Journal of Arid Environments 219 (2023) 105094



Contents lists available at ScienceDirect

Journal of Arid Environments

journal homepage: www.elsevier.com/locate/jaridenv





Karoo dwarf tortoises (*Chersobius boulengeri*) prefer and disperse doll's roses (*Hermannia* spp.)

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ARTICLE INFO

Keywords:
Forage
Habitat requirements
Homopus
Reptile
Seed dispersal
Trophic interaction

ABSTRACT

Tortoises inhabit arid regions where they may represent a significant herbivorous component, but are among the most threatened of tetrapod groups. Studying tortoise diets can help predict potential trophic disruptions following extinctions of tortoise populations, and inform conservation. We studied the diet of Karoo dwarf tortoises (*Chersobius boulengeri*), an endangered endemic of the arid Nama and Succulent Karoo biomes in South Africa, using vegetation analysis, DNA metabarcoding, morphological evaluation of faeces, a germination trial, and focal feeding observations. DNA metabarcoding was highly successful in the identification of dietary items. Karoo dwarf tortoises fed on a broad diet, including approximately 10 taxa eaten frequently. Specifically, doll's roses (five species in the genus *Hermannia*) were eaten more frequently than would be expected based on their availability, whereas grasses were rejected, for a dietary Proportional Similarity Index of 0.31–0.38. Dietary selection was likely related to water and nutrient contents of plant taxa. At least four plant species (including doll's roses) germinated from faecal samples, suggesting mutualistic relationships with Karoo dwarf tortoises. The habitat preference of Karoo dwarf tortoises (i.e., well-structured rocky terrain) may provide germination microsites with ample shade and run-off water to benefit seedling recruitment in an arid environment.

1. Introduction

Trophic interactions are an important topic in ecological studies (Dáttilo and Rico-Gray, 2018; Van der Putten et al., 2004), partially due to their interplay with the distribution and abundance of species (Wisz et al., 2013), and ultimately ecosystem integrity and resilience (McCann et al., 1998). For example, trophic cascading effects of the disappearance of large apex consumers can destabilize ecosystems with knock-on effects including wildfires, infectious disease, and biodiversity loss (Estes et al., 2011). In response to human-induced trophic downgrading, studies have addressed mitigation and restoration of lost trophic interactions (Svenning et al., 2015). Substitution with non-native taxa might restore some trophic interactions, but is unlikely to reinstate all original ecological dynamics (Heinen et al., 2023). Thus, revealing and preserving existing trophic relationships can contribute to effective nature conservation.

Basal trophic interactions occur between plants (primary producers) and herbivores (primary consumers). These interactions may be unidirectional (i.e., herbivores benefiting from water, nutrients and energy at the expense of plants), but can be mutual such as when they assist pollination or seed dispersal, or enhance seed germination (Agrawal, 2000). Consequently, loss of trophic interactions between plants and herbivores may trigger various changes in the ecosystem. This is illustrated by declines of tortoise (Testudinidae) populations in arid ecosystems. Tortoises are a significant herbivorous faunal component of many arid regions (Lovich et al., 2018; Turtle Taxonomy Working Group, 2021), but are among the most threatened of tetrapod groups (Cox et al., 2022; Turtle Taxonomy Working Group, 2021). Grazing pressure posed by abundant Aldabra giant tortoises (*Aldabrachelys gigantea*) can alter plant communities, while giant tortoises in general (*A. gigantea*, *Chelonoidis niger*), Texas tortoises (*Gopherus berlandieri*), leopard tortoises (*Stigmochelys pardalis*) and spur-thighed tortoises (*Testudo graeca*) may affect plant communities through seed dispersal and germination enhancement (Lovich et al., 2018). Some plant taxa (e.g., large-seeded island taxa, some grasses) may depend on tortoises for seed dispersal (Falcón et al., 2020; Lovich et al., 2018). Understanding the diets of tortoises is essential to help predict ecosystem effects of potential tortoise extinctions, and to provide conservationists with information about requirements of tortoise habitats. More than a quarter of extant tortoise species occur in South Africa (Turtle Taxonomy

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https://doi.org/10.1016/j.jaridenv.2023.105094
Received 23 July 2023; Received in revised form 28 October 2023; Accepted 30 October 2023
Available online 10 November 2023
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Working Group, 2021) hence tortoise natural diet studies in this region are of particular importance, but are biased towards leopard tortoises (Mason et al., 1999; Milton, 1992; Rall and Fairall, 1993).

Karoo dwarf tortoises (*Chersobius boulengeri*) are endemic to South Africa, where they inhabit a relatively small, arid (<200–400 mm per annum; Esler et al., 2010) range in the Eastern, Northern and Western Cape provinces (Boycott and Bourquin, 2000). Within their range, Karoo dwarf tortoises are restricted to rocky terrain, especially exposed dolerite sills (Loehr, 2023), and spend most of the time in retreats under and among rocks (Loehr et al., 2021). In 2017, the species was categorized Endangered, mainly due to ongoing habitat degradation (Hofmeyr et al., 2018). Because there are no published records of their natural diet, information is lacking about trophic interactions among Karoo dwarf tortoises and the plants in their range, including mutualistic relationships.

This study highlights which plant species are eaten by Karoo dwarf tortoises, and assesses how this might benefit plants. Because active Karoo dwarf tortoises spent 80% of the time walking and scanning, in part to find food (Loehr et al., 2021), we hypothesized that tortoises would select specific plant species rather than feeding according to prevalence. We also expected to find dietary differences between summer and spring, reflecting rainfall and plant phenological patterns. Males and females were expected to have similar diets, given their largely similar behaviours (Loehr et al., 2021). In addition, analogously to other tortoises that disperse seeds (Falcón et al., 2020; Lovich et al., 2018), we hypothesized that Karoo dwarf tortoises would eat fruits and seeds, and that seeds passed through the digestive tract would still be viable.

2. Materials and methods

2.1. Study site and field recordings

We conducted our study at a 16-ha site, with tortoises using adjacent areas up to 250 m, in the Northern Cape Province of South Africa (coordinates recorded on the biodiversity database of CapeNature, Western Cape Province, South Africa). The site was situated on a north-eastern mountain slope (altitude 1,373–1,445 m) with several plateaus in the Nama Karoo biome (Mucina et al., 2006), and had sandstone and dolerite bedrock, boulders and rocks (Loehr, 2023). There are no long-term weather recordings for this region, but weather simulations (Meteoblue, https://www.meteoblue.com/en/weather/forecast/modelclimate, last accessed 07-07-2023) indicate that the climate is arid (average 162 mm per annum) with rainfall peaking in late summer and autumn (i.e., February until April). Average monthly minimum and maximum temperatures range between 1–14 and 14–30 °C, respectively. We placed dataloggers (Hobo S-THB-M00x, RS3-B and S-RGF-M002 connected to H21-USB; Onset Computer Corporation, Bourne, MA, USA) in the centre of the site to record rainfall and ambient temperatures during this study.

