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Exploring the dietary niche of *Atlantolacerta andreanskyi* (Lacertidae) using DNA metabarcoding

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Abstract

Determining the dietary niche is an essential part of any conservation strategy, and for modeling the community responses to climate change. DNA metabarcoding methods are revolutionizing such approaches, allowing higher taxonomic resolution than typically possible using microscopy. However, few studies have compared directly the approaches to evaluate the differences in methodology. Here we assess the dietary niches of two genetically diverse populations of *Atlantolacerta andreanskyi*, a lizard endemic to the Atlas Mountains, Morocco, using DNA metabarcoding of faecal samples, and for one of these we compared the results to two previously published assessments of diets obtained using microscopy of pellets and stomach contents respectively. While results at the Order level were similar, the higher taxonomic resolution obtained in this study provided new insights into the dietary niche of this species. Comparisons between the two populations further highlighted how ecologically distinct these are.

Key Words

16S rRNA, dDNA, lizards, metabarcoding, Morocco

Introduction

Species interactions form the backbone of any ecosystem, and as these relationships underpin food webs, it is essential to elucidate them in order to predict how ecosystems respond to disturbances. Dietary studies have traditionally relied on visual identification of prey, either from stomach contents or fecal matter, but such approaches are time consuming, require substantial taxonomic expertise – especially when dietary ranges are broad – and may fail to detect more easily digested prey (Taberlet et al. 2012). High-Throughput-Sequencing (HTS) of diet contents provides a powerful alternative tool to provide rapid assessments of large numbers of samples, as well as greater taxonomic resolution, but has its own limitations (reviewed in Sousa et al. 2019) and potential errors such as risk of contamination (Furlan et al. 2020). Nevertheless, dietary studies based on HTS are becoming routine in studies focusing on single species, as well as in broad-scale studies, aiming to illuminate levels of ecosystem resilience to climatic change (e.g. Berry et al. 2019).

In the specific case of ectothermic species, more studies providing baseline dietary data are urgently needed, for example in cases of species restricted to remote high mountain areas, where predator-prey dynamics are more susceptible to climate change (Manes et al. 2021). In lizards, few studies have employed HTS approaches for dietary assessments, despite early works demonstrating the potential

advantages of the methodology (e.g. Brown et al. 2012). Recent appraisals of island specialists have highlighted how greater prey diversity is apparent when using HTS, due to the higher taxonomic resolution obtained, while also identifying the need for further dietary studies to evaluate across-season changes (Gil et al. 2020; Tercel et al. 2022). A recent assessment of generalist lizards from North Africa highlighted differences between results obtained from HTS and microscopy (Pereira et al. 2019), as did an assessment of a ground-dwelling gecko (Kurita and Toda 2022) but there are still too few comparative studies to generalize regarding differences between the approaches.

There are multiple reasons why dietary assessments would be particularly enlightening for *Atlantolacerta andreanskyi*, a lizard species endemic to the Atlas Mountains of Morocco (Martínez del Mármol et al. 2019). Firstly, global biodiversity assessments show there is a bias towards research on other vertebrates, such as birds, with lizards receiving disproportionately less attention. This is particularly problematic, because lizard biodiversity hotspots demonstrate limited overlap with other taxa (Roll et al. 2017), while catastrophic vertebrate population declines are driven by clusters primarily among amphibians and reptiles (Leung et al. 2020). At a more local level, climate change is predicted to particularly impact Moroccan endemic reptiles, with montane forms expected to be especially affected (Martínez-Freiría et al. 2013). Furthermore, *A. andreanskyi* appears to constitute a cryptic species complex, encompassing deeply divergent genetic lineages with major clades separated since the Miocene and multiple potential species occupying distinct montane massifs since the Plio-Pleistocene (Barata et al. 2012). Additionally, while different populations of *A. andreanskyi* are morphologically similar, some are significantly larger than others, and the degree of sexual dimorphism varies appreciably, with females typically being larger than males (Barata et al. 2015). Dietary breadth is often correlated with body size, so variation in prey diversity between populations could be associated with this aspect, even if larger lizards may optimize prey-capture strategies by targeting larger, more profitable prey items, and thus counter-intuitively decrease dietary niche breadth (Costa et al. 2008). Although dietary patterns have not been elucidated in most *A. andreanskyi* lineages, two assessments using microscopy have been performed on the population at Oukaimeden in the High Atlas Mountains, where females were found to be bigger than males (Busack 1987; Carretero et al. 2006). However, results were not consistent; Busack (1987) analyzing stomach contents reported that males consumed mainly Coleoptera (30% of dietary items identified) and females Hymenoptera (42%), Carretero et al. (2006) analyzing pellets identified primarily Hemiptera (40%) and then Coleoptera (19%) as common prey, with no differences between males or females. Thus, while in both studies sampling was carried out in the spring (May and June in Busack 1987; and April in Carretero et al. 2006), results were quite different.

