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Natural Diet of European Green Lizards, *Lacerta viridis* (Squamata: Lacertidae): A Comparison of Macroscopic and Molecular Identification Methods

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Abstract: An analysis of the diets of reptiles is essential for understanding the role of reptiles in the ecosystem and the employment of successful conservation management plans. For this purpose, noninvasive and invasive methods to identify consumed prey have been used. Here, we investigated the diet of male and female European Green Lizards (*Lacerta viridis*) by sampling fecal pellets across 2 yr in the spring and late summer at a single site. We used the following two methods for identifying prey remnants from fecal samples: the classical macroscopic approach that requires competent expert knowledge and the molecular approach based on the dietary metabarcoding of nondegraded prey remnant DNA. According to both methods, lizards consumed mainly insects belonging to 13 orders, with Coleoptera as the dominant prey. The number of prey taxa was similar between the sexes, but the prey composition at the genus level was significantly different, with males capturing some coleopterans more than females. The diets also differed significantly between season. In the spring, lizards consumed many more prey types and many more coleopteran specimens than in late summer. The proportion of identified prey taxa was significantly different between the identification methods. From the total of identified prey, macroscopic identification yielded only about 50% of taxa, whereas molecular identification yielded more than 80% of taxa. Our results show that molecular identification can recover a much higher number of prey than the macroscopic method, yet not all prey. Thus, the integration of both methods best described the natural diet and complex trophic interactions of European Green Lizards.

Key words: Fecal analysis; Metabarcoding; Predator; Prey; Reptile conservation

Trophic studies are crucial for understanding the life history, evolution, and ecology of animals (Pianka 1986). In the current circumstances of a rapidly changing environment, the study of the trophic ecology of lizards can help us to understand population dynamics and devise better conservation management plans to protect their populations (Drago et al. 2020). There are several methods for examining lizard diets, each with advantages and limitations. Direct observation of feeding or hunting in the field is a traditional but time-consuming method. The identification of prey being captured at a distance is reliable only when the prey is apparent, and the capture is not cryptic (Nielsen et al. 2017; Leu and Petrovan 2022). Therefore, invasive approaches have been frequently used (Luiselli and Amori 2016). For example, the dissection of gut contents in museum-stored individuals is effective; however, it is limited to past specimens (Shine et al. 1996) and thus cannot be used to address questions on the current trophic status. When doing ecology and behavior research, it is critical to use live animals, while limiting the risk of injury to them. For this purpose, a stomach flushing method can be used (Legler and Sullivan 1979; Herrel et al. 2006). Yet, even if all safety precautions are taken, it is possible to harm or even kill investigated individuals (Pietruszka 1981; Barreto-Lima 2009; Akani et al. 2011). Other methods include the investigation of stomach contents by means of the doubly labelled water technique (Peterson et al. 1998) and by means of stable isotopes (Seminoff et al. 2006). The major disadvantage is the limited capacity of such methods to identify prey at low taxonomic levels.

One of the most effective and least invasive extraction methods, with a reduced risk of harming investigated animals (Akani et al. 2011; Böhm et al. 2013), is fecal pellet analysis (Pérez-Mellado et al. 2011). The identification of prey from pellets is, however, challenging, and taxonomic expertise is essential. When investigating lizards' diets, the consumed prey species, which are mostly arthropods, differ in their level of body sclerotization. Consumed species with a more sclerotized exoskeleton have a higher chance of having body remnants in the fecal pellet and thus a higher chance of being identified macroscopically, which is not the case for soft-bodied species (Piñol et al. 2014; Jeanniard-du-Dot et al. 2017). This can lead to biased results and misinterpretations of trophic interactions. Thanks to recent developments in molecular methods, gut-content analysis based on metabarcoding has become more frequent and reasonably priced (Ando et al. 2020). As molecular methods depend on remnant prey DNA that is present in fecal pellets, it should minimize the bias mentioned above.

Lacerta viridis is one of the largest European lizard species and occurs in several types of habitats (Nettmann and Rykena 1984). Adults can reach lengths of almost 40 cm, including the tail (Moravec 2015). The species appears to be an euryphagous generalist predator (sensu Pekár and Toft 2015). Arthropods, such as Araneae, Coleoptera, Hymenoptera, and Orthoptera, appear to be their primary prey (Korsós 1984; Maier et al. 2020). Due to its large body size, the lizard can occasionally even catch small vertebrates, including other lacertids and conspecifics (Nettmann and Rykena 1984; Leu and Petrovan 2022), which is also typical for other taxonomically related species (Angelici et al. 1997; Rugiero et al. 2021). Previous dietary studies conducted on related species showed that diet composition changes with body size, age, and season

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(Angelici et al. 1997; Crovetto and Salvidio 2013; Sagonas et al. 2018). Based on the macroscopic identification of prey remnants from stomach contents, Mollov et al. (2012) observed seasonal differences in diet, as follows: in spring, *L. viridis* captured mainly Orthoptera, while during summer, it preyed mostly on Coleoptera and Diptera. On the other hand, Sagonas et al. (2018) did not find seasonal effects on diet. Generally, for an opportunistic predator, prey composition can depend on the type of habitat where it occurs (Fischer and Rehák 2010).