We visited the site in summer 2018 (14 February–22 March), 2019 (12 February–20 March) and 2020 (11 February–17 March), and in spring 2018 (2 October–8 November) and 2019 (8–11 October). All plant species that we encountered at the site were preliminarily identified based on field guides (e.g., Court, 2010; Esler et al., 2010; Manning, 2009) and internet sources (e.g., iNaturalist, 2023; South African National Biodiversity Institute, 2023). Two independent specialists (S. Dean and D. Gwynne-Evans, pers. comm.) used photographs to supplement and verify preliminary identifications. Species that continued to have uncertain identifications were prepared as herbarium specimens and identified by Compton Herbarium (Cape Town, South Africa). We recorded for each species its growth form (above-ground bulb, dwarf shrub <1 m, fern, forb, geophyte, grass, parasite, sedge, shrub >1 m, succulent, or tree) and life cycle (annual or perennial). In summer 2018, we randomly positioned 10 permanent 1 m^2 plots at the study site. Plots were marked with rebar rods at the corners, and evaluated once in each sampling period (i.e., 25 February 2018, 25 October 2018, 11 March 2019, and 23 February 2020), except in spring 2019. At each evaluation, we identified all plants in the plots and estimated the proportion of the plot surface that was covered by plants (0%, 0–25%, 25–50%, 50–75%, or 75–100%). In addition, we noted if plants were flowering or carried fruits. Occasional unidentifiable seedlings and woody remains of dead plants were ignored. In summer 2020, we extended the permanent plots with 38 randomly positioned 1 m^2 plots, and evaluated those between 25 February and 15 March following the same procedure that we had used for the permanent plots.

Each sampling period, we searched for hiding Karoo dwarf tortoises under and among rocks. When we found a tortoise, we recorded its sex, with individuals having plastral concavities and large tails being males, smaller individuals than the smallest with male characteristics being juveniles, and remaining individuals being females (Boycott and Bourquin, 2000). We then measured straight carapace length (SCL, to the nearest 0.1 mm) with digital sliding callipers, and body mass (BM, to the nearest 1 g) with a digital balance. If tortoises produced faeces during handling, we collected and air-dried (summer and spring 2018, and summer 2019) or silica-dried (spring 2019 and summer 2020) faeces at room temperature. In summer 2018, we glued radiotransmitters (type RI-2B with internal antenna; Holohil Systems Ltd., Carp, ON, Canada) onto the posterior costal scutes of nine adult male and 16 adult female Karoo dwarf tortoises (total equipment mass ≤9.7% of body mass). Males and females were tracked in summer 2018, spring 2018 and summer 2019, and females subsequently in spring 2019 and summer 2020, when faeces were collected and dried if any were produced during handling. On eight days in summer 2020, we tracked females in random order until an active individual was found, which was left undisturbed and followed until it was seen feeding. Plants eaten were recorded during a 1 h observation period. We followed and observed an opportunistically encountered active male according to the same procedure.

2.2. DNA barcoding

We used the chromosomal internal transcribed spacer 2 (ITS2) region to identify plant taxa in tortoise faeces, but several plants at the study site lacked reference sequences for the ITS2 region in the public DDBJ/ENA/GenBank and BOLD databases. Therefore, we DNA barcoded nineteen plant species. Other species without reference sequences could be identified based on genus- or family-level sequences in the databases (i.e., only one species in a genus or family was present at the study site), or were tall or rare taxa that were unlikely food items, and were not barcoded. Samples for DNA barcoding consisted of fresh leaf cuttings taken at the study site, dried on silica at room temperature.

DNA barcoding was performed by AllGenetics & Biology (Oleiros [A Coruña], Spain), comprising the following procedures: DNA isolations were carried out using the ISOLATE II Plant DNA Kit (Bioline, Memphis, TN, USA), following the manufacturer's instructions. DNA was resuspended in a final volume of 100 μL. A negative control that contained no sample was included in the DNA isolation procedure to check for contamination. The complete ITS2 region was amplified by PCR using the primers ITS2-S2F (5′ ATG CGA TAC TTG GTG TGA AT 3'; Chen et al., 2010) and ITS-p4_mod (5′ CCG CTT AKT GAT ATG CTT AA 3′), modified by AllGenetics from the reverse primer ITS-p4 (Cheng et al., 2016). The PCR reactions were carried out in a final volume of 25 μL, containing 12.5 μL of Supreme NZYTaq Green PCR Master Mix (NZYTech, Lisbon, Portugal), 0.5 μM of each primer, 2.5 μL of the template DNA solution, and PCR-grade water up to 25 μL. The thermal cycling conditions included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 47 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. A negative control that contained no DNA was included in every PCR round to check for cross-contamination. PCR products were run on 2% agarose gels stained with GreenSafe (NZYTech) and imaged under UV light to verify the amplicon size. PCR products were bi-directionally

sequenced using the PCR primers.

Electropherogram analysis and overlapping was conducted in Geneious 8.1.9 (Biomatters, Auckland, New Zealand). During electropherogram analysis, the primer annealing regions and the low-quality regions at both ends of each electropherogram were trimmed (error probability limit of 0.03). Sequence reads were manually checked for sequencing errors or ambiguous base calls, and ambiguities and heterozygous positions were coded using the IUPAC ambiguity code (e.g., K: G or T). Samples that failed initially at the sequencing step were repeated to remove ambiguous base calls and improve sequence length. The samples were identified to the lowest possible taxonomic level by comparing the sequences obtained with reference sequences in the DDBJ/ENA/GenBank and BOLD databases, by using either the NCBI BLASTn tool against the Nucleotide database (nr/nt) or the Classify Sequences tool in Geneious. A sequence similarity-based approach was applied for taxonomic assignment, using different threshold values for the different taxonomic ranks (>97%: species level; 96–90%: genus level; 89–85%: family level; 84–75%: order level; <75%: class level). When all hits above a given similarity threshold matched the same taxonomic group, the query sequence was confidently assigned to this taxon. Conversely, when hits with equal similarity values belonged to different taxa, the query was only assigned to the lowest common taxonomic level.

