoding is an increased detection rate of consumed items and better taxonomic resolution in some cases (Ruppert et al., 2019). The quantitative ability of DNA metabarcoding remains in question, as there is still uncertainty around whether it can accurately describe the relative amounts of dietary items consumed by a target species (Deagle et al., 2019; Pompanon et al., 2012; Stapleton et al., 2022). Several studies have reported a positive correlation between the RRA of a dietary item and the feeding and foraging time undertaken by a species on that item (Mallott et al., 2018) or its mass percentage in a tissue mixture when a bias correction is applied (Thomas et al., 2016). Using fecal samples collected from several large mammalian herbivores within a similar savanna ecosystem, Kartzinel et al. (2015) reported a significant positive correlation between the consumption of C_4 grasses estimated using DNA metabarcoding and stable-isotope analysis. A meta-analysis of 22 different metabarcoding dietary studies exploring relationships

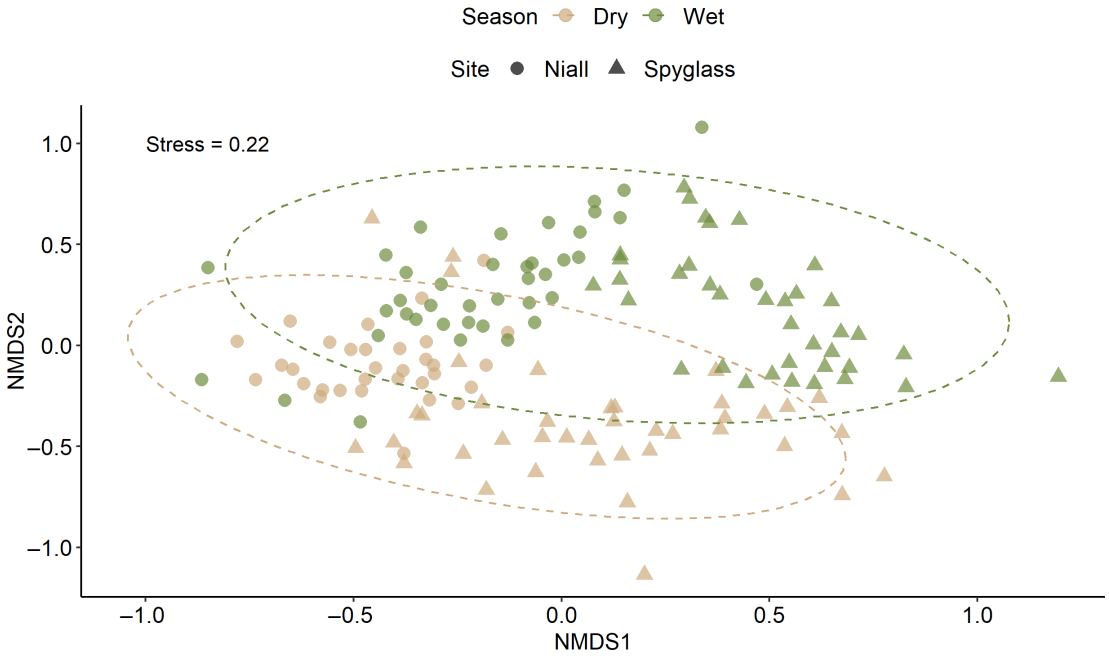

FIGURE 6 Similarity in composition of chital deer fecal pellet samples collected across two seasons (dry and wet) and from two different sites (Niall and Spyglass). Each point represents an individual animal, and dashed lines represent 95% confidence ellipses. NMDS, nonmetric multidimensional scaling.

between the composition of items in a test community (as biomass, number of items, or concentration of DNA) and the proportion of sequence reads corresponding to the respective items in the test community reported a positive relationship, although with large uncertainty (Lamb et al., 2019). While there is some evidence to suggest that sequence read depth can reflect the amount of an item consumed, especially when utilizing the *trnL* intron P6 loop gene region (Soininen et al., 2009; Willerslev et al., 2014), the capacity for DNA metabarcoding to detail quantitative dietary information is still an active area of research. In the absence of further experimentation and validation, our use of RRA to estimate the plant biomass consumed by chital should still be treated with some caution.