In lacertids, the diet can differ between the sexes (Crovetto and Salvidio 2013; Santamaría et al. 2020). Males of *L. viridis* have larger bodies and heads and stronger jaws than females (Nettmann and Rykena 1984; Urošević et al. 2013). Therefore, they can hunt prey of larger size and/or with harder armor. This was observed based on macroscopic identification of prey remnants from stomach contents (Sagonas et al. 2018). However, because this information was gathered using a macroscopic analysis of prey remnants, there is a chance the data could be biased.

Here, we employed the molecular identification of prey remnants in European Green Lizards for the first time. We investigated the prey composition of one population of *L. viridis* in spring and late summer over 2 yr. Our primary aim was to compare the efficacy of two prey identification methods using fecal pellets—specifically, the macroscopic and dietary metabarcoding of the cytochrome oxidase I (COI) marker. We predicted that the molecular identification method would reveal more prey taxa than the macroscopic one, particularly those belonging to soft arthropods. With the obtained data, we then investigated the trophic niche in detail and tested the hypothesis of sexual diet partitioning and a seasonal shift in the diet composition.

Materials and Methods

Sampling and Macroscopic Identification of Prey in Fecal Pellets

Sampling was performed in the Pálava hills (South Moravia region, Czech Republic), which host a dense population of *L. viridis*. Sampling was performed in spring (May/June) and late summer (August/September) in both 2019 and 2020. In spring, we collected feces from 9 (2019) and 30 (2020) lizard individuals, and in late summer, we collected feces from 8 (2019) and 28 (2020) individuals. Lizards were captured by lassoing. Their feces were collected by abdominal massage or after spontaneous defecation during manipulation. Feces were immediately placed singly in Eppendorf tubes with pure ethanol. The sex of each collected lizard was recorded. All lizards were released back onto the site after sampling. Pellets were stored in a refrigerator until processing.

In the laboratory, pellets were first processed macroscopically. The pellets were disintegrated with a sterile pincer on a sterile cotton disc, and prey remnants were identified to the lowest taxonomic level possible, usually to an order, using the stereo microscope Leica EZ5 (Leica Microsystems GmbH). A few prey remnants with apparent coloration were identified to species (*Pyrrhocoris apterus* [Linnaeus], *Graphosoma italicum* [O.F. Müller], *Cercopis sanguinolenta* [Scopoli], and *Eresus* spp.). Between each pellet, we sterilized the pincer and placed the new pellet on a new sterile cotton disc. For each pellet, we recorded the number of remnants of each taxon so that if two same wings or two same legs were found, then this was considered to belong to a single prey individual.

DNA Extraction

As the fecal samples were stored in pure ethanol, they were placed on sterilized cotton pads for 30 s before extraction in order to partially dry them—i.e., to remove any excess ethanol. The fecal samples weighed between 0.2 and 1 g, and our Dneasy PowerSoil Kit (Qiagen) allowed us to extract up to 0.3 g of sample for each PowerBead tube provided. Therefore, we split each fecal sample into as many as three equal portions and extracted them in separate PowerBead tubes. This way, all prey DNA from the whole fecal sample was obtained, and no prey remnants that might have been present only in one part of the fecal sample were lost. Afterward, the fecal samples were extracted using the Dneasy PowerSoil Kit (Qiagen) according to the manufacturer's protocol. We added only 50 μL of C6 solution in the final step to increase the concentration of prey DNA. Additionally, for every 23rd sample we extracted, we tested a negative extraction control, where no tissue was added to the PowerBead tube at the beginning of the extraction. These negative controls went further through the same process as the fecal samples with extracted DNA. The extracted DNA and negative controls were stored at −20°C until further use.

PCR Amplification, Library Preparation, and Sequencing

To analyze the lizard's diet, PCR amplification of the COI gene fragment (225 bp) was performed. The universal primers MiteMiniBarF (5′-CATGCNTTYRTNATRATTTTTTTY ATAG) and MiteMiniBarRmodif2 (5′-GGRTAAACWGTT CAHCCWGTHCC) were used to identify all possible arthropod prey (Groot et al. 2016). In fecal samples, we found only arthropod remnants; therefore, we did not apply primers for the identification of other types of prey (e.g., plants or vertebrates). To prepare the samples for Illumina sequencing, in the first PCR, we appended overhang adapters to our COI locus-specific sequence (Appendix I in Supplemental Materials, available online). These adapters were needed for the second index PCR, when the sequencing adapters with identification indexes were added. To add complexity to the beginning of sequencing and enhance Illumin'a cluster calling, we introduced heterogeneity spacers between the overhang adapters and the locus-specific sequence (Lundberg et al. 2013; Fadrosh et al. 2014). The three forward or reverse primers, which varied only in the heterogeneity spacers, were combined in equimolar concentrations (10 μM each) into one forward or reverse primer solution to avoid any possible primer batch effects during sequencing.