2.3. Processing of faecal samples

Faeces that had been collected in summer and spring 2018 were used for morphological evaluation, faeces collected in summer 2018, spring 2018 and summer 2019 for a germination trial, and faeces collected in spring 2019 and summer 2020 for DNA metabarcoding. Morphological evaluation was performed using a dissecting scope (magnification 7–100×) to compare morphological characteristics and coloration of plant remains in faeces, soaked in water for 30 min, with dried reference plants from the study site. We recorded plant species or genera, and the presence of flowers and fruits for each taxon. Faeces used for the germination trial were broken into small pieces and placed on a moist, sterile sand and peat mixture near a window at room temperature. We grew seedlings until they could be identified or died. DNA metabarcoding was performed by AllGenetics & Biology, comprising the following procedures: DNA from the faecal samples was isolated upon arrival at the laboratory using the DNeasy PowerSoil DNA isolation kit (QIAGEN), following the manufacturer's instructions. A mock community (22 DNA samples pooled in equimolar amounts) containing plant species that had been DNA barcoded in the current study was processed along with the faecal samples to evaluate the amplification efficiency of the selected ITS2 primer pair in a composite sample. DNA from all samples was resuspended in a final volume of 100 μL. DNA extraction blanks (Bex1, Bex2) were included in every DNA extraction round and treated as regular samples to check for cross-contamination. For library preparation, the complete ITS2 region was amplified using the same primers as had been used for DNA barcoding, and the Illumina sequencing primer sequences were attached to the 5′ ends of primers ITS2-S2F and ITS-p4_mod. PCRs were carried out in a final volume of 25 μL, containing 2.5 μL of template DNA, 0.5 μM of the primers, 10 μM of the blocking primer, 6.5 μL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 25 μL. The reaction mixture was incubated including an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 47 °C for 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min. The oligonucleotide indices that are required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions, but including only five cycles and 60 °C as the annealing temperature. A negative control that contained no DNA (BPCR) was included to check for contamination during library preparation. The libraries were run on 2% agarose gels stained with GreenSafe (NZYTech) and imaged under UV light to verify the library size. Libraries were purified using the MagBind RXNPure Plus magnetic beads (Omega Biotek, Norcross, GA, USA), following the instructions provided by the manufacturer, and were pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA USA). The pool was sequenced in a fraction (1/4) of a MiSeq PE300 run (Illumina). Illumina paired-end raw files consist of forward (R1) and reverse (R2) reads sorted by library and their quality scores. The indices and sequencing primers were trimmed during the demultiplexing step, and the quality of the FASTQ files was checked using the software FastQC (Babraham Bioinformatics, Cambridge, UK).

The obtained ITS2 amplicon reads were processed using QIIME2 (release 2019.10) (Bolyen et al., 2019). DADA2 (Callahan et al., 2016), implemented in QIIME2, was used to remove the PCR primers, filter the reads according to their quality, denoise, merge the reads, remove the chimeric reads, and cluster the resulting sequences into amplicon sequence variants (ASVs). First, DADA2 trimmed and filtered the data to avoid low-quality scores. After checking the read quality profiles, reads were truncated at position 299 for forward reads and at position 245 for reverse reads. Error rates were learned from the dataset to denoise, using the parametric error model implemented in DADA2. Before the inference of sequence variants, dereplication of the dataset was carried out, combining all identical sequencing reads into unique reads to reduce the computational time. The core sample inference algorithm (Callahan et al., 2016) was applied to the dereplicated forward and reverse reads. Consecutively, R1 and R2 reads were merged with DADA2 by overlapping a minimum region of 12 identical base pairs. Artefacts such as chimaeras, generated during PCR and sequencing, may appear in the raw data, and can overestimate the number of merged sequence variants. The DADA2 pipeline included a final processing step to reduce the impact of such artefacts in the dataset. Finally, a table with the number of times that each ASV was observed in each sample was generated.

For taxonomic assignment, a reference database was created by combining the ITS2 sequence data available for the class Magnoliopsida in the DDBJ/ENA/GenBank and BOLD databases, and the ITS2 reference sequences obtained from plants that we barcoded in this study. Identical sequences in the reference database were collapsed to a single entry and their taxonomic information was checked to remove potentially misidentified records. The resulting reference database was used as input to train a Naive Bayes classifier employing the QIIME2 feature-classifier plugin with the fit-classifier-naive-bayes method. Finally, the taxonomy was assigned to ASVs using the pre-trained Naive Bayes classifier and the feature-classifier classify-sklearn approach implemented in QIIME2 (Bokulich et al., 2018). Unassigned sequences were directly queried against the NCBI nr/nt (non-redundant nucleotide) database using BLASTN 2.10.1+. Sequences assigned to non-target organism groups, as well as those sequences that remained unidentified after the BLASTN search, were removed from the ASV table to perform the downstream analysis. Singletons (i.e., ASVs containing only one member sequence in the whole dataset) were excluded from the ASV table. In addition, ASVs occurring at a frequency below 0.01% in each sample were removed to correct for misassignment of the indices during library preparation, sequencing, and/or demultiplexing steps. To verify that sequence depth sufficed to retrieve most of the plant diversity in faecal samples, we constructed alpha rarefaction plots of the number of unfiltered and filtered ASVs obtained with a rarefied number of sequences in each sample, and assessed whether curves tended towards saturation.

2.4. Statistics

We calculated monthly average minimum and maximum temperatures for the study site and compared them with simulated temperatures. This comparison was made using linear mixed models (LMM), with temperatures as dependent variables, time period (February–January simulated, February 2018–January 2019, February 2019–January 2020, and February 2020–March 2020) as factor, and month as random effect.

Frequencies of plant taxa in permanent vegetation plots (i.e., occurrence in one or more plots), as well as frequencies of taxa with flowers or fruits and vegetation cover, were compared among sampling periods with Friedman repeated-measures analysis of variance (RM ANOVA).

Chi-square tests compared frequencies of taxa in faeces as identified through DNA metabarcoding or morphological evaluation. We used Fisher's exact tests to compare taxa frequencies between sampling periods for data from DNA metabarcoding, morphological evaluation, and the germination trial. The relatively large DNA metabarcoding dataset enabled us to compare taxa frequencies between sexes, and between faeces and vegetation plots for summer 2020 (including temporary vegetation plots) by means of Fisher's exact tests. A Fisher's exact test also compared taxa frequencies from DNA metabarcoding with taxa frequencies from morphological evaluation. The dataset originating from morphological evaluation provided frequencies taxa with flowers or fruits, which were compared between sampling periods with Fisher's exact tests. To ensure independence of samples in chi-square and Fisher's exact tests, tested samples consisted of individual tortoises that lacked repeated measures, supplemented with randomly selected recordings of tortoises with repeated measures (Table 1). The full DNA metabarcoding dataset was used to compare frequencies of growth forms among seasons with a Poisson generalized linear mixed model (P-GLMM). Frequencies were used as dependent variable, growth form as factor, and tortoise as random factor.

We summed for each taxon the number of vegetation plots (permanent and temporary plots combined), and the number of faecal samples (DNA metabarcoding, morphological evaluation, and germination trial combined) and focal records in which it occurred, and used Feinsinger's (Feinsinger et al., 1981) Proportional Similarity Index (PSI) to estimate if Karoo dwarf tortoises are dietary generalists or specialists:

$$\mathrm{PSI} = 1 - 0.5\sum_{i}|p_i - q_i|$$

where p_i is the proportion of taxon i relative to all taxa eaten by the population, q_i is the proportion of taxon i relative to all taxa available to the tortoises, and PSI can range from 1 (ultimate dietary generalists) to the smallest value of q_i (dietary specialists). To account for variation in species- and genus-level identifications in vegetation plots and dietary analyses, we summed proportions at genus level if a genus contained identifications at species and genus level. We repeated PSI calculations for the independent dataset for summer 2020.

