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ORIGINAL ARTICLE

Metabarcoding analysis of different portions of the digestive tract of scorpions (Scorpiones, Arachnida) following a controlled diet regime shows long prey DNA half-life

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Abstract

Molecular analysis of gut content is one of the widest used methods to investigate diet in arthropods. Stomach content analysis in some arthropods is particularly difficult, e.g., in arachnids, because they have external digestion and a low foraging frequency. Scorpions have a particularly low feeding frequency, and their diet information is scarce. In this work we explore a DNA metabarcoding approach to detect prey DNA in Vietnamese forest scorpions (*Heterometrus laoticus*) under a controlled diet regime. A different type of prey (crickets, mealworms, and cockroaches) was offered once every 3 weeks for a total of 9 weeks. To assess the most suitable part of the digestive system and extraction method to use for molecular diet analysis, we separately analyzed three different portions of the digestive tract of scorpions (mid-gut, hepatopancreas, and hindgut) using two different extraction methods (salt-out method and a customized beads-based protocol). We calculated the detectability half-life of the prey DNA for each digestive tract section. We detected all three targeted prey items, showing that in scorpions multiple predation events can be distinguished in the same specimen within its last 9 weeks of foraging activity. The hepatopancreas was the portion of the digestive tract that provided the best prey detection and the longest DNA detectability half-life (51 days), followed by the mid-gut (22 days) and the hindgut (16 days). We found no significant difference between the extraction methods used. However, the salt-out method was less effective in some of the PCRs and is therefore not recommended for molecular diet analysis.

KEYWORDS
diet, hepatopancreas, *Heterometrus laoticus*, metabarcoding, scorpions

1 | INTRODUCTION

For many years, direct observations of predation events and the analysis of the remains of consumed prey were the most used approaches to investigate diet composition in arthropods (reviewed in Sunderland, 1988). These methods require years of investigation to gather a comprehensive number of records/samples, as well as taxonomic expertise. Due to the quality of

the samples, species-level identification is not always possible (Nielsen et al., 2018). Moreover, these methods insufficiently provide information about diet in liquid feeders (Symondson, 2002) and in species living under water (Hyslop, 1980) or beneath the soil (Pompanon et al., 2012).

Several molecular techniques have been developed to study diet (reviewed in Nielsen et al., 2018). Recently, DNA-based diet analysis of stomach or scat content has been the most widely used tool to analyze diet and map trophic interactions (Cristescu, 2014; King et al., 2008; Liu et al., 2020; Nielsen et al., 2018; Pompanon et al., 2012; Sousa et al., 2019).

One of the main challenges of DNA-based diet analysis is the undesired amplification of the DNA of the consumer taxon. The co-amplification of the consumer DNA can be limited using a combination of exclusion and universal primers (Cuff et al., 2021; Lafage et al., 2020). When the consumer and the ingested item are phylogenetically distant, species-specific primers are used to detect a specific targeted consumed item. This approach may be useful for conservation purposes (Egeter et al., 2015, 2019) or to investigate herbivore diets (De Barba et al., 2014).

Consumer DNA amplification can also be reduced by using consumer-specific blocking primers (Deagle et al., 2009; Toju & Baba, 2018; Vestheim & Jarman, 2008). However, the use of blocking primers can prevent the amplification of prey species that are phylogenetically close to the consumer (Piñol et al., 2014, 2015). Other studies have suggested extraction strategies based on the retention of short DNA fragments, presumably belonging to digested item, and the exclusion of longer fragments most likely belonging to the consumer (Krehenwinkel et al., 2017).

The detectability of DNA of the ingested items also depends on how long the ingested DNA resides in the digestive tract of the consumer. One of the parameters to assess the detectability of ingested DNA is its half-life (D_{50}) (Greenstone et al., 2014; Uiterwaal & DeLong, 2020). The D_{50} is the time at which sequences belonging to a consumed meal are detected in half of the consumers tested. However, the D_{50} parameter is sensitive to the type and size of the ingested meal (Schattanek et al., 2021), the digestion rates (Uiterwaal & DeLong, 2020), and the analyzed portion of the digestive tract (Macías-Hernández et al., 2018).

Molecular sequencing of stomach contents has been used to assess the diet composition of many arthropods, and has proven to be a successful tool to investigate the diet of liquid-feeding arachnids like spiders (Krehenwinkel et al., 2017; Lafage et al., 2020; Macías-Hernández et al., 2018; Sierra Ramírez et al., 2020), mites (Heidemann et al., 2014; Pérez-Sayas et al., 2015), and ticks (Kirstein & Gray, 1996; Pichon et al., 2003). The diet of scorpions has to date not been studied using this method.