The PCR reaction mixture's total volume of 25 μL was made of 10.6 μL of Multiplex master mix (from Multiplex PCR kit; Qiagen), 0.8 μL of forward and reverse universal primers (10 μM each), 1.8 μL of Q buffer, 6 μL of ultraclean water, and 5 μL of extracted gut DNA (<15 μg mL^{-1}). The following conditions were used for PCR amplification: initial denaturation at 95°C for 15 min; 35 cycles of 94°C for 30 s, 50°C for 90 s as an annealing temperature, and 72°C for 90 s; and a final extension at 72°C for 10 min. The PCR products were detected on agarose gels stained with 2% GoodView Nucleid Acid Stain (Renwik Bioinnovations) and immersed in

0.5 × Tris-borate-EDTA (TBE) buffer and afterward cleaned using the QIAquick PCR Purification Kit (Qiagen). Using an Invitrogen Qubit Fluorometer (Thermo Fisher Scientific), we measured the concentration of each PCR product from the sample and diluted it to 2 ng/L (as per instructions by the sequencing company). The PCR products from each sample and negative controls were placed in labelled 96-well plates and, within each plate, a space was left for two internal controls from the sequencing company. The plates were sent to SEQme s.r.o. (Prague, Czech Republic) for further library preparation (PCR II with Nextera indexes, DNA concentration measurements on Qubit, the pooling of samples, and purification using Agencourt AMPure X beads; Beckman Coulter, Inc.) and paired-end read sequencing with 150 cycles (PE150), which was performed on an Illumina NovaSeq6000 instrument. The expected sequencing depth was 100,000 reads per sample to increase the possibility of the recovery of prey reads and to account for the high variability in the recovery of reads in every sample (Krehenwinkel et al. 2016). Raw sequencing data were automatically processed by the Basespace cloud interface (Illumina, Inc., San Diego, CA) in default settings. The base calling, adapter clipping, and quality filtering were carried out using bcl2fastq v2.20.0 Conversion Software (Illumina, Inc.).

Bioinformatic Analysis and Data Clean-up

The sequencing output was processed using Geneious Prime® 2022.1.1 software (Biomatters, Inc., Aukland, New Zealand). The paired reads of each investigated sample were provided as separate forward and reverse read lists and, during upload into Geneious Prime, were paired into a single file. The reads in each file were then trimmed of remaining Illumina adaptors, their bases on the ends with a quality lower than 20 were cut out, and reads shorter than 120 bp were removed using the BBDuk plugin from BBtools (Joint Genome Institute 2023). After trimming, the forward and reverse reads were merged using the BBmerge plugin (Bushnell et al. 2017). Using Geneious Prime's De Novo Assemble option, the merged reads were clustered into molecular operational taxonomic units (MOTUs) with a minimum overlap identity of 97%. All obtained MOTUs were classified using BLAST in the NCBI database (Altschul et al. 1990) and the BOLD database (Ratnasingham and Hebert 2007). The percentage identification match determined the level of assigned taxonomic identity to MOTU, as follows: family level required ≥90% and genus level required ≥95% identity.

The clean-up of artefacts in the results was based on suggestions from Drake et al. (2022) and Cirtwill and Hambäck (2021). MOTUs belonging to the lizard, as well as low abundance MOTUs with <5 reads, were removed from each sample. We assumed that some possible artefacts might arise from the bleeding of prey sequences with a high read number into other samples. Therefore, we estimated a prey-specific threshold based on Cirtwill and Hambäck (2021) with a 2% error rate and removed reads in each sample that amounted to less than 2% of the overall number of prey reads (i.e., of each MOTU). Afterward, as we had sequencing results from the five negative controls that went through the same whole process as that of the samples, we removed all MOTUs that had a lower number of reads than the highest read count within a negative control for that specific MOTU. This way, we eliminated potential extraction and PCR contaminations, as well as any possible tag-jumping contaminations.

Sequences that were likely contaminations (human, pig, and mouse DNA) were removed, as well as sequences belonging to bacteria, microbial fungi (the orders Microstromatales and Mucorales), oomycetes (the order Albuginales), parasitic nematodes (the genus *Bursaphelenchus*), and parasitic mites (the orders Sarcoptiformes or Trombidiformes), of which all were too small to be a real part of the lizard's diet and were most likely consumed accidentally. Additionally, MOTUs belonging to parasitoid wasps (the subfamilies Bembicinae, Microgastrinae, Tiphiinae, and Tryphoninae) and parasitoid flies (the subfamilies Dexiinae and Tachninae) were excluded due to the uncertainty of them being real, chosen prey or a parasitoid inhabiting the eaten prey. The prey results from fecal samples that had to be split into subsamples during extraction were placed together, and MOTUs that were present in more than one subsample were fused into one prey event (PE). Three lizard samples were removed due to the absence of any dietary detections, leaving 72 samples to be used in the statistical analyses. After these filters were applied, read counts were transformed into presence–absence data for each sample.