Linear mixed models (LMM and P-GLMM) were performed using the GAMLj module, and chi-square analyses and Fisher's exact tests using the jmv module, in jamovi 2.4.1.0 (The jamovi project, https://www.jamovi.org/). We performed chi-square tests with df = 1 in Microsoft Excel, applying Yates' correction. Significant LMM and P-GLMM tests were followed by Bonferroni-corrected post-hoc tests. Friedman RM ANOVA, followed by Student-Newman-Keuls post-hoc tests when significant, was completed in SigmaPlot 12.0 (Systat Software, San Jose, CA, USA). We report means with standard errors (SE), and considered results statistically significant when $P < 0.05$.

3. Results

3.1. Weather and tortoises

Average monthly minimum and maximum temperatures differed among time periods (LMM, $F_{3,\geq 23.0} \geq 26.7$, $P < 0.001$). Post-hoc tests showed that minimum temperatures recorded by our datalogger were similar among February 2018–January 2019, February 2019–January 2020 and February–March 2020 ($t_{\leq 23.3} \leq 2.66$, $P \geq 0.084$), but exceeded simulated minimum temperatures with 3.0–4.7 °C for all time periods ($t_{\geq 23.0} \geq 4.34$, $P \leq 0.001$). Recorded maximum temperatures were similar between February 2018–January 2019 and February–March 2020, and between February 2019–January 2020 and February–March 2020 ($t_{\leq 23.1} \leq 1.21$, $P = 1.00$), but were 1.7 °C higher in February 2019–January 2020 than in February 2018–January 2019 ($t_{23.0} = 4.02$, $P = 0.003$). Also, recorded maximum temperatures were 2.0–3.7 °C higher than simulated maximum temperatures for all time periods ($t_{\geq 23.0} \geq 3.54$, $P \leq 0.011$).

Rainfall differed greatly from simulated rainfall. In February–September 2018, rainfall exceeded simulated rainfall (148 mm and 102 mm, respectively), but a drought (9 mm, compared to 108 mm in simulations) occurred from the start of the spring 2018 sampling period until the end of the summer 2019 sampling period. Thereafter, rainfall continued to be low (58 mm, compared to 98 mm in simulations) until December 2019, after which rainfall exceeded simulated rainfall in January–March 2020 (122 mm and 64 mm, respectively).

Our search for hiding Karoo dwarf tortoises yielded 14 unique adult males (SCL 84.7 ± 0.81 mm, BM 95.9 ± 2.54 g) and 19 unique adult females (SCL 94.6 ± 0.94 mm, BM 158.3 ± 3.60 g). Each individual was encountered in one or more sampling periods, and some individuals were encountered multiple times in single sampling periods (Table 1).

3.2. Vegetation

We field-recorded 112 plant species in 44 families, 102 of which were identified to species level. DNA metabarcoding of faecal samples revealed nine additional species, five of which might represent field-recorded taxa that could not be identified at species level. In addition, DNA metabarcoding revealed five additional genera that each added at least one species, for a grand total of at least 124 species in 44 families at

Table 1
Sample sizes, including repeated measurements between brackets, for dietary recordings of 14 male (M) and 19 female (F) Karoo dwarf tortoises (*Chersobius boulengeri*) in summer 2018 to 2020, and in spring 2018 and 2019.

Sample	Sex	DNA meta-barcoding	Morphologi-cal evaluation	Germination trial	Focal feeding observation
Summer 2018	M	0	1 (1)	0	0
	F	0	10 (16)	9 (10)	0
Spring 2018	M	0	1 (1)	0	0
	F	0	9 (11)	10 (11)	0
Summer 2019	M	0	0	0	0
	F	0	0	5 (5)	0
Spring 2019	M	3 (3)	0	0	0
	F	9 (9)	0	0	0
Summer 2020	M	11 (11)	0	0	1 (1)
	F	12 (16)	0	0	7 (8)
2018 combined	M	–	2 (2)	–	–
	F	–	15 (15)	10 (10)	–
2019 + 2020	M	12 (12)	–	–	1 (1)
	F	13 (13)	–	–	8 (8)
2018 + 2019	M	–	0	0	–
	F	–	0	13 (13)	–

the site (Supplemental Table 1). Excluding potential overlaps between species recorded in the field and species revealed by DNA metabarcoding, most species were dwarf shrubs (38 species), succulents (23 species), geophytes (19 species) or forbs (18 species), whereas there were eight species of grass, seven species of shrub, two species of sedge, and only one species of above-ground bulb, fern, parasite and tree (Supplemental Table 1). Most of these species (108) were perennials, and 12 were annuals (Supplemental Table 1). Although our permanent vegetation plots made up less than 0.01% of the study site, they contained 28% of the species present at the site (Supplemental Table 1). Including the temporary plots (0.02% of study site), 44% of the species at the site were represented (Supplemental Table 1). Species in vegetation plots were usually found in few plots (1.0 ± 0.63 to 2.6 ± 0.57 permanent plots, and 5.6 ± 0.92 temporary plots). In contrast, frequent species were *Aristida diffusa* (8–10 permanent and 36 temporary plots), *Salsola calluna* (5–7 permanent and 16 temporary plots), and *Aptosimum spinescens* (4–5 permanent and 12 temporary plots). In the temporary plots, *Limeum aethiopicum* (17 plots), *Enneapogon scaber* (15 plots), an undescribed *Hermannia* sp. (13 plots), and *Fingerhuthia africana* (12 plots) were also relatively frequent.

The number of species, and the number of flowering species, in permanent vegetation plots differed among sampling periods (Friedman RM ANOVA, $X^2_3 \geq 12.07$, $P \leq 0.007$). Post-hoc tests showed that plots had more species, and more flowering species, in summer 2020 than in other periods. The number of species with fruits, and vegetation cover, also differed among periods (Friedman RM ANOVA, $X^2_3 \geq 9.92$, $P \leq 0.019$), but differences were not distinguishable in post-hoc tests. Overall, permanent vegetation plots usually had no plants with fruits, and 0–25% cover (Supplemental Fig. 1). In summer 2020, almost all temporary plots (36) contained flowering plants, and 25 temporary plots contained plants with fruits. The most frequent flowering species (both in 14 plots) were *Hermannia* sp. and *L. aethiopicum*, and the most frequent fruit-bearing species (18 plots) was *A. diffusa*. Fourteen flowering species were not recorded in any other sampling period than summer 2020.

Most (90%) of the plant species recorded at the site had ITS2 sequences in the DDBJ/ENA/GenBank and BOLD reference databases, either at species (63 species) or genus (49 species) level. Nineteen species were successfully DNA barcoded in the current study, 15 of which lacked references at species level in databases, leading to reference ITS2 sequences for 78 of the recorded species at species level, and 40 of the recorded species at genus level, for a total of 119 (96%) species with reference sequences. The following species lacked references: *Empodium flexile*, *Microloma armatum*, *Roepera lichtensteiniana*, *Schizobasis intricata*, and *Walafrida geniculata*. Our DNA barcoding detected two potential errors in the GenBank database; database accession number MF349147.1 (*Neohenricia sibbettii*) gave a 100% match (321 base pairs) with *Stomatium mustellinum*, and database accession number HQ615128.1 (*Albuca rupestris*) gave a 100% match (340 base pairs) with an unidentified *Albuca* sp. The former match was unlikely because the plant at our study site had leaves that were toothed and keeled towards the leaf ends, and not flat leaves covered with warts (Court, 2010), so we maintained *S. mustellinum*. The latter match was unlikely because the species in the GenBank database did not occur in the study area or in similar habitat (South African National Biodiversity Institute, 2023), so we maintained identification at genus level.