Scorpions are important predators in many different biomes, ranging from deserts to humid rainforests. In arid environments in particular, scorpions can represent the highest fraction of the animal biomass, second only to termites (Polis & Yamashita, 1991). Consequently, in these environments, their high densities and their predatory habits may have a great impact on arthropod population dynamics (Polis & McCormick, 1986; Shachak, 1980).

Studying scorpion diet is particularly challenging because, besides being liquid feeders, scorpions have a particularly slow metabolic rate when compared to similar-sized arthropods (Lighton et al., 2001). As a consequence, these animals feed infrequently and are able to survive several months without eating (Pimenta et al., 2019; Stahnke, 1966; Vachon, 1957). Because of these factors, investigating scorpion diet using traditional methods is difficult and time-consuming. Information about their natural diet is known only for a few of the approximately 2600 scorpion species currently recognized. Moreover, works on this topic report only a small subset of the potential prey items these species may consume (for reviews, refer to Polis, 1990; Simone & van der Meijden, 2021). The most complete scorpion diet assessment was done for *Paruroctonus mesaensis* (Stahnke) (Polis, 1979). It was conducted using direct observation and recognition of the remains of the prey left in the scorpion burrows and took 5 years to complete.

Like many other arachnids, scorpions have an external predigestion of the prey. Specifically, prey is chewed through chelicerae, while digestive enzymes secreted from coxal glands of the legs I and II act on prey tissues (Alexander, 1972; Polis, 1990; Venkateswararao, 1967). Pre-digested prey fluids are sucked into the oral cavity through the pumping action of the muscles of the pharyngeal chamber wall (Pavlovsky & Zarin, 1926). Once the prey fluids are pumped into the pharyngeal chamber, they are then conveyed to the central digestive tube (the mid-gut) running almost the whole length of the scorpion body. The mid-gut is strongly branched, and diverticula extend through the whole body of the animal, and even reach the legs (Pavlovsky & Zarin, 1926; Snodgrass, 1952). The diverticula end in different parts of the hepatopancreas, an organ responsible for the internal digestion, absorption, and storage of the ingested food (Fuzita et al., 2015; Warburg, 2012). The last part of the digestive tract leads the food to its expulsion through the anus, which is located in the metasoma or "tail," just before the venom vesicle.

In scorpions, particles of food are found both in the mid-gut lumen and in the cells composing the epithelium of the peripheral branches running in the interstitial tissue of the hepatopancreas (Goyffon & Martoja, 1983; Zouari et al., 2006). The digestion process happens in two different types of cells covering the epithelium of the mid-gut diverticula: the basophilic cells (or zymogen cells) and the digestive cells. The basophilic cells perform the extracellular digestion while the digestive cells absorb the fluid containing food from the lumen of the diverticula. The latter, through intracellular digestion, stores the nutrients in characteristic vacuoles (Goyffon & Martoja, 1983; Zouari et al., 2006). The hepatopancreas is composed of interstitial cells that surround the mid-gut diverticula and apparently its function is limited mainly to food storage. The waste products of both extra- and intracellular digestion are expelled into the lumen of the caudal portion of the mid-gut, conveyed into the hindgut, and then expelled through the anus.

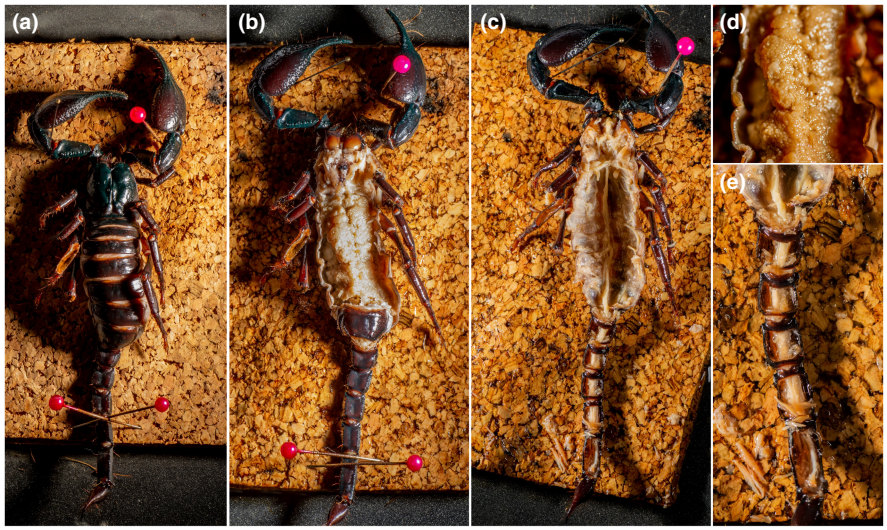

FIGURE 1 (a) Scorpion prepared for dissection. (b) The hepatopancreas is exposed once the mesosomal and prosomal cuticle of scorpion is removed. (c) The mid-gut is exposed after removal of the hepatopancreatic mass, ovaries, book lungs, and the ventral nervous chord. The removal of the dorsal cuticle and the muscles of the metasoma exposes the hindgut. (d) Detail of the hepatopancreatic mass with its typical granular texture. (e) Close-up of the hindgut