Statistical Analyses

All statistical analyses were performed in R v4.2.0 (R Core Team 2022). The standardized Levin's index (B_A) was used to calculate the trophic niche breadth at the prey order level (Hurlbert 1978). Raphidioptera were excluded from all subsequent analyses due to very low abundance.

At first, we compared the efficacy of the two prey identification methods (macroscopic and molecular) by means of generalized estimating equations (GEE), which is an extension of generalized linear model (GLM) for correlated data (Pekár and Brabec 2018). Such data arose due to the nested design of the investigations (several prey in the same individual). GEE from the geepack package (Yan and Fine 2004) with binomial (GEE-b) were used. The working correlation structure was exchangeable. Cohen's h was used to estimate the effect size.

Then we compared the diet diversity between sexes and seasons by combining data from both prey identification methods. The numbers of prey taxa at order level per specimen were compared between sexes (female, male) and seasons (spring, late summer) using GLM with Poisson error structure (GLM-p; Pekár and Brabec 2016). The prey proportions (at order level) were compared between sexes and seasons using GEE with Poisson (GEE-p) errors because of the nested design. The linear predictor in each model included (two- and three-way) interactions between all explanatory variables. The interactions were removed if not significant. The quality of the fit was inspected using standard diagnostic plots.

To investigate the differences in more detail, i.e., at genus level, we combined binary (presence/absence) data obtained from both identification methods and subjected them first to the detrended correspondence analysis available from the vegan package (Oksanen et al. 2022). The data matrix was reduced by excluding rare taxa (represented by less than 1%) and subjected first to detrended correspondence

TABLE 1.—List of prey orders and their percentages in the feces ($n = 72$) of European Green Lizards (L. viridis) at the population level. Results of both methods (molecular and macroscopic) are combined.

Class/Order	% individuals
Gastropoda	3.49
Araneae	13.70
Ixodida	0.54
Opiliones	0.54
Isopoda	3.22
Diplopoda	2.69
Orthoptera	15.86
Hemiptera	9.68
Coleoptera	34.95
Lepidoptera	8.06
Raphidioptera	0.27
Diptera	2.42
Hymenoptera	4.57

analysis to find the length of the gradient along the first axis. Then, we subjected the data to canonical correspondence analysis (CCA) with sex as an explanatory variable.

In addition, we estimated the species accumulation curve, using all taxa at genus level, by means of Mao Tau implemented within the specaccum function from the vegan package.

RESULTS

The Illumina run generated 22,284,902 reads (average number of reads per sample = 210,234; min = 636 [negative control]; max = 523,754). After bioinformatical processing and data clean-up, 72 samples had at least one predation event (i.e., MOTU). On average, each sample had 3.42 predation events (min = 0, max = 8, median = 3). The macroscopic analysis yielded 5 samples without any macroscopic results, while the average number of predation events detected was 1.80 (min = 0, max = 4, median = 2).

Overall, using both the molecular and macroscopic approach, we identified 77 taxa at genus level and 13 taxa at order level (Appendix II in Supplemental Materials, available online). The prey of lizards was composed mainly of insects (75.8%) and arachnids (13.7%), with the former being represented particularly by Coleoptera (Table 1). The Levin's index of trophic niche breadth at order level was estimated to be $B_A = 0.36$.

The proportion of identified taxa was different between identification methods (GEE-b, $\chi^2_1 = 39.0$, $P < 0.0001$). With macroscopic methods, only about 50% of total taxa were identified at least to order level, whereas with molecular methods, the proportion was more than 80% of the total (Fig. 1). The effect size shows a large difference between proportions (Cohen's $h = 0.72$). The differences were found particularly in Diplopoda, Diptera, Gastropoda, Isopoda, and Lepidoptera that all failed to be identified macroscopically (Fig. 2). On the other hand, some taxa (e.g., Orthoptera and Hemiptera) failed to be detected molecularly (Appendix II in Supplemental Materials). The proportion of identified taxa was not different between sexes (GEE-b, $\chi^2_1 = 0.9$, $P = 0.34$) and between seasons (GEE-b, $\chi^2_1 = 0.2$, $P = 0.67$). The accumulation curve of prey taxon richness did not achieve a plateau, indicating that more samples should be taken to achieve a more complete diet composition estimate (Fig. 3).

When using results of both identification methods, the overall number of prey taxa identified in the feces was similar between sexes (GLM-p, $F_1 = 0.7$, $P = 0.40$) but differed between seasons (GLM-p, $F_1 = 11.5$, $P = 0.0011$); there were almost twice as many taxa (from different orders) per pellet in spring than in late summer (Fig. 4). The interaction between season and sex was not significant (GLM-p, $F_1 = 1.4$, $P = 0.24$).