3.3. DNA in faecal samples

DNA analysis retrieved plant DNA from all faecal samples. Moreover, alpha rarefaction plots showed that ASVs before and after filtering tended towards saturation for all samples, indicating that sequencing depth sufficed to retrieve most of the plant diversity in the samples. Nevertheless, two species in the mock community (*Ledebouria apertiflora* and *Ornithoglossum parviflorum*) appeared to have relatively low primer binding affinities, as they were not retrieved (i.e., PCR dropout). Furthermore, several identifications assigned to ASVs indicated contaminations (e.g., celery, common walnut, tomato) and were removed from the dataset. After ASV filtering and removal of contaminants, samples contained 33 species. In addition, 28 and 5 taxonomic assignments were at genus and family level, respectively. Sixteen of these could be reassigned to species (some already recorded) because only one species within the genus or family occurred at the study site, for a total of 43 species (Supplemental Table 1). Two identifications at species level were problematic; database accession numbers HQ615108.1 (*Albuca bracteata*) and EU347814.1 (*Lotononis glabra*) gave 100% matches (7–1260 base pairs), but these species did not occur in the study area or in similar habitat (South African National Biodiversity Institute, 2023). Consequently, we maintained identifications for these taxa at genus level.

Our independent dataset (i.e., each tortoise individual represented only once, $n = 25$) showed that each faecal sample contained on average 14.0 ± 0.85 taxa. Taxa were retrieved at different frequencies (chi-square test, $X^2_{56} = 405$, $P < 0.001$). Most frequent taxa (>20 faecal samples) were *Hermannia* sp., *Hermannia coccocarpa*, *Hermannia spinosa*, and *Hermannia desertorum*. *Hermannia grandiflora* and *Hermannia* spp. had relatively high frequencies (15 and 13 faecal samples, respectively) too. In spring 2019, summer 2020 and for both periods combined, male and female faecal samples contained taxa at similar frequencies (Fisher's exact test, $P \geq 0.96$, $n = 12$–25), but taxa frequencies for both sexes combined differed between spring 2019 and summer 2020 (Fisher's exact test, $P < 0.001$, $n = 25$). Most *Hermannia* spp., *Euphorbia inaequilatera*, *A. spinescens* and *Melolobium candicans* were well-represented in both seasons (Fig. 1). In summer, *Dipcadi viride*, *Tribulus terrestris* and Poaceae had relatively high frequencies, whereas spring had relatively high frequencies of *Crassula* spp., *Crassula muscosa* and *Oxalis* spp.

For all samples combined, taxa frequencies related to growth form (P-GLMM, $X^2_6 = 216.06$, $P < 0.001$) and season ($X^2_1 = 6.85$, $P = 0.009$), with significant interaction between these factors ($X^2_6 = 24.86$, $P < 0.001$). Post-hoc tests showed that dwarf shrubs and forbs were more frequent in faeces than any other growth form ($z \geq 3.57$, $P \leq 0.008$). Succulents were more frequent than sedges and shrubs ($z \geq 4.08$, $P \leq 0.001$), whereas grasses, shrubs and succulents had similar frequencies, as did sedges and shrubs ($z \leq 2.94$, $P \geq 0.068$). Geophytes were the only growth form with different frequencies between seasons ($z = 3.48$, $P = 0.046$); frequencies were higher in spring than in summer.

Plant taxa in faecal samples that had been collected in summer 2020 (independent sample) occurred at different frequencies than they occurred in vegetation plots (permanent and temporary plots combined) in the same period (Fisher's exact test, $P < 0.001$; Fig. 2). The most frequent plant species in vegetation plots, *A. diffusa* and *S. calluna*, were not present in faecal samples, whereas all *Hermannia* spp., *E. inaequilatera* and *M. candicans* were frequent in faeces yet much rarer in vegetation plots. *Dipcadi* spp. and *Crassula* spp. had not been recorded in any vegetation plot, yet were relatively frequent in faecal samples. The PSI for this dataset was 0.31. For all vegetation plots, faecal samples (DNA metabarcoding, morphological evaluation, and germination trial), and focal observations combined, Karoo dwarf tortoises had a PSI of 0.38.

3.4. Morphological evaluation and germination trial

Morphological evaluation of plant remains in faeces failed for some faecal matter, due to lack of distinct morphological characteristics that could reliably be related to dried plants. Nevertheless, our independent sample ($n = 17$) contained 24 taxa. After removal of four taxa (*Aptosimum indivisum*, *Asparagus* sp., *Kewa salsoloides*, and *Pleiospilos compactus*) with single observations to generate expected frequencies >5, remaining taxa were retrieved at different frequencies ($X^2_{16} = 37.6$, $P = 0.002$). The most common taxa, each found in 10 faecal samples, were *Haworthiopsis venosa tessellata*, *Hermannia* sp., *H. desertorum*, and *Albuca* sp. Frequencies of taxa in faecal samples differed between summer and