In this work, we test a DNA-based approach to assess scorpion diet for the first time, using scorpions fed under a laboratory-controlled diet regime. The two main objectives of this work are as follows: (i) identifying the best portion of the digestive tract to select to gather the highest amount of prey DNA, and (ii) estimating the D_{50} of prey DNA across different portions of the digestive tract. Secondarily, different extraction methods have been compared to assess whether a bead-based method should be preferred to a salt-out one.

2 | METHODS

2.1 | Feeding experiment

Fourteen subadult specimens of the Vietnamese forest scorpion, *Heterometrus laoticus* (Couzijn, 1981), were subjected to a controlled diet regime for 9 weeks. All the tested scorpions were bred in our laboratory and kept in captivity for several years with humid substrate, a photoperiod of 12:12 light/dark at 24–26°C and fed only with fresh crickets (*Acheta* sp.) every 3 weeks prior to the study commencement. The experiment consisted of three feeding sessions. During each session one prey type was provided to each scorpion. The first prey type offered was an adult cricket, *Acheta* sp. (week 0), followed by larvae of mealworms, *Tenebrio molitor* (week 3), and adult Argentinian cockroaches, *Blaptica dubia* (week 6). The total mass of each prey type offered was controlled to standardize the food intake per feeding session (see Appendix S1). After each feeding session, a period of starvation of 3 weeks followed. This is the same starvation time used in the only available published work about food detection in the scorpion digestive tract (Quinlan et al., 1993). Three weeks after the last feeding session (week 9), scorpions were anesthetized using isoflurane, and subsequently euthanized by freezing (−20°C). Only 6 scorpions of the 14 completed the feeding trials successfully. The other scorpions that refused to eat at least one of the offered prey were discarded from the experiment.

2.2 | Dissection protocol

Euthanized scorpions were labeled and defrosted before the dissection procedure. First, scorpions were rinsed in distilled water and then placed into a container filled with 500 ml bleach at 5% for 40 min to remove external contaminant DNA (Greenstone et al., 2012; Linville & Wells, 2002). Scorpions were then rinsed in distilled water to remove any excess bleach and placed on a sterilized dissection plate. To minimize cross-contamination, a new set of gloves and new scalpel blade were used for each specimen. All reusable tools used during the dissection were rinsed in 60% bleach, then in 70% ethanol, and finally flamed between specimens. The dissection was performed under a stereomicroscope (Motic SMZ-168) and started with carefully removing the dorsal portion of the exoskeleton from prosoma to the end of the metasoma. The heart was carefully removed and the hepatopancreas underneath was moved laterally until it was possible to observe the mid-gut. The latter was then carefully removed and stored in a 2 ml cryotube and labeled as mid-gut (MG). The whole hepatopancreatic mass was stored in a 15 ml tube and labeled as HP. To extract the last part of the digestive tract, all the muscles in the five metasomal segments were removed and the last part of the digestive tube was stored in a 2 ml cryotube and labeled as hindgut (HG). Figure 1 shows three different stages of the dissection process. The content of the dissected gut portions was homogenized and the whole volume divided in half. The DNA from each half was subsequentially extracted using two different methods.

2.3 | DNA extraction

Two different extraction methods were used. The first is a salt-out method and the second one uses Agencourt AMPure XP® beads (Beckman Coulter, Ma, USA) and Qiagen® (Qiagen, Germany) buffers (for detailed protocols, see Appendix S2). DNA extracted

through the salt-out method was stored in 100 µl of pure demineralized water, while DNA extracted through the bead-based method was stored in 70 µl of the AE buffer (Qiagen®).

2.4 | Blocking primer design and PCR optimization

Sequences of COI of *Heterometrus laoticus*, along with representative sequences of different species of scorpions and other arthropods possibly included in scorpion diet, were retrieved from the NCBI website (see Appendix S3).

Universal primers for terrestrial arthropods fwhF2 (forward) and fwhR2n (reverse) (Vamos et al., 2017) were chosen for this study for their high efficiency in targeting arthropod DNA in environmental studies (Elbrecht et al., 2019). This set of primers amplifies a 205 bp portion of the Folmer fragment (Folmer et al., 1994).