In the present study we performed an assessment of the diet of *A. andreanskyi* from two locations, Oukaimeden and Ait Bouguemez, employing an HTS approach. These populations correspond to different genetic lineages that also differ in average body size (Barata et al. 2012, 2015). Our main objectives are to: 1) compare the results with those of previous diet studies based on microscopy analysis; 2) determine whether the diets of these genetic lineages differ; 3) assess diet variability across seasons; 4) test whether increased taxonomic resolution will enable us to identify dietary differences between sexes. Finally, these data will be essential for proposing conservation strategies for these endemic lizards, but will also give further baseline information regarding the prey found in these unique habitats, which are under ever-increasing anthropogenic pressures.

Methods

Eighty-five adult specimens of *A. andreanskyi* were captured by hand from two localities in the High Atlas Mountains in Morocco, 1) Ait Bouguemez (Central High Atlas; 31°45'45.8"N, 6°16'50.6"W, altitude: 2,723 m), and 2) Oukaimeden (South limit of Central High Atlas; 31°12'09.5"N, 7°51'24.4"W, altitude: 2,600 m), which belong to different lineages of the *A. andreanskyi* complex (Barata et al. 2012, where the first population is called "Jebel Azourki"). Both localities feature a mountain climate, with cold winters, and were characterized by vegetation predominantly composed of xerophilous thorny bushes, especially *Alyssum spinosum*, which provide refuge against predators for these lizards. It has been previously shown that specimens from Ait Bouguemez are significantly larger on average than those from Oukaimeden (Barata et al. 2015). Only adult specimens were considered, with those with snout-vent length (SVL) over 36 mm considered adults following Schleich et al. (1996). Sex was determined by the presence of developed femoral pores and head robustness in males (Barata et al. 2015). Faecal samples were collected by gentle massaging of the individual's abdomen when necessary, and were stored in 96% ethanol. All specimens were then released at the collection sites. The populations at Oukaimeden were sampled in both 2016 and 2017 (25 and 36 individuals respectively), while the population at Ait Bouguemez was sampled only in 2017 (24 individuals). Considering seasonal variation, Ait Bouguemez was sampled in summer and autumn, while Oukaimeden was sampled in autumn 2016, and spring and summer 2017. Full details are given in Suppl. material 1: table S1.

DNA extraction was performed in a dedicated positive controlled pressure room designed to prevent contamination. Prior to extraction, samples were dehydrated at 37 °C overnight. Extraction was performed using the PureLink™ Genomic DNA kit (Thermo Fisher Scientific) following the manufacturer's protocol, but with triplication of lysis buffer and proteinase K quantities. All

samples were vortexed to disrupt the faecal mass and digested overnight. Extracted DNA was stored at -20 °C prior to library preparation.

A two-step PCR (Polymerase Chain Reaction) approach was performed. For the first PCR, a 16S rRNA fragment was amplified using the primers Ins16S_1F and Ins16S_1R (Clarke et al. 2014) at 55 °C annealing temperature for 35 cycles. Platinum Taq Polymerase (Invitrogen) (2U/µL) was mixed in a 10 µL reaction volume with 0.5 µL of DNA. PCR reactions contained BSA (Bovine Serum Albumin) 25 mM and 2.5 mM $MgCl_2$ concentration. PCRs were run in triplicate for each sample with a negative control (blank). Amplified fragments were examined in a 2% agarose gel and replicate PCRs were pooled. PCR purification was performed using the Agencourt AMPure XP (Beckman Coulter) system with a proportion of 0.8 µL of magnetic beads to 1 µL of PCR product. To attach a unique tag to each sample, a second PCR was performed in a 10 µL reaction volume using the same conditions as the first PCR for 10 cycles. A final purification was performed using the ratio of 1.2 µL of beads to 1 µL of PCR. Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) was used to quantify amplicon concentration and all samples were normalized to 5 nM and then pooled, with 2 µL of each sample. Sequencing was outsourced to a commercial company (Genewiz, Germany) and conducted using an Illumina MiSeq sequencer, with 2×150bp paired-end configuration and ≤30% of PhiX spiked-in to increase sequencing diversity.