Differing sampling strategies might explain some of the discrepancies observed between the results of this study and the previous work of Watter, Baxter, Brennan, et al. (2020). Factors including digestibility, water content, and nutrient composition of food, as well as the size and age of the consumer, can significantly affect the passage rate of food through the digestive system of ruminants (Baker & Hobbs, 1987; Reid & Brooks, 1994). Easily digested dietary items may become increasingly difficult to detect visually through macroscopic analysis of the rumen, while remaining readily detectable over a longer period using DNA from fecal samples (Nichols et al., 2016). Although the rumen characteristics and retention times of various deer species are well understood (Prins & Geelen, 1971), estimates of DNA persistence time from initial consumption to final detection in fecal samples are currently limited to a few studies on carnivores, with results suggesting that certain food items are still detected multiple days after ingestion (Deagle et al., 2010; Thuo et al., 2019). Because the dietary diversity and digestive systems of carnivores and herbivores are fundamentally different, it may be unrealistic to apply these DNA detection times to chital (De Cuyper et al., 2020). At the very least, it is possible that the work of Watter, Baxter, Brennan, et al. (2020) represented a snapshot of the most recent items consumed by chital deer, whereas the DNA metabarcoding method undertaken in this study represents chital diet over multiple days. This would explain the increased detection of shrub and tree species in the diet of chital through DNA metabarcoding, as these items are generally digested less completely than forbs and grasses (Holechek et al., 1982) and may remain detectable in fecal samples for a longer period. Equally, this may have also inflated the importance of these items in the diet. Further research into the persistence time of dietary item DNA in fecal samples, particularly for herbivores, may help clarify the time periods from plant species and growth form consumption to final detection with fecal DNA.

Body condition and habitat associations

Three plant families were relatively overrepresented in samples from chital individuals in poor condition during the dry season (Combretaceae, Anacardiaceae, and