The gathered sequences were aligned in Geneious using the MUSCLE algorithm (Edgar, 2004). The blocking primer we designed, Simone_2021_HIBlk, 5′- CCYCCTTTGTCTTCTAGTATATTTC 3C – 3′, shares the first five bases with the last five of primer fwhF2 and matches the next 21 bases of the sequence of *H. laoticus* (AY156573) without ambiguities.

To assess the specificity of the blocking primer, we tested the pair Simone_2021_HIBlk / fwhR2n in the NCBI Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and retrieved all the possible targets within the invertebrate dataset having up to three mismatches with at the least one of the aforementioned pair of primers.

Subsequently, blocking primer influence on non-targeted taxa was tested by performing PCRs using DNA extracted from tissue of fresh specimens of *H. laoticus*, crickets, mealworm larvae, and Argentinian cockroaches as templates. DNA extracted from these samples was first quantified using a Nanodrop 1000 (Thermo Scientific). To avoid having different DNA concentrations among samples biasing the result of the amplification process, we diluted all samples to 1 ng/µl concentration. The PCRs were carried out with and without blocking primers in volumes of 10 µl, comprising 5 µl Qiagen Multiplex PCR Master Mix (Qiagen, Germany), 0.4 µl of each primer (10 pM), and 1 µl extracted DNA. PCRs done with the blocking primer also included 0.4 µl of Simone_2021_HIBlk at 100 pM. Cycling conditions used initial denaturing at 95°C for 15 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 30 s, with a final extension at 60°C for 10 min.

Increasing concentrations of blocking primer were also tested. Using the DNA extracted from fresh leg tissue of *H. laoticus*, blocking primer efficiency was tested at 10×, 15×, and 20× the concentration of the amplification primers. PCR conditions were the same as indicated above.

Optimization of annealing temperature was performed. The same template DNA was used to perform PCRs with and without blocking primers using a temperature gradient in eight steps from 42°C to 60°C. The other PCR conditions were kept unchanged.

All PCR results were checked by visually inspecting 2 µl of each PCR product on a 2% agarose gel stained with GelRed (Biotium, USA).

2.5 | Dual-step Illumina library preparation

Based on the optimization tests mentioned above, we selected an annealing temperature of 56°C and a blocking primer concentration of 10x. Three PCR replicates per sample were prepared using 5 µl of Qiagen Multiplex PCR Master Mix, with 0.4 µl each of primers fwhF2 and fwhR2n at 10 pM, 0.4 µl of primer Simone_2021_HIBlk at 100 pM, and 1 µl of extracted DNA for a total volume of 10 µl. To test the efficiency of the blocking primers, PCRs of six arbitrary selected samples, amplified without the addition of blocking primers, were also sequenced. A negative PCR was included to control for possible contaminants. For all PCRs, the universal primers already had the adaptors for the Illumina sequencing. Library preparation, including indexing PCR (second PCR), fragment purification, normalization, and final pooling, followed the steps indicated in Chapter 3.3 from Paupério et al. (2018) with some minor modifications, which are as follows: before the second PCR all three replicates of the first PCR were pooled together per sample; total volume for the second PCR consisted of 14 µl, including 7 µl KAPA HiFi PCR kit (KAPA Biosystems, USA), 0.7 µl of each "index," and 2.8 µl of diluted PCR product (only strong PCR products were diluted). Lastly, bead purification was carried out using Agencourt AMPure XP® beads (Beckman Coulter, MA, USA) for an amount of 0.75% of the final library volume. The final library was then sequenced on a MiSeq machine using an Illumina Reagent Kit v3 600-cycle (Illumina, California, USA). The run, aiming for a coverage of 30,000 reads per sample, was shared with other projects on freshwater macroinvertebrates and bat samples.

2.6 | Bioinformatic pipeline

Sequence data were processed using the MBC pipelines package (Galhardo et al., 2018, commands used are provided in Appendix S4) following Peixoto et al. (2020). Shortly, reads produced on the MiSeq platform were demultiplexed according to the sample-specific indexes using BASESPACE (basespace.illumina.com). Paired-end reads were aligned using FLASH2 (Magoč & Salzberg, 2011) and primers were removed with cutadapt (Martin, 2011). Using VSEARCH (Rognes et al., 2016), sequences were dereplicated, singletons were removed, and sequences outside the expected amplicon lengths of 70–130 bp (without including primers) were removed. Default settings were used for all of the commands. The amplicons were mapped against the NCBI nucleotide database using the BLAST algorithm. Taxonomy was assigned to each query using a lowest common ancestor (LCA) approach. For each query, relatively stringent percentage identity thresholds were used: 99% for species level, 97% for genus level, 95% for family level, and 93% for higher-than-family level. Data were filtered to

reduce false positives, tag jumping, and other contaminants. In order to have a reliable minimum sequence copy threshold, we further filtered followed Drake et al. (2022). We applied the "Max contamination" filter together with different "Sample %" thresholds (ranging from 0.08% to 1%). The optimal "Sample %" threshold of 0.25% was selected in order to maximize the removal of contaminants while preserving most of the reads of the target prey items (see Appendix S5).