Samples were de-multiplexed and adaptors removed. Fastq files were analyzed using USEARCH v11.0.667 (Edgar 2010), to merge, quality filter and cluster reads into Zero-radius Operational Taxonomic Units (ZOTUs). After overall sequencing quality control, primers were removed and paired-end reads were merged using the command -fastq_mergepairs. Samples were filtered by quality with -fastq_filter, which was first set to discard reads shorter than 100 bp (Yu et al. 2012) and then for overall quality score filtering. Maximum expected sequencing errors was set to 1 due to the high quality of the sequences (Edgar and Flyvbjerg 2015), and replicated reads and singletons (unique reads present across the entire dataset) were discarded with –fastx_uniques. Unique sequences were de-noised (sequencing errors were corrected and chimeras removed) and then clustered into ZOTUs using –unoise3. Finally, -otutab was used to establish a ZOTU table showing the frequency of all ZOTUs per sample.

Sequences were compared against the GenBank database using the BLAST algorithm (Altschup et al. 1990). Sequences were identified to order, family, genus or species when similarities were ≥85%, 90%, 98%, and 99% respectively, following Pereira et al. (2019). When equal similarity to two or more taxa was found, sequences were identified to the higher taxonomic level that included both taxa. When a match-up to the order level was not found, ZOTUs were considered unidentified. Sequences that were 1) present in extraction or PCR blanks; 2) classified as the host; or 3) not considered a prey item (e.g. bacteria, fungi) were removed. Additionally, samples with fewer than 100 sequences were removed and ZOTUs representing less than 0.1% of the total number of reads of each sample were not considered for that sample (Deagle et al. 2019).

Possible differences in SVL between sexes in each population (i.e. Oukaimeden 2016; Oukaimeden 2017 and Ait Bouguemez 2017) were tested with the Kruskal-Wallis test due to the non-normality of the data. For the populations sampled in several seasons, the non-significant effect of season in SVL was first confirmed to avoid misleading results regarding differences between sexes. Differences in diet between sexes and seasons (for the samples collected in 2017) at each locality were estimated with a Permutation Multivariate Analysis of Variance (PERMANOVA) using the adonis function implemented in the vegan package in R software, Oksanen et al. 2008) and the Jaccard dissimilarity index calculated both at ZOTU and family levels. Samples from Oukaimeden 2016 were not included in direct comparisons between localities because of the possible variation in diet between 2016 and 2017 and because those samples were sequenced in a different Miseq run from the rest of the samples. Given that there were significant differences associated with season (see results), to assess differences in prey at the family level between the two populations sampled 2017, the PERMANOVA was performed with the factor season nested within locality and using the strata option for season (diet composition ~ locality/season, strata = season). All sequences were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (PRJNA 1019328).

Results

Of the 85 samples collected, 84 gave informative sequences – 61 from Oukaimeden (25 from 2016 and 36 from 2017) and 23 from Ait Bouguemez. One sample from Ait Bouguemez was discarded because it had less than 100 sequences after filtering. Nine orders of prey were detected, Coleoptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera, Diptera, Dermaptera, Plecoptera and Thysanoptera. Six orders were identified in both localities, while Dermaptera was only identified in Ait Bouguemez, and Plecoptera and Thysanoptera only in Oukaimeden (only in 2017) (Fig. 1). Within these orders, at least 16 prey families were identified at Ait Bouguemez, 11 in Oukaimeden 2016 and 32 in Oukaimeden 2017, but potentially more were present, since some prey items were only identified to order level (Suppl. material 1: table S2). In total, 22 prey items were identified to the species level (Suppl. material 1: table S2). There were significant differences in diet composition between individuals sampled in the two localities in 2017 when considering prey families (PERMANOVA: $F_{57}= 2.66$; $p = 0.001$). There were also differences between seasons in both localities when comparing ZOTUs, as well as seasonal differences