With respect to prey composition at order level, the sexes were similar (GEE-p, $\chi^2_1 = 2.1$, $P = 0.14$), but the seasons differed (GEE-p, $\chi^2_9 = 23.9$, $P = 0.004$). Coleoptera were consumed about three times more frequently in spring than in late summer (Fig. 5). At genus level, however, there was a difference in the prey composition between sexes (CCA, $F_2 = 4.3$, $P = 0.001$). As seen from the ordination plot, some coleopterans were consumed more by males than by females (Fig. 6).

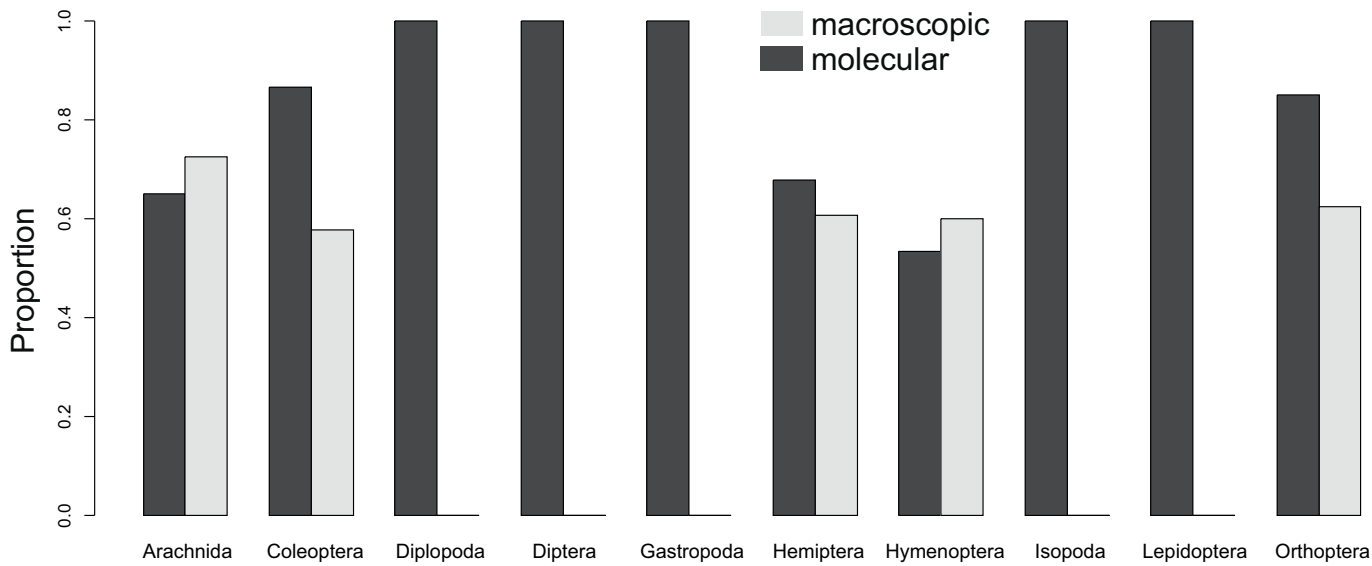

FIG. 1.—Comparison of the proportion of the number of identified taxa (at order level) between macroscopic and molecular methods in the fecal pellets of *L. viridis*. Bars are estimated means. Raphidioptera were excluded due to the low abundance.

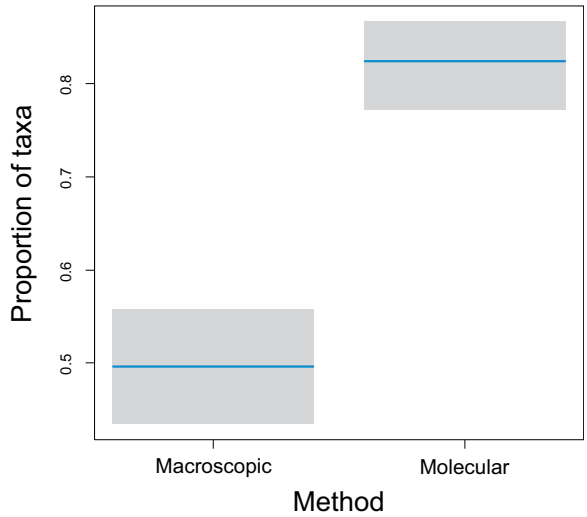

Fig. 2.—Comparison of the proportion of the number of prey taxa (at genus level) identified by two methods in the fecal pellets of *L. viridis*. Blue lines are estimated means, grey boxes are 95% confidence intervals. A color version of this figure is available online.