2.7 | Statistical analysis

We used three methods to analyze the effect of gut portion and extraction method on each prey type separately and then on all prey types pooled. The first method converts all the filtered reads into a binary presence/absence type of data. This dataset was analyzed through generalized linear mixed models (GLMMs) with the prey presence/absence data as the response variable and the gut portion and the extraction method as fixed variables. Specimens were included as random factors. The data were modeled following a logistic distribution.

In the second and third method, in order to account for the large variance in total number of reads among samples, we modeled the response variable as a ratio of the taxon reads to the total number of reads per sample.

The second method consists of a GLMM model where gut portion and extraction method are the fixed variables and the ratio of prey read count/total number of reads per sample is the response variable. In this method, the response variable follows a quasibinomial distribution.

The third method differs from the second in using a zero-inflated beta regression.

We used the "lme4" package (Bates et al., 2015) for binary and binomial GLMMs and the package "glmmTMB" (Brooks et al., 2017) for the zero inflated beta regression.

Likelihood ratio tests (LRT) were used to evaluate all the models using a nested approach (from null to full model, adding each variable sequentially to a previous model) through the function *lrtest* of the package "lmtest" (Zeileis & Hothorn, 2002).

The same three methods were also applied to the samples sequenced with and without the blocking primer. The response variable and the random factor are the same described for each method, while the fixed variable in these models is the presence of the blocking primer.

For all the models generated, the corrected Akaike criterion index (AICc) was used to select the best fitting model using the function *AICc* from the package "MuMIn" (Burnham & Anderson, 2002).

To fit the detection against the time from ingestion of the three targeted prey types for each gut portion type, we used probit regression. From these models we calculated the probability of detection for each day and the day at which the probability of detection is 50% (D_{50}).

Datasets are in Appendix S7 and all analyses were performed in R 4.1.2 (R Development Core Team, 2021).

3 | RESULTS

After removing the singletons, sequencing generated a total of 852,788 reads for which 45,943 (5.4%) belong to the three targeted prey. Of the total reads, 805,068 (94.4%) belong to *Heterometrus laoticus*, while only 1777 (0.2%) reads came from contaminants. Five PCRs of 41, namely 2-HP-SALT, 3-HG-SALT, 10-HG-SALT 8-HP-SALT, and 10-HP-SALT, were excluded from the statistical analysis. The first three because they accounted for a total of zero reads, while the last two had a read count below that of the negative. The "Max contamination" filter alone cleaned all the negatives. The "Sample %" threshold of 0.25% removed the 90% of all the artifacts. Figure 2 shows the great variance in the detection of the three target prey, and that the majority of the reads come from just two specimens out of the six sequenced.

The presence or absence of the blocking primer for the six samples tested did not significantly affect the amplification of the target prey: Binary, $Primer\_Chisq_1 = 9.07E{-}02$, p-value = 0.732; Quasibinomial, $Primer\_Chisq_1 = 4.91E{-}02$, p-value = 0.825; Beta-regression, $Primer\_Chisq_1 = 0.157$, p-value = 0.693.

The likelihood ratio tests on the nested logistic and the quasibinomial models, respectively, provided a significant and a nearly significant effect of the gut portion on read count of the three pooled target species. The same analysis found no significant effect of the extraction methods. The effects of both gut portion and extraction method were not significant in the zero-inflated beta regression model. In Table 1 we report only the results of the methods applied to the pooled target prey because their higher combined numbers provide more statistical power. Per target species model likelihood ratio tests are provided in the Appendix S8. Note that these have to be cautiously interpreted given their limited statistical power.

The half-life estimated from the probit regressions is 21.8 days for mid-gut, 51.5 days for the hepatopancreas, and 15.8 days for the hindgut (see Figure 3).

4 | DISCUSSION

4.1 | Gut portion selection

This study reports the results of a metabarcoding-based approach to identify how DNA sequences of partially digested prey are distributed across the three main sections of the scorpion digestive tract. The hepatopancreas retained prey DNA sequences longest, followed by the mid-gut. This result finds support in similar studies conducted in spiders, where the opisthosoma (the tagma containing the mid-gut and the hepatopancreas) was the best body part to detect DNA of the consumed prey (Krehenwinkel et al., 2017; Macías-Hernández et al., 2018; Miller-ter Kuile et al., 2021).