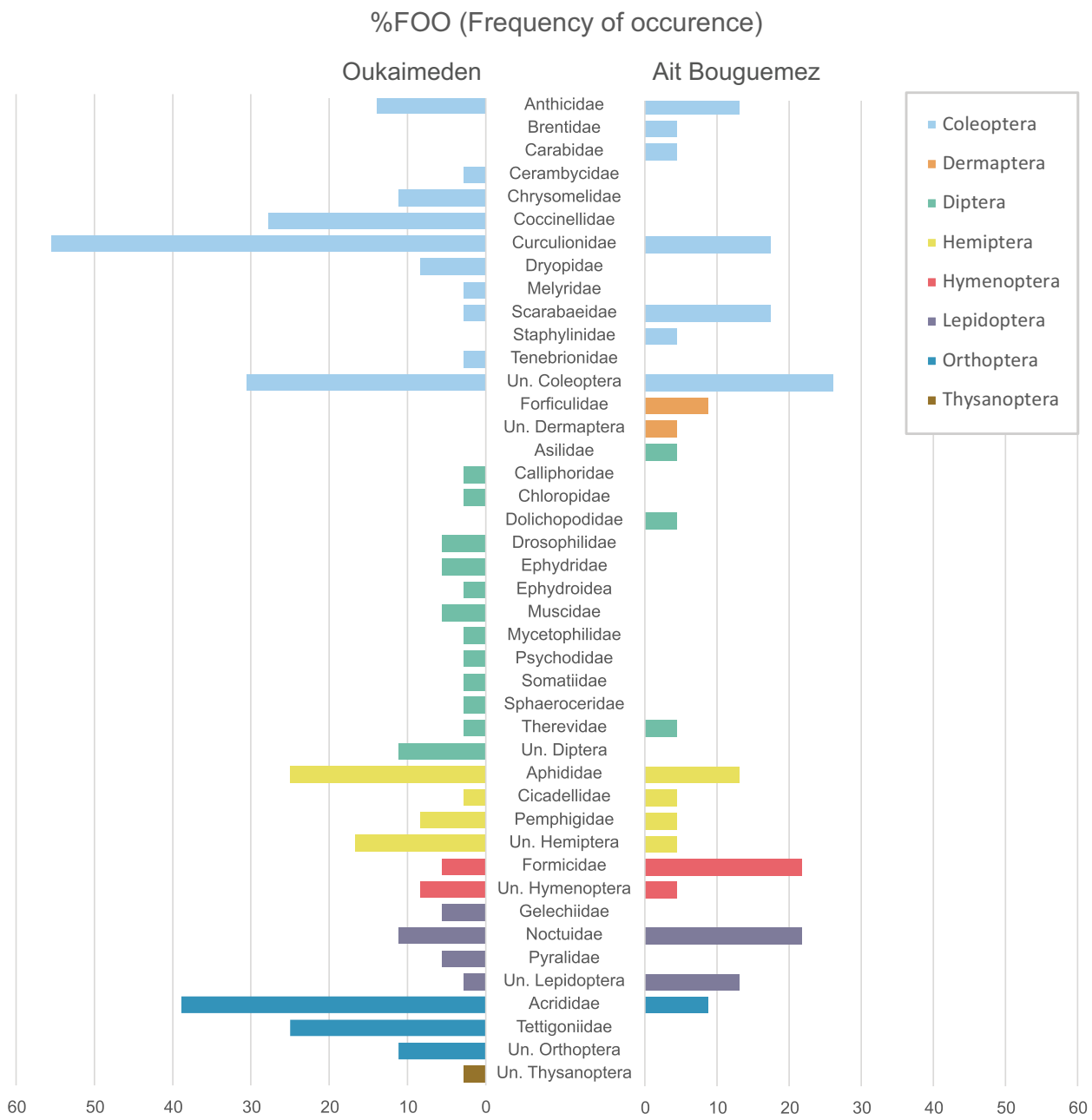

Figure 1. Comparison of the percentage of Frequency of Occurrence (%FOO – the number of samples that contain a given food item, expressed as a percentage, following Deagle et al. 2019) of prey taxonomic families between the samples collected in Oukaimeden and Ait Bouguemez in 2017. Families belonging to the same order are represented in the same color.