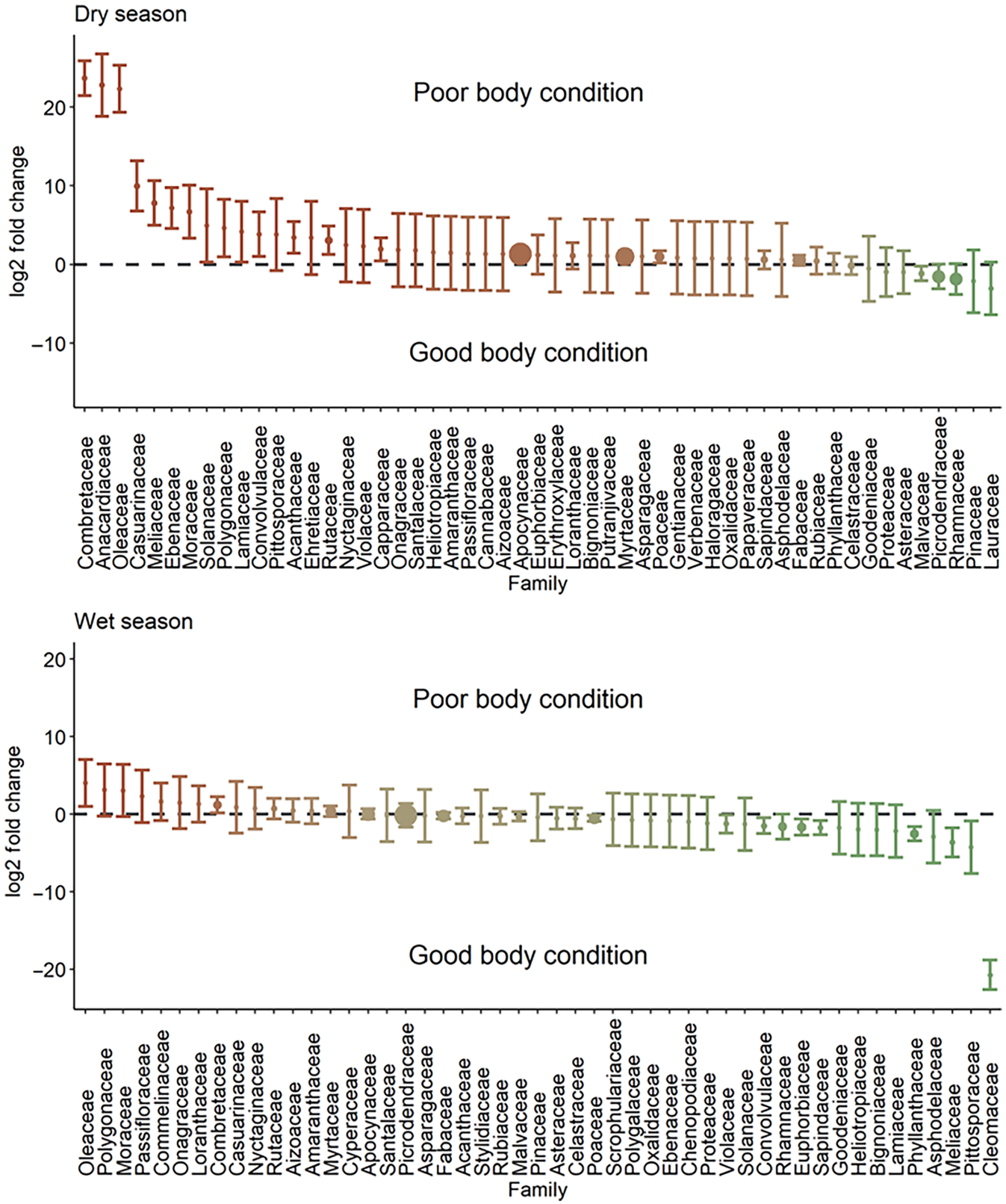

FIGURE 7 The log2 fold change (LFC) for plant families detected in the chital deer diet across the dry and wet seasons. A positive LFC above the dashed line indicates that a plant family was detected more in the diets of animals considered to be in poor body condition (kidney fat index <30%), and a negative value indicates that a family was detected in diets of animals in good body condition. The symbol point size indicates the mean sequence read depth for each family in the analysis, and bars represent SE. A gradient of colors is used to differentiate plant families associated with individuals in poor (brown) or good (green) body condition.

Oleaceae; Figure 7). This suggests that variation in body condition of individuals is linked with the consumption of these taxonomic groups. Whether increased consumption of these plant items causes poor body condition, or whether poor body condition or limited food availability leads to an increased consumption of these plant items is difficult to determine. Either way, we found that chital deer in poor body condition are using a different collection of food resources compared to individuals in better condition during the dry season.

There was no clear relationship observed among the dietary items that influenced body condition and any specific habitats or vegetation communities, as the few genera associated with body condition are known to occur in many different habitats. *Terminalia* is largely associated with brigalow (*Acacia harpophylla*) dominated open woodlands common to northern Australia, the *Hakea* genus is generally observed along creeklines or low-lying areas, and *Disopyros* and *Jasminum* are found in tropical rainforests, dry woodlands, and rocky savannas (Jessup, 2014; Peeters & Butler, 2014). Given the lack of a connection between each of these genera and one or few habitats exclusively, our results suggest that the effect of individual plant items on chital body condition is greater than any effects of where chital forage.

Some of the dietary items associated with poor body condition are known to detrimentally affect other mammal species. The *Terminalia* genus detected in this study is likely represented by *Terminalia oblongata*, a native Australian species that poses significant risks to livestock, including sheep and cattle (Filippich & Cao, 1993; McKenzie, 1991). Frequent consumption of the species can cause liver and kidney injury, which can negatively impact the body condition of an affected animal (Filippich & Cao, 1993). Excessive consumption has resulted in serious losses to livestock, particularly in dry or drought years when declines in the quality and quantity of pastures are observed (Murdiati et al., 1992). Additionally, the genus *Diospyros* contains several species of fruit-bearing persimmon and ebony trees. In mammals, and especially ruminants, overconsumption of persimmon fruit may form concretions of fibers within the gastrointestinal tract, known as phytobezoars (Banse et al., 2011). These formations occur when the tannins contained within many *Diospyros* species polymerize with acids in the stomach, trapping seeds and fibers from the persimmon fruit and resulting in gastric impaction, ulceration, and significant mass loss (Cummings et al., 1997). While these items might be considered harmful with significant levels of consumption, no observations attributable to *Terminalia* toxicity, such as jaundice and visible discoloration of the liver, kidneys, and intestines, were noted during necropsy, nor were any phytobezoars in the gastrointestinal tract substantial enough to cause malabsorption of nutrients.