Presumably due to the highly degraded nature of the waste products of digestion (Goyffon & Martoja, 1983; Kanugo et al., 1962; Yokota, 1984), the hindgut was the gut portion from which the

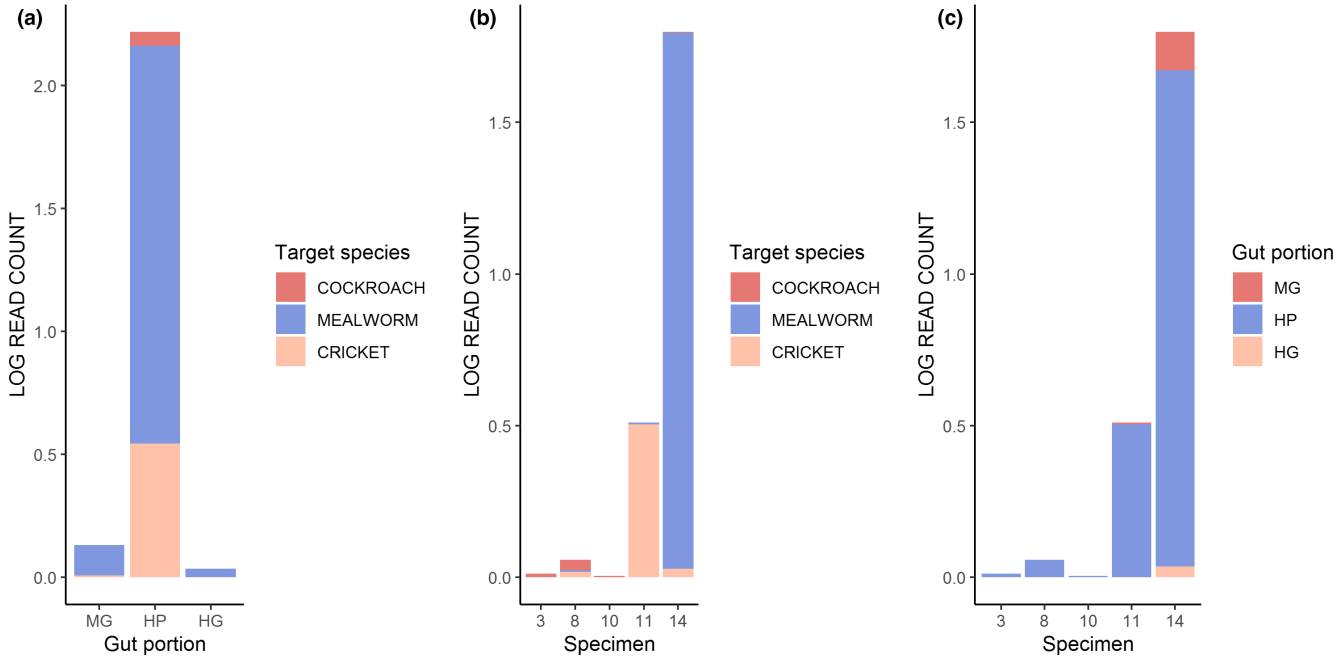

FIGURE 2 (a) Abundance of the three prey species per extraction method. (b) Abundance of the prey species per specimen. (c) Abundance of reads of the target prey per gut portion for all the specimens

TABLE 1 Likelihood ratio test of nested models using different response variables and statistical analysis. Significant *p*-values (<0.05) are highlighted in bold. Nearly Significant *p*-values are highlighted in italics

	Model description	AICc	#df	LogLik	df	Chisq	Pr(>Chisq)
Binary (0,1)							
Null Model	detection ~1 + (1 | Specimen)	94.8	2	−45.3			
Model 1	detection ~ Gut_portion + (1 | Specimen)	76.7	4	−34.1	2	22.4	**1.36E−05**
Full model	detection ~ Gut_portion + Extr_method + (1 | Specimen)	78.6	5	−33.9	1	0.293	0.588
Binomial (0 ≤ X < 1)							
Null Model	Prey_reads/Total_sample_reads ~1 + (1 | Specimen)	23.4	2	−9.63			
Model 1	Prey_reads/Total_sample_reads ~ Gut_portion + (1 | Specimen)	22.9	4	−7.25	2	4.74	*0.0932*
Full model	Prey_reads/Total_sample_reads ~ Gut_portion + Extr_method + (1 | Specimen)	25.0	5	−7.18	1	0.14	0.708
ZINF Beta Regression (0 ≤ X < 1)							
Null Model	Prey_reads/Total_sample_reads ~1 + (1 | Specimen)	43.6	4	−17.6			
Model 1	Prey_reads/Total_sample_reads ~ Gut_portion + (1 | Specimen)	47.9	6	−17.5	2	0.2303	0.891
Full model	Prey_reads/Total_sample_reads ~ Gut_portion + Extr_method + (1 | Specimen)	50.1	7	−17.4	1	0.1866	0.666

lowest number of prey DNA reads was detected, and is therefore less suitable for metabarcoding diet analysis.