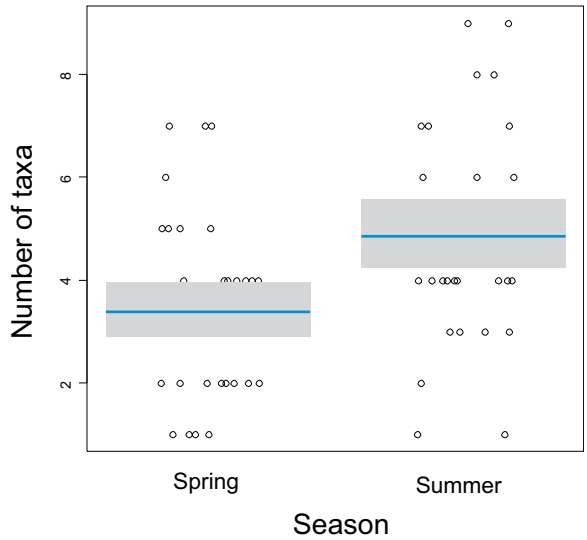

Fig. 4.—Comparison of the number of prey taxa (at genus level) identified in the fecal pellets of *L. viridis* by both methods (pooled) between two seasons. Blue lines are estimated means, and gray boxes are 95% confidence intervals. A color version of this figure is available online.

Discussion

We found that European Green Lizards are opportunistic predators of arthropod prey with a moderate breadth of trophic niche (in the taxonomic dimension), which agrees with former studies (Korsós 1984; Maier et al. 2020). Using both methods of prey detection, we found 13 arthropod orders, which is similar to findings of other studies using macroscopic prey identification in the same (Mollov et al. 2012) or related lacertids (Hódar et al. 1996; Mollov and Petrova 2013). Representatives of the family Lacertidae are mostly carnivorous, catching particularly invertebrates, with a tendency to omnivory in the case of a shortage of standard prey (Iverson 1982; Valido and Nogales 2003; Herrel et al. 2004). However, some of the larger species of the genera *Timon* and *Lacerta* will occasionally prey on vertebrates, such as the offspring of birds, rodents, lizards, and snakes (Hódar et al. 1996; Leu and Petrovan 2022). Even cannibalism has been recorded, although rarely (Nettmann and Rykena 1984; Elbing 2001). We did not find vertebrate prey remnants in the fecal pellets. Except for mouse DNA, the molecular approach did not find vertebrate DNA. However, the primers we used were more effective at amplifying mainly invertebrate DNA (Groot et al. 2016), which may explain the relative lack of vertebrate DNA. Lastly, cannibalism is impossible to detect by dietary metabarcoding, as the predator and prey sequences do not have sufficient

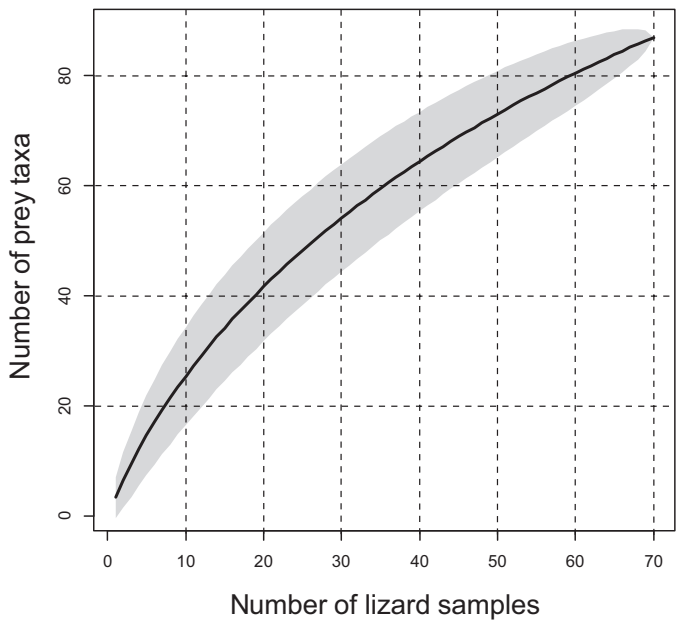

Fig. 3.—Accumulation curve of prey taxa identified (by molecular and macroscopic method at genus level) in lizard fecal pellets with a 95% confidence band (grey).

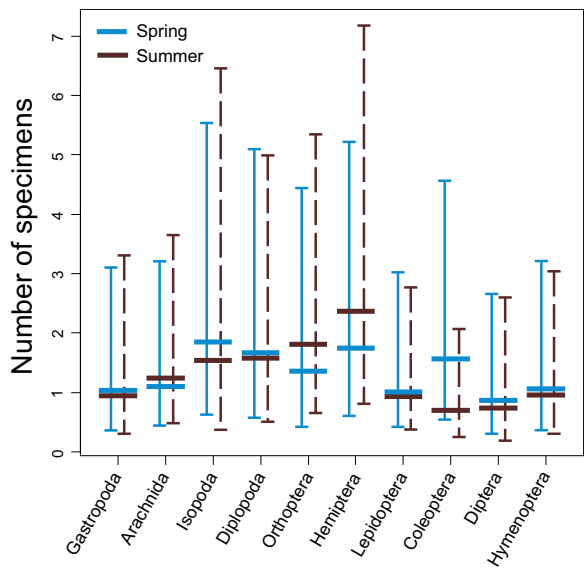

Fig. 5.—Comparison of the number of taxa (at order level) identified in the pellets of *L. viridis* by both methods (pooled) between two seasons. Horizontal lines are estimated means, and whiskers are 95% confidence intervals. Raphidioptera were excluded due to the low abundance. A color version of this figure is available online.