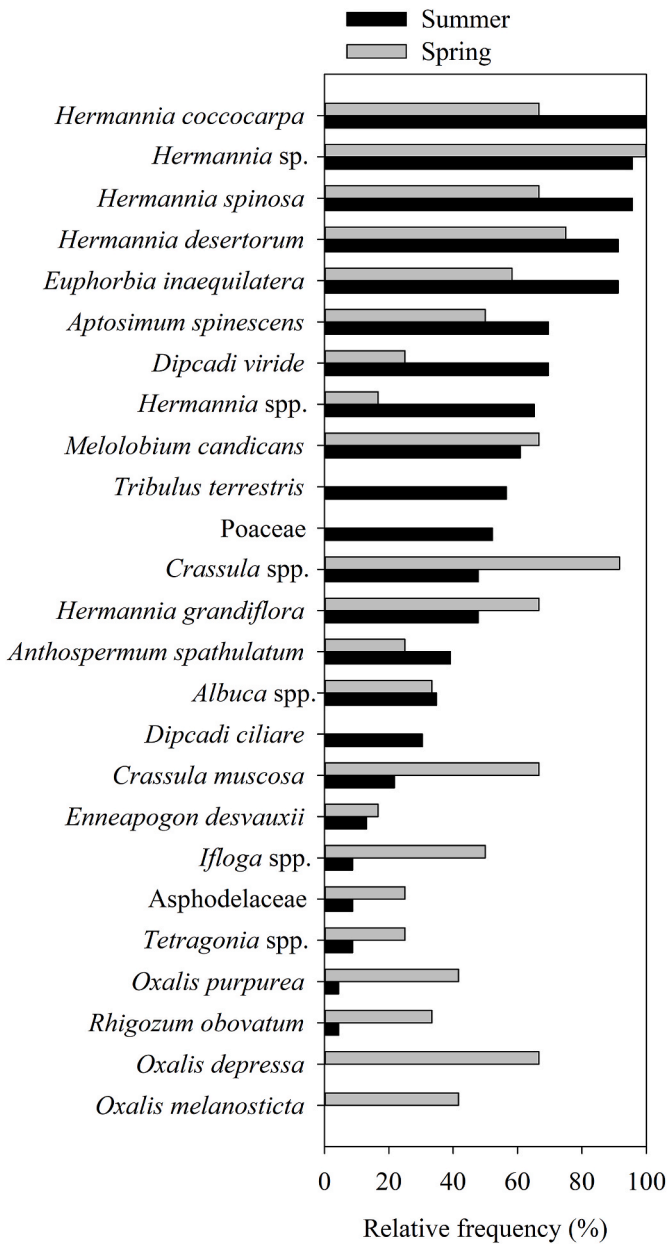


Fig. 1. Relative frequencies of plant taxa recovered as DNA from 17 and 8 faecal samples of Karoo dwarf tortoises (*Chersobius boulengeri*; males and females combined) in spring 2019 and summer 2020, respectively. Absolute frequencies range from 0 to 23, and rare taxa encountered in less than five faecal samples in total are not depicted.

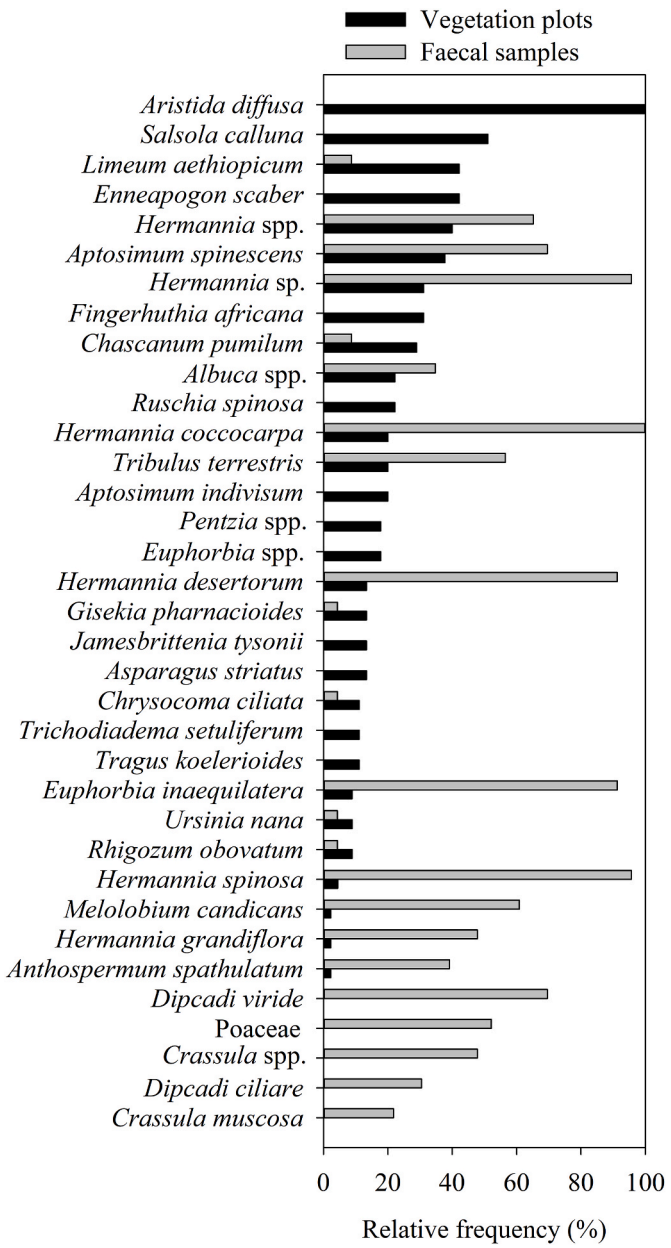


Fig. 2. Relative frequencies of plant taxa recovered as DNA from 48 vegetation plots and 24 faecal samples of Karoo dwarf tortoises (*Chersobius boulengeri*; males and females combined) in summer 2020. Absolute frequencies range from 0 to 45, and rare taxa encountered in less than five samples in total are not depicted.

spring (Fisher's exact test, $P < 0.001$). *H. venosa tessellata*, *H. desertorum*, *H. coccocarpa* and *L. aethiopicum* were relatively frequent in summer as well as in spring. *Pegolettia retrofracta* and *Albuca* sp. were more frequent in summer than in spring, whereas *Hermannia* sp. was common in spring.

Frequencies of taxa revealed by DNA metabarcoding (spring 2019 and summer 2020) and morphological evaluation (summer and spring 2018) differed for spring, summer, and both seasons combined (Fisher's exact tests, $P < 0.001$). Frequencies appeared quite similar for several *Hermannia* spp., *E. inaequilatera*, *A. spinescens* and *M. candicans*, but morphological evaluation lacked 42 taxa found by DNA metabarcoding, and *Albuca* spp. were more frequently found by morphological evaluation (Fig. 3).

Morphological evaluation of faecal samples revealed the presence of flowers and fruits. Samples contained flowers of 13 species, and fruits of six species (Table 2). Flowers and fruits were present in summer and spring, and our independent dataset showed that frequencies of species with flowers and fruits did not differ between seasons (Fisher's exact tests, $P \geq 0.36$). Nine of the 26 samples used for the germination trial produced a total of 27 seedlings in six species (Table 2). Most notably, five samples yielded a total of 14 *A. spinescens*. Frequencies of germinating species did not differ between summer and spring (Fisher exact test, $P = 0.60$).

3.5. Focal feeding observations

Focal feeding observation directly confirmed that Karoo dwarf tortoises ate 12 species that had been found in faecal samples (Supplemental Table 1). Additional species were not recorded. All observed tortoises, except one, fed on four *Hermannia* species. *Melolobium candicans*, *E. inaequilatera* and *Albuca virens* were eaten by respectively, 3, 2