at the level of prey families in Ait Bouguemez (Table 1). There were no differences in diet between males and females, either when considering ZOTUs, or when comparing prey taxonomic families (Table 1). For the size comparison between males and females, SVL was obtained from 61 adults at Oukaimeden (26 females, 35 males) and 32 at Ait Bouguemez (14 females and 18 males). In both populations there was no significant difference in SVL between sexes ($\chi^2 < 3.22$; $p > 0.07$), while the population at Ait Bouguemez was substantially larger on average than the one from Oukaimeden (average SVL 48.90 mm versus 43.05 mm respectively).

Discussion

There were clear similarities in the identity of insect prey items at the order level between the earlier studies based on microscopy of pellets (Carretero et al. 2006) and stomach contents (Busack 1987) and the present study using HTS at Oukaimeden. All studies recorded Hemiptera, Lepidoptera, Coleoptera, Hymenoptera and Diptera as the prey of *A. andreanskyi*. Orthoptera were also recorded by Busack (1987) and the present study, but not in Carretero et al (2006). Busack (1987) further reported Odonata and Neuroptera, although notably these were recorded in low numbers (between 1 and 5 records each), none of

Table 1. Differences in diet composition between sexes and seasons at each locality. Differences in SVL between sexes were tested with a linear model (significance obtained with ANOVA). Differences in diet composition were estimated with a PERMANOVA of the Jaccard dissimilarity index with the presence/absence matrix of each OTU and also with the presence/absence matrix of prey families. Significant values are in bold.

		Oukaimeden 2016	Oukaimeden 2017	Ait Bouguemez 2017
		χ^2/F (p-value)	χ^2/F (p-value)	χ^2/F (p-value)
SVL	Sex	3.22 (0.07)	0.07 (0.79)	0.92 (0.34)
Diet (ZOTUs)	Sex	1.08 (0.32)	1.14 (0.11)	0.90 (0.69)
	Season	–	**1.46 (0.003)**	**1.62 (0.005)**
	Sex*Season	–	0.94 (0.68)	1.04 (0.36)
Diet (prey families)	Sex	1.41 (0.14)	0.83 (0.67)	0.54 (0.93)
	Season	–	1.28 (0.17)	**2.90 (0.002)**
	Sex*Season	–	0.91 (0.58)	0.41 (0.99)

which were detected in the present study, while Thysanoptera was only recorded in the present study. Most of these differences are unlikely to represent methodological artifacts, since for example Carretero et al. (2006) identified Orthoptera in pellets of *Podarcis vaucheri* from Oukaimeden but these were absent from the pellets of *A. andreanskyi*. Rather, given the low numbers of prey items from these orders, dissimilarities likely reflect differences in prey availability or sampling effects. The percentage of occurrence of prey items from the different orders varied greatly between studies, with Busack (1987) identifying predominantly Hymenoptera (particularly in females), Carretero et al. (2006) Hemiptera (within the suborder Homoptera), and in this study the order with the greatest number of ZOTUs identified was Coleoptera. Again, these differences may reflect seasonal differences in availability of prey, this being supported by the significant variation in prey item ZOTUs found across seasons in both populations examined in the present study. On the other hand, the presence of thrips (Thysanoptera) and aphids (Hemiptera) could potentially be due to secondary predation, since ladybirds (Coccinellidae) – which were also detected – are known to feed on them. Identification of DNA via secondary predation has been demonstrated experimentally in dietary metabarcoding studies, but it is difficult to set rules that are not arbitrary for eliminating this potential source of detected DNA (Ando et al. 2020). In this case, many thrips and aphids are large enough to have been preyed on directly by lizards, but still small enough to have possibly been overlooked in previous diet assessments based on microscopy. However, based on the current evidence, the identification of such DNA in the pellets due to secondary predation cannot be excluded, and the possible inflationary effect on the proposed diet should not be disregarded.