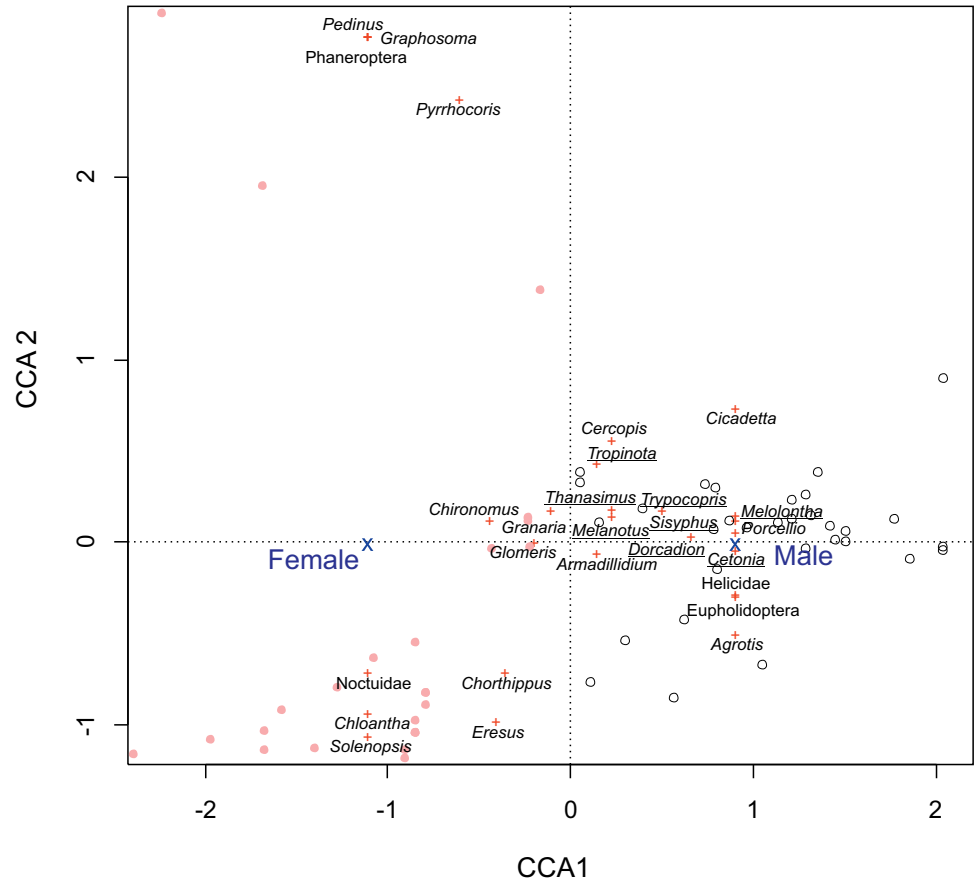

FIG. 6.—Ordination CCA diagram of prey identified at genus level (using both methods) between lizard sexes. The eigenvalue of the first unconstrained axis is 0.84 and the second is 0.79. Coleopteran genera are underlined. Male samples are represented by open points and female samples are by filled pink points. A color version of this figure is available online.

intraspecific variation in the short metabarcoding markers (Cuff et al. 2023).

Our results confirmed that the most frequent prey of *L. viridis* were Coleoptera, which corresponds to the findings of other authors for this species (Korsós 1984; Maier et al. 2020) and to the diets of other large European lacertids (Castilla et al. 1991; Angelici et al. 1997; Sagonas et al. 2015). Whether coleopterans are preferred prey or the most profitable prey for lacertids remains to be investigated. Lacertids seem to be adapted to hunting large and strongly sclerotized prey (beetles), which may be possible due to the large size of the lizard's body and head and its strong jaws (Urbani and Bels 1995; Mateo and López-Jurado 1997).

In sexually dimorphic species, larger adult males with larger heads and stronger jaws should be adapted to capturing larger and harder prey, resulting in a more variable prey composition (e.g., Schoener 1977). Indeed, we found the following differences in prey composition (at genus level) between the sexes: some genera of Coleoptera, either large (such as *Melolontha*) or heavily sclerotized (e.g., *Dorcadion*), were captured more by males than by females of *L. viridis*. Similarly, Liang et al. (2022) found significant differences between sexes of lizards in prey composition, leading to a broader trophic niche for males. However, Crovetto and Salvidio (2013) failed to find support for the niche divergence hypothesis in *Lacerta agilis*. Instead, they found more prey taxa in feces of adults than that in juveniles. In contrast, Kartzinel and Pringle (2015) found a similar prey composition between the sexes at the population level, whereas female lizards exhibited broader prey richness than males at the individual level, which could be due to differences in reproductive investment.