It is interesting that the last provided item (cockroach) can only be detected in the mid-gut and the hepatopancreas, suggesting that perhaps three weeks are not sufficient for the food to reach the last portion of the intestine (hindgut) in well-nourished scorpions. Digestion rate is affected by the feeding state of the consumer (Secor, 2001). Studies conducted in carabid larvae show that food detectability in the digestive tract decreases with an increased starvation period (Lövei et al., 1985). Similar results have been also found in other species of insects (Agustí et al., 1999). However, since our scorpions were fed regularly and the experimental regime was

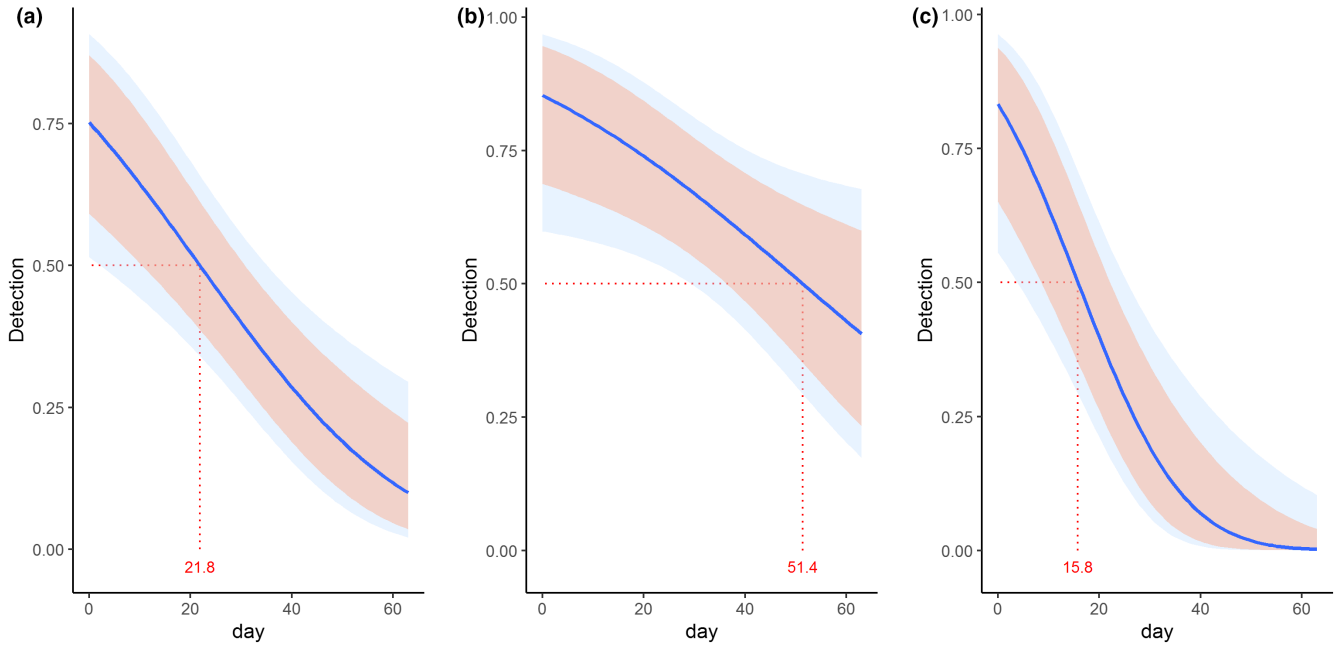

FIGURE 3 Probit regressions of prey DNA decay over time per section of the digestive tract (blue line) in (a) mid-gut and (b) hepatopancreas (c) hindgut. The dotted red lines indicate the D_{50} of prey DNA (in days) and the light blue band indicates the 95% confidence interval, while the 83% confidence interval is shown in salmon

similar to the prior normal feeding regime, we expect that they were not starving or overly satiated. Another unexpected result was the high number of reads of the first prey item detected in the mid-gut, contrasted with an almost total absence in the hindgut. Since our tested scorpions have been maintained exclusively on a diet of crickets prior to the beginning of the treatment, cricket permanence in the mid-gut and its consequent detection may be a result of prolonged exposure to that prey type in the past. Alternatively, as already observed in mites, the mid-gut is readily filled with food along its length, but the distribution of nutrients in the absorbing and storing tissues is not totally synchronous, and the most caudal portions of the mid-gut may be the last to be emptied (Bowman, 2017, 2019). This possibility is corroborated by the detection of asynchronous digestive processes across diverticula in scorpions fasting for 1 month (Goyffon & Martoja, 1983). Although we cannot draw robust conclusions due to our limited sample size, the lack of DNA of the oldest prey type in the hindgut may be explained by repeated scorpion defecations within the 9 weeks of treatment. In other *Heterometrus* species, the first defecation event is within 3 days after the last meal (Kanugo et al., 1962).