Given that the fecal sample collection over multiple years coincided with a period of drought, the observed consumption of dietary items with detrimental impacts is most likely explained by a severe decline in the availability of dietary resources. Two equally plausible hypotheses might further explain why consumption of *Terminalia* and *Diospyros* was particularly evident during the dry season. First, as the quantity and quality of forage decline, competition among individuals may force the consumption of plant species that are less preferred or previously avoided. On the Indian subcontinent, the native range of chital, numerous sympatric ungulates such as four-horned antelope (*Tetracerus quadricornis*) and sambar deer (*Rusa unicolor*) avoid consuming several *Terminalia* species (Haleem & Ilyas, 2022a, 2022b), likely because of its low palatability and toxicity in large quantities. In northern Australia, the nutritional bottleneck placed on the availability of plant resources during dry and pronounced drought conditions may have driven chital to consume *Terminalia* regardless of unfavorable effects (Fritz et al., 1996; Owen-Smith & Cooper, 1987). Second, the consumption of harmful plant species may be explained by their sudden availability. During extended dry periods, *Terminalia* drops leaves, branches, and bark, which become available for consumption by chital at ground level (Bradshaw & Waller, 2016; Filippich & Cao, 1993; Gill, 1992; Nakahama et al., 2021). Similarly, the fruits of *Diospyros* species develop throughout the year, yet mature in the period that coincided with the fecal sample collection during the dry season (Jessup, 2014). Therefore, the increased detection of *Terminalia* and *Diospyros* DNA in the diet of individuals in poor condition during the dry season might be explained by the consumption of suddenly available fruits and leaves by browsing chital. We recommend feeding trials to explore and detail the toxicity effects of various native plant species on chital and other non-native deer, as well as highlight patterns of preference or avoidance among different plant species when all are equally available, and not limited by the effects of drought or the dry season.

CONCLUSIONS

Using a fecal DNA metabarcoding approach, we detected significantly more plant items consumed by invasive chital than previously described by macroscopic analysis of rumen contents. Given that the rumen and fecal samples were collected from the same individuals, our results suggest that DNA metabarcoding can be a more efficient and effective option for characterizing herbivore diet. One important caveat is that this study was conducted

during a period of drought, which would have influenced the diet of chital through restricted availability of many plant species. We therefore suggest further assessment of diet and body condition across a larger geographic area during non-drought periods to further clarify the patterns described here.

Contrary to previous research, our results indicated that the diet of chital in northern Australia is dominated by browse items. Preferential browsing by deer can reduce the abundance of highly preferred species in the environment, which may result in the gradual encroachment of less preferred or avoided species (Davis et al., 2016; Wills et al., 2023). In Australia, competition with livestock is considered a major agricultural impact of introduced deer (Davis et al., 2023), including chital (Watter, Baxter, Pople, & Murray, 2020); however, our results suggest that these competitive interactions are less likely to occur in the study area.

Our observation that poor body condition was associated with the consumption of certain plant species during the dry season suggests that during resource-poor seasons, nutritionally stressed individuals may be forced to consume non-preferred, detrimental, or novel dietary items. It is unknown whether the consumption of any one or more of these unfavorable plant items could play any role in slowing the rate of expansion of chital deer individuals or populations into new environments.

The discrepancy observed between the macroscopic results presented previously and the metabarcoding results in this study requires further investigation. Future research should directly compare the macroscopic and metabarcoding methods in similar locations of the gastrointestinal tract to address the reliability of RRA as a proxy for biomass consumed. Additionally, given the coarse nature of macroscopy, it is advisable to contrast metabarcoding with microscopic techniques to directly compare the detection and prevalence of dietary items in degraded fecal samples (Forsyth & Davis, 2011; Sparks & Malechek, 1968). We anticipate that further experimental studies will advance our understanding of DNA metabarcoding as a quantitative method and therefore improve interpretation of sequence reads arising from dietary analysis in the future.

ACKNOWLEDGMENTS

We would like to thank the Ecological Society of Australia, Holsworth Wildlife Research Endowment, Sporting Shooters' Association of Australia, Australasian Wildlife Management Society, Australian Research Council (LP180100267), and James Cook University for supporting this research. Steve Anderson and Sean Reed facilitated access to Spyglass Beef Research Station, and Dan Lyons is thanked for providing access to Niall Station. Thanks to Michael Brennan, Keith Staines, and Glen Harry for field collection of samples on Spyglass and Niall. We thank Dr. Sally Lau for her help and guidance in the laboratory, and Zoe Taulli for assisting with the identification of ASVs. Lastly, we thank Prof. Joe Holtum, Leesa Baker, and Dr. Danielle Sisson for their advice regarding plant traits and toxic impacts on browsing animals. Open access publishing facilitated by James Cook University, as part of the Wiley - James Cook University agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data (Quin et al., 2025) are available from Dryad: https://doi.org/10.5061/dryad.gqnk98sxd.

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SUPPORTING INFORMATION

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

How to cite this article: Quin, Matthew J., Ben T. Hirsch, Lin Schwarzkopf, Kurt Watter, Anthony Pople, and Jan M. Strugnell. 2025. "DNA Metabarcoding Provides New Insight into the Diet of Invasive Chital Deer (*Axis Axis*) in a Tropical Savanna Landscape." *Ecosphere* 16(6): e70288. https://doi.org/10.1002/ecs2.70288