The season may affect food composition markedly. In a similar species, *Timon lepidus*, Castilla et al. (1991) observed a decrease in prey taxa richness in autumn compared with that in spring. In the same species, Hódar et al. (1996) also found differences in diet composition between seasons. In spring, the lizards captured mostly ants; in summer, the lizards switched to Coleoptera. The shift corresponded to prey availability during each season. In our study, we found a different pattern, as follows: there were more beetles captured by lizards in spring than in late summer. We did not study the prey availability in the two seasons, so it is impossible to state whether the shift to fewer beetles in late summer was due to availability or prey selection, as suggested by Maier et al. (2020). We assume that, in our case, the decrease in beetle consumption is most likely due to a seasonal change in their occurrence. Although beetles mainly occur as active imagoes searching for mates in spring, in late summer, they are in the larval stage, often hidden in soil and wood, among other locations, and are thus unavailable for epigeic predators like lizards.

The diet compositions of different lacertid species have so far been studied using the following two approaches: direct observations and analysis of ingested prey. The first approach provides direct evidence of capture, but it is mostly anecdotal, and prey identification can be biased due to short observation times. Thus, it cannot give a complex picture of food composition (e.g., Leu and Petrovan 2022). The second approach provides only indirect evidence of capture but has a higher potential for the correct identification of prey remnants (Pérez-Mellado et al. 2011; Santamaría et al. 2020).

An analysis of ingested prey can use macroscopic or molecular methods. As usual, each method has its pros and cons. Macroscopic identification requires an experienced person (or several experts)—in particular, when the prey is soft-bodied (Hódar 1996, 1997; Pérez-Mellado et al. 2011; Luiselli and Amori 2016). Sometimes it might be impossible to identify soft-bodied prey because it was completely consumed. Additionally, the precision of the identification of prey remnants using the macroscopic approach varies across several taxonomic levels, from species to phylum. Although Korsós (1984) was able to identify some remnants to family level and Hódar et al. (1996) was able to identify some to species, most frequently, identification remains at order level (Korsós 1984; Hódar et al. 1996; Angelici et al. 1997; Mollov et al. 2012; Crovetto and Salvidio 2013; Maier et al. 2020; Santamaría et al. 2020). Obviously, molecular identification provides much higher precision in taxonomic identification if DNA information on the prey is available. This agrees with results of our study. Furthermore, we expected that molecular identification would be superior to macroscopic identification in terms of the number of taxa that can be identified because the digestion state of samples does not influence the success of molecular identification as much as the macroscopic kind. Not surprisingly, a significant number of prey taxa were recovered by the molecular method but not by the macroscopic one. Specifically, the molecular method identified 30% more of the total taxa at the order level than the macroscopic one. Molecular methods increased the identification potential of prey belonging to Diplopoda, Diptera, Gastropoda, Isopoda, and Lepidoptera, which were completely absent in the macroscopic results.

An advantage of macroscopic identification is that the sex, size (volume), and ontogenetic categories of the prey can be determined (Hódar et al. 1996; Angelici et al. 1997; Mollov et al. 2012; Crovetto and Salvidio 2013; Maier et al. 2020; this study), which is impossible when using molecular identification. Another drawback of molecular methods can be false-positive results arising from secondary predation (Sheppard et al. 2005), i.e., the identification of prey consumed by the primary predator. Such prey would unlikely be detected macroscopically but could potentially be detected with molecular methods (Tercel et al. 2021). This, in theory, should be less of a problem when working with fecal samples, where the primary prey DNA is already drastically degraded and secondary prey DNA should be minimal (Cuff et al. 2023).

In our study, some prey were detected only macroscopically. Even with our small sample size, 44 taxa in 28 fecal samples were identified using macroscopic methods but were not detected through molecular methods. The lack of detection of these prey remains using the molecular method could have resulted from the complete degradation of prey DNA either during degradation in the stomach (Cuff et al. 2023), due to storage conditions in the refrigerator (Marquina et al. 2021), or due to the use of universal primers that were biased toward the amplification of some prey DNA more than others (Pompanon et al. 2012; Deagle et al. 2013). We tried to reduce primer bias by testing many published primers in silico before ordering them and by increasing the sequencing depth to obtain as many prey reads as possible (Krehenwinkel et al. 2016). It needs to be emphasized that few prey taxa we detected macroscopically failed to be found through metabarcoding. As we detected them through metabarcoding in other fecal samples, the primer bias did not have a strong influence on the detection of this prey. Nevertheless, using several universal primers for arthropod detection could provide potentially more information and could result in better alignment with the results of the macroscopic method (Gil et al. 2020; Cuff et al. 2023). However, this would increase the cost of the molecular method, which, compared to the macroscopic method, is already significantly more costly.

Therefore, integrating multiple methods allowed us to describe complex trophic interactions with high resolution, as found by others (Luiselli and Amori 2016; Nielsen et al. 2017). In our case with lizards, determining a reliable diet composition with noninvasive methods was achieved by combining macroscopic and molecular methods.

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Supplemental Material

Supplemental material associated with this article can be found online at https://doi.org/10.1655/Herpetologica-D-23-00017.S1.

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