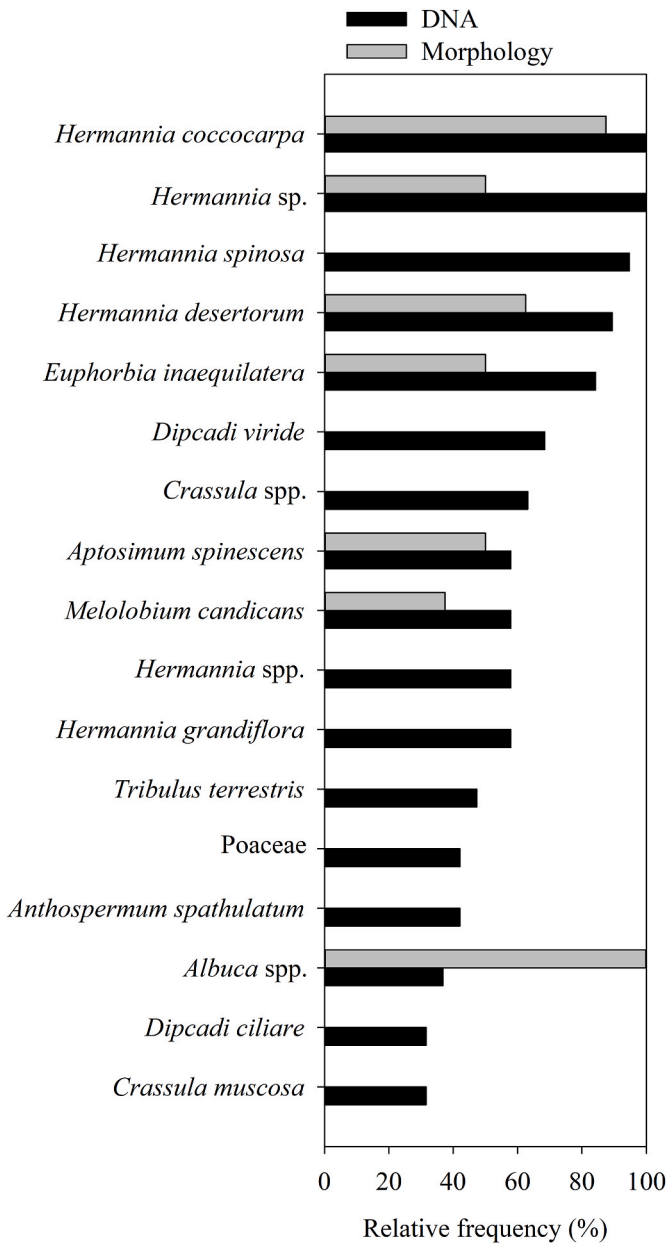


Fig. 3. Relative frequencies of plant taxa recovered as DNA (n = 20) or by morphological examination (n = 11) of faecal samples of Karoo dwarf tortoises (*Chersobius boulengeri*; males and females combined). Faecal samples for DNA and morphological evaluation were obtained in spring 2019 and summer 2020, and in summer and spring 2018, respectively. Absolute frequencies range from 0 to 19, and rare taxa encountered in less than five samples in total are not depicted.

and 2 tortoises, whereas the remaining plant species were single recordings.

4. Discussion

Although several dietary studies on aquatic chelonians have used DNA metabarcoding (Ducotterd et al., 2021; Sarkis et al., 2022), the technique has not been applied much in studies of tortoise diets (Moorhouse-Gann et al., 2022). This study demonstrates the technique's power, because 56 out of 68 dietary taxa (excluding family-level identifications) were revealed by DNA metabarcoding, and 27 species were revealed exclusively by DNA metabarcoding. Moreover, DNA metabarcoding identified nine species that had been overlooked or that could not be identified in the field. In contrast, the morphological evaluation of faecal samples that we performed revealed dietary components that were too digested or difficult to identify, problems that might have been mitigated to some degree by epidermal cell evaluation (El Mouden et al., 2006; Hichami and Znari, 2023; Mason et al., 1999). Direct focal observation of Karoo dwarf tortoises resulted in a small sample size, due to the inactive nature of the species (i.e., individuals spend on average < 6 min/day feeding, less than any other tortoise species; Loehr et al., 2021). Regardless of the power of DNA metabarcoding, the technique had drawbacks too. Public databases with reference sequences did not contain all plant taxa occurring at the study site, some species may have been overlooked due to low primer binding affinities, and DNA metabarcoding was unable to distinguish among plant parts ingested (e.g., leaves, flowers, fruits). Supplementation of DNA metabarcoding with other methods was necessary to test our hypotheses.

Table 2
Frequencies of faecal samples of Karoo dwarf tortoises (*Chersobius boulengeri*; males and females combined) that contained flowers or fruits in summer and spring 2018, or that produced seedlings in a germination trial (numbers of seedlings between brackets) using faeces from summer and spring 2018, and summer 2019.

Species	Flowers (n = 29)	Fruits (n = 29)	Germination (n = 28)
Annual forbs			
Euphorbia inaequilatera	1	2	0
Gisekia pharnacioides	1	0	0
Hermannia coccocarpa	5	1	0
Hermannia sp.	5	4	1 (2)
Tribulus terrestris	0	2	0
Perennial dwarf shrubs			
Aptosimum spinescens	0	2	5 (14)
Asparagus striatus	0	0	1 (3)
Drosanthemum lique	1	0	0
Hermannia desertorum	6	0	0
Hermannia spinosa	1	0	0
Jamesbrittenia tysonii	1	0	0
Limeum aethiopicum	5	0	0
Melolobium candicans	1	0	0
Pegolettia retrofracta	4	0	0
Trichodiadema setuliferum	2	0	0
Perennial geophytes			
Albuca virens	0	0	1 (4)
Albuca sp.	5	0	0
Perennial succulents			
Haworthiopsis venosa tessellata	0	1	0
Stomatium mustellinum	3	0	0
Undetermined			
Hermannia spp.	0	0	2 (3)

Karoo dwarf tortoises had a similar diet structure to other tortoises. Tortoises have broad diets, often comprising dozens of plant taxa, with 3–8 taxa usually making up the bulk (Attum et al., 2021; El Mouden et al., 2006; Lagarde et al., 2003; Loehr, 2006; Mason et al., 1999; Rall and Fairall, 1993). Bulk taxa may reflect abundant availability (Macdonald and Mushinsky, 1988), but tortoise species are able to actively select specific plants (Bjorndal, 1989; Del Vecchio et al., 2011; Jennings and Berry, 2015; Mason et al., 1999). Consequently, tortoise dietary PSIs may vary around 0.5 (Lagarde et al., 2003; Macdonald and Mushinsky, 1988; Mason et al., 1999), midway between dietary generalists and specialists, with spur-thighed tortoises being relatively specialistic at a PSI of 0.17–0.25 (El Mouden et al., 2006). The diet of Karoo dwarf tortoises contained 68 plant taxa, but approximately 10 taxa were frequently eaten. Its PSI of 0.31–0.38 suggests that Karoo dwarf tortoises have slightly specialised diets as we had hypothesized. Thus, the large time investment of male and female Karoo dwarf tortoises walking and scanning (Loehr et al., 2021) might indeed relate to selection of specific food items.