While two previous dietary assessments of *A. andreanskyi* have been performed at Oukaimeden, the present study is the first to assess the population at Ait Bouguemez. At the order level the results are very similar to those from Oukaimeden, with six orders found in both populations. Only Plecoptera and Thysanoptera were found at Oukaimeden but not at Ait Bouguemez, and Dermaptera at Ait Bouguemez but not at Oukaimeden. However, the composition of diets at the family level was significantly different between the populations, with prey items belonging to 32 families identified in Oukaimeden 2017, and only 21 at Ait Bouguemez, despite the specimens from the latter population being larger on average. It is clear that the dietary niches are different between these two - genetically distinct but morphologically similar - populations of *A. andreanskyi*. Furthermore, these results highlight again how the higher taxonomic resolution achieved using a DNA metabarcoding approach relative to microscopy analysis allows additional inferences to be made. However, the notable differences between the results from 2016 and 2017 from Oukaimeden highlight the effects of season and the need for caution when interpreting differences between populations.

Interestingly, we found no difference in SVL between males and females in either population, or any significant differences in diet. In the assessment of Carretero et al. (2006) at Oukaimeden, females were larger than males although the significance level was marginal (Student T-test, t_{23}=-2.14, p=0.04), while Busack (1987) reported similar average SVLs for males and females, although the maximum size of females was larger. Thus, it seems that these populations have minimal sexual dimorphism, and this is reflected by similarity in the diet of both sexes. Other populations of *A. andreanskyi*, such as those at Jbel Ayache in the Middle Atlas range, have much higher levels of sexual dimorphism (Barata et al. 2015). Hence, it would be useful to assess diet variation in these other populations, both to confirm that dietary niches change between genetic lineages, but also to determine how sexual dimorphism and diet are related.

The use of dietary DNA as part of biodiversity assessments of prey items has been proposed before, especially in the case of remote and inaccessible regions (Boyer et al. 2015; Sousa et al. 2019). The primers used herein enable the identification of various insects to the species level (Pereira et al. 2019, 2021) allowing data collection on the distributions of these prey items. The data provided by the present study can be useful in this regard as well. For example, the cross-backed grasshopper of the genus *Dociostaurus* includes various cryptic species, so the identification of *D. jagoi* in Oukaimeden adds to the understanding of the distribution of these species (González-Serna et al. 2017).

Our findings provide important information for conservation of the focal lizard. *Atlantolacerta andreanskyi* is the only representative of its genus, and considered as Near Threatened in the Red List of IUCN due to its high habitat specificity and the small and fragmented geographic range (Geniez 2006). Furthermore, since the genetic lineages are potentially distinct species, some could be considered endangered due to the small known ranges. The observed niche conservatism in the thermal ecophysiology of the species complex reduces the expected evolutionary response to fast environmental shifts (S'khifa et al. 2022), and these authors highlight how a nuanced approach including water availability and microhabitat will be needed to better model the impact of climate change. Our data, which highlight the dietary differences between populations, also underline the complexity of the relevant food web dynamics, that in a changing environment need to be understood for effective management and conservation (Roslin and Majaneva 2016).

Dietary studies are inevitably point estimates of a more complex continuum, varying with season and size of the organisms. It is therefore not surprising that different studies made at different times will produce somewhat different results. This work highlights how, in *A. andreanskyi*, diet varies extensively along the year, and between years, likely reflecting prey availability, and the same is almost certainly true for other lizards from these localities. Dietary niche also differed between the two populations, despite both being high montane habitats, separated by only a little over 50km in a straight line. This study provides additional evidence for the cryptic differences within this apparent species complex, and with recent assessments of ecophysiological differences (S'Khifa et al. 2022) demonstrate how distinct these populations actually are. Assessment of additional populations, and years, is needed to determine how much these reflect consistent differences. On the other hand, even with greater taxonomic resolution of prey items, differences between sexes were not identified. Finally, the study demonstrates again how the identification of the prey items can be useful not just for determining the diet of these endemic lizards, but also for assessing the distribution of the insect prey species themselves.

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Supplementary material 1

Information for the samples and prey item identification

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