4.2 | Food permanence in digestive tract

While cytophysiological aspects of scorpion digestion have been well explored, studies on food permanence in scorpions' digestive tracts are lacking. The only data available on this topic can be found in Quinlan et al. (1993). Here, using an antibody-based technique, the authors detected prey antigens in the hepatopancreas of two species of scorpions that had undergone up to 32 days of food deprivation. Similar studies have been conducted in spiders, obtaining much lower detection times (up to 13 days) (Sopp & Sunderland, 1989). In ticks, a PCR-based analysis of the composition of remnants of larval blood meals obtained from mid-gut of nymphs allowed the detection of host DNA up to 270 days after the molt (Kirstein & Gray, 1996; Pichon et al., 2003). In recent years, due to improvement of high-throughput sequencing techniques, consumed meal detectability has been estimated through the calculation of its D_{50}. This parameter was used to show differences in prey detectability time across different families of spiders. The highest half-life value for spiders (16 days) was obtained in the family Lycosidae (Uiterwaal & DeLong, 2020). Our data suggest a high value for D_{50} of at least 51 days for the hepatopancreas, showing that it stores food in an undigested or partly digested state for a long time. However, these results have to be considered cautiously because, even if the mass of the prey items ingested was roughly the same, we could not take into account the detectability half-life of each prey type because of our experimental design.

4.3 | Primer and tissue biases on target prey amplification

None of the three prey species was present in the list of taxa that could be blocked by the blocking primer. The blocking primer has high specificity for scorpions, spiders, and wasps (see Appendix S6). Additionally, when the blocking performance of the blocking primer was tested on different arthropod species, the amplification of the three prey species was unaffected or no evident attenuation was observed (see gel reported in the Appendix S3).

The performance of the universal primer Fwh2 in amplifying the orders Blattoidea, Ortopthera, and Coleoptera was evaluated by Tournayre et al. (2020). In that study, the power of the amplification of the primer is very similar across the targeted taxa of our work.

A possible tissue-specific inhibition cannot be excluded to explain lack of detection of prey DNA in the hindgut and in the midgut. Tissue-specific inhibition has been observed in some portions of the digestive tract of the ants of the genus *Tetramorium*, specifically when the crop is not removed from the whole gaster (*Penn* et al., 2016). More experiments are needed to better address this possible methodological issue.

4.4 | Extraction method

Our results show no significant difference between the two extraction methods. However, considering that the only PCRs that have failed were associated with DNA obtained using the salt extraction method, we do not recommend this extraction method. If salt extraction is considered during the design of a project including metabarcoding-based diet analysis, we suggest to increase the quality of the extracted DNA by implementing post-extraction cleaning procedures to reduce the amount of PCR inhibitors (Schrader et al., 2012). However, kit extraction protocols are high yielding and provide quality DNA, and are therefore preferable to salt extraction methods (Dell'Anno et al., 2015).

5 | CONCLUSIONS

The main objective of the present work was to develop and validate a protocol for a DNA barcoding approach using scorpions fed under controlled conditions, and to study the detectability of DNA in the gut content. This work represents the starting point for a more comprehensive characterization of the diet of scorpions sampled in the field and for which diet is mainly unknown. Deeper knowledge on the dietary composition of scorpions is an important step to understand how these predators affect arthropod communities. Differently from deserts, where the remains of consumed prey are better preserved and observational methods have thus been possible, in tropical or more humid biomes there is a total lack of knowledge about the roles of scorpions in the food network. Alternatively, thanks to the long D_{50} of scorpions, the composition of their gut content may be used to obtain information to assess the composition of local prey species communities.

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AUTHOR CONTRIBUTIONS

YS performed the experiments, analyzed the data and wrote the manuscript. CC prepared the DNA-libraries and contributed with the preparation of the sequencing process. BE and AvdM conceived and designed the study. All the authors contributed to the improvement of the first draft and accepted the final version.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships.

DATA AVAILABILITY STATEMENT

Data available in article supplementary material

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