Hermannia spp. (dwarf shrubs and forbs) were the clearest example of plants selected by Karoo dwarf tortoises, and consistently appeared in

all analyses (i.e., DNA metabarcoding, morphological evaluation, germination trial, and focal observation), followed by *E. inaequilatera*, *Dipcadi* spp., *Crassula* spp., and *M. candicans*. In contrast, Poaceae and *S. calluna* were clearly avoided. There is little information why tortoises prefer specific plants. Hichami and Znari (2023) showed that food selection by arid-region spur-thighed tortoises favours plants high in water and protein, but low in potassium contents, corroborating avoidance of potassium by captive desert tortoises (*Gopherus agassizii*; Oftedal and Allen, 1996). Nitrogen content also appeared to be a selection criterion for desert tortoises (Jennings and Berry, 2015), and for South African leopard tortoises and Kalahari tent tortoises (*Psammobates oculifer*; Rall and Fairall, 1993), but not for radiated tortoises (*Astrochelys radiata*; Rasoma et al., 2013). Leopard tortoises and Kalahari tent tortoises also selected succulents, possibly to avoid dependency of free water (Rall and Fairall, 1993), although desert tortoises lost energy when feeding on succulent annual plants and required supplementation with drier forage and free water (Peterson, 1996). Published information about water and nutrient contents of plants selected by Karoo dwarf tortoises is not available. *Euphorbia inaequilatera* and *Crassula* spp. appeared to contain a relatively large amount of water, and *M. candicans* is in the Fabaceae family and might contain considerable nitrogen. *Hermannia* spp. are highly palatable species, eaten by micromammals, antelopes, goats and sheep (Esler et al., 2010; Gwynne-Evans, 2015; Jansen van Vuuren et al., 2022). Moreover, *Hermannia* spp. dominated in the diet of leopard tortoises and Kalahari tent tortoises when available (Rall and Fairall, 1993), and were also present in the faeces of speckled dwarf tortoises (*Chersobius signatus*; Loehr, 2006). Our results confirm the wide use of *Hermannia* spp. by herbivores including tortoises.

The diet of Karoo dwarf tortoises differed between summer and spring, as we expected based on other tortoise species that have seasonally different diets due to annual rainfall and plant phenological patterns (Attum et al., 2021; Del Vecchio et al., 2011; Jennings and Berry, 2015; Mason et al., 1999; Milton, 1992). However, the temporally unbalanced design of our methods and small sample size in spring 2019 disabled rigorous analysis in the context of plant availability. Moreover, the spring 2019 sampling period was extraordinarily dry and had little plant growth, confounding possible effects of season and drought. *Oxalis* spp. eaten in spring, and *Dipcadi ciliare* eaten in summer, might reflect the respective growth periods of these geophytes (Manning, 2009). We are reluctant to interpret the frequent intake of *Crassula* spp. in spring 2019 as a response to drought conditions (Milton, 1992), because we did not detect a dietary shift towards succulents, and *Crassula* spp. may simply have been one of the few plant taxa available during the drought. The marked presence of *Albuca* spp. in morphological evaluation (especially in summer 2018), compared to much smaller frequencies detected in DNA metabarcoding (spring 2019 and summer 2020), might reflect availability because of rainfall variation, or relatively low primer binding affinity for *Albuca* spp.

In summer and spring, Karoo dwarf tortoises ate fruits of several plant taxa, and consumption of flowers of additional taxa suggests that the number of taxa consumed as fruits was underestimated. For example, we did not identify *Albuca* spp. fruits in faecal samples, but flowers were eaten, and seedlings germinated from faecal samples. Relatively long gut retention times in tortoises (mean 2.5–23.8 days) and minimal seed damage during digestion provide opportunities for plant species to disperse viable seeds through tortoises (Falcón et al., 2020). The short straight-line daily movement distances (mean 28 m) and small home ranges (mean 1.1 ha) of Karoo dwarf tortoises (Loehr, 2023) limit their dispersal capability, but preference of Karoo dwarf tortoises for well-structured terrain with boulders and rocks (Loehr, 2023) might provide microsites with ample shade and run-off water that could benefit seedling recruitment in an arid environment. We established that Karoo dwarf tortoises dispersed viable seeds of at least four plant species, including preferred taxa (e.g., *Hermannia* spp.), and *Asparagus striatus* that had not been detected in DNA metabarcoding, morphological analysis, or focal observation. Faecal samples of 51 leopard tortoises from the southern Karoo contained seeds of at least 43 plant species (Milton, 1992). Leopard tortoises are much bigger than Karoo dwarf tortoises and have up to 400-fold larger home ranges (McMaster and Downs, 2009), which might translate to more and a broader variety of food items being eaten. Surprisingly, the germination trial conducted by Milton (1992) did not produce *Hermannia* spp. seedlings, although seeds had been identified in faecal samples. Leopard and Karoo dwarf tortoises may interact with *Hermannia* spp. differently. *Hermannia* spp. dispersal is primarily through endozoochory, given their tough, tannin-rich seeds that may remain viable in excess of 20 years, and known dispersal by eland, goats and sheep (Gwynne-Evans, 2015). Moreover, Gwynne-Evans (2015) found that *Hermannia* spp. fitted eight of the 10 criteria related to Janzen's (1984) "foliage is the fruit" hypothesis, dictating that plants promote incidental ingestion of fruits by providing palatable leaves. This study is the first to report a mutualistic relationship between *Hermannia* spp. and tortoises.

5. Conclusion

This study provides new evidence highlighting the role that tortoises may play in the dispersal of seeds in arid regions. Karoo dwarf tortoises were able to frequently locate and eat relatively rare plant taxa, and passed through the digestive tract viable seeds of several taxa. Interestingly, seeds ingested by Karoo dwarf tortoises had no features (e.g., fleshy fruits) to make them particularly attractive as food, and ingestion probably occurred when tortoises targeted leaves or flowers. Specifically, there was a strong relationship between Karoo dwarf tortoises and *Hermannia* spp., which warrants further investigation of interspecific dependencies. Previous tortoise extinctions have resulted in cascading effects in their ecosystems. Given the rapid and ongoing decline of Karoo dwarf tortoise populations, effects on plant communities may already occur. Reversely, the potential of Karoo dwarf tortoises as seed dispersers should be considered when planning rehabilitation or restoration of overgrazed and damaged land.

CRediT authorship contribution statement

Victor J.T. Loehr: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Visualization, Supervision, Project administration, Funding acquisition. **Toby Keswick:** Validation, Investigation, Writing – review & editing. **Nicole Barten:** Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We would like to thank Sheryl Gibbons, Courtney Hundermark, Andreas Iosifakis, Mark Klerks, On Lee Lau, Clara Lemyre, Esther Matthew, Olda Mudra, Sharon Pavoni, Susannah Peel, Bonnie Schumann, Paul van Sloun and Carlos Voogdt, for their help in the field, and Ralf Mullers en Marcella Dobbelaar for their student supervision. Special thanks are extended to Sue Dean, David Gwynne-Evans, and Sérgio Silva for (verification of) plant identifications. We are also grateful to Henry Kuipers, who provided valuable input for statistical analyses. The Northern Cape Department of Environment and Nature Conservation and the Senate Research Committee of University of the Western Cape are thanked for providing research permits (FAUNA 0950/2017, FLORA

0066/2017 and FLORA 0067/2017) and ethical clearance (registration no: 04/4/11), respectively. This work was supported by Zoo Knoxville; Turtle Conservation Fund/Conservation International; Holohil Grant Program; Turtle Survival Alliance Europe; Dutch-Belgian Turtle and Tortoise Society; British Chelonia Group; Crocodile Zoo Prague; and several private individuals.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaridenv.2023.105094.

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