Trofička niša mirmekofagnog predatora tijekom ontogenetičkog razvoja

Gajski, Domagoj

University of Zagreb

Faculty of Science

Department of Biology

Domagoj Gajski

Trophic niche of an ant-eating predator during its ontogenetic development

Graduation Thesis

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This thesis was made during an internship at the Masaryk University (Brno, Czech Republic) under the supervision of Prof. Mgr. Stanislav Pekár, Ph.D. and Assoc. Prof. Dr. sc. Damjan Franjević from University of Zagreb, and submitted for evaluation to the Department of Biology, Faculty of Science, University of Zagreb in order to acquire the title Master of molecular biology.

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TROPHIC NICHE OF AN ANT-EATING PREDATOR DURING ITS ONTOGENETIC DEVELOPMENT

Domagoj Gajski

Roosevelt Square 6, 10000 Zagreb, Croatia

The realized trophic niche describes the natural diet of a species and is mostly limited by the prey availability. The natural diet can be assessed through various methods, but the most commonly used method is DNA-based diet analysis. *Zodarion rubidium* (Simon, 1914) is, unlike most spider species, described as a stenophagous predator specialized in foraging ants. Still, his natural diet is for the most part unknown and there is no knowledge of prey preferences during ontogenetic development. The main goal of this research is to determine whether there is a shift in prey preferences during *Z. rubidum's* ontogenesis and if there is a difference in the diet between sexes due to sexual dimorphism. In addition, a feeding trial was performed to assess the potentials of DNA-based diet analysis and to estimate the time at which prey-DNA can be detected.

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Supervisor: Dr. Stanislav Pekár, Prof.

Reviewers:

1) Dr. Damjan Franjević, Assoc. Prof.
2) Dr. Sofia Ana Blažević, Assist. Prof.
3) Dr. Tomislav Ivanković, Assist. Prof.

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TROFIČKA NIŠA MIRMEKOFAGNOG PREDATORA TIJEKOM ONTOGENETIČKOG RAZVOJA

Domagoj Gajski

Rooseveltov trg 6, 10000 Zagreb, Hrvatska

Ostvarena trofička niša opisuje prehranu jedne vrste u prirodnom okruženju i uglavnom je ograničena dostupnošću hrane. Prirodna prehrana može se procijeniti različitim metodama, ali najčešće se koristi analiza prehrane temeljena na DNA. *Zodarion rubidum* (Simon 1914) je, za razliku od većine vrsta paukova, opisan kao stenofagni predator specijaliziran za prehranu baziranu na mravima. Ipak, njegova prirodna prehrana uglavnom je nepoznata i ne postoje saznanja o prehrambenim preferencijama tijekom ontogenetskog razvoja. Cilj ovog istraživanja je utvrditi postoji li promjena u prehrambenim preferencijama tijekom ontogeneze Z. rubiduma i postoji li razlika u prehrani između spolova. Osim toga, proveden je pokus kontroliranog hranjena kako bi se odredio potencijal metode analize prehrane temeljene na DNA i kako bi se procijenilo vrijeme u kojem se DNA plijena može detektirati unutar predatora.

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Mentor: prof. dr. sc. Stanislav Pekár

Ocjenitelji:

1) izv. prof. dr. sc. Damjan Franjević

2) doc. dr. sc. Sofia Ana Blažević

3) doc. dr. sc. Tomislav Ivanković

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List of abbreviations

ANOVA – analysis of variance

BOLDsystems – Barcode of Life Data Systems

COI – cytochrome c oxidase subunit I

DNA – deoxyribonucleicacid

MID – molecular identifiers

MOTU – molecular operational taxonomic unit

NCBI – National Center for Biotechnology Information

NGS – next generation sequencing

PCR – polymerase chain reaction

Table of contents

1. Introduction

1.1 Realized trophic niche of predators

In Ecology, a niche describes the tolerance of a species to a range of environmental conditions and its need for various resources while interacting with other species (Begon et al., 2006). Each species has a different number of conditional ranges forming a multidimensional hypervolume and this ranges usually vary according to biotic and abiotic factors. The trophic niche is part of the total species's niche space, an aspect established over its evolutionary history. As part of the environmental niche, the trophic niche is determined by numerous factors as well, such as environmental conditions, food resources (size of food, quality of food), and interactions with other species (Soberon and Peterson, 2005).

Predation is a type of feeding behavior where the predator kills and eats another organism (Lafferty and Kuris, 2002). Pekár et al. (2017) described the trophic niche of predators via a simple Venn diagram (Figure 1) inspired by the environmental niche concept proposed by Hutchinson (1957). They show three distinct dimensions of the trophic niche: prey type (P_T), prey size (P_s), and prey availability (P_A). These dimensions depend on each other and consequently overlap. The fundamental trophic niche describes the area where the prey type and prey size overlap. Overlap of all three dimensions is defined as the realized trophic niche. The realized trophic niche area is smaller than the fundamental trophic niche as it depends on the spatial and temporal co-existence of predator and prey. Other factors that can affect the realized trophic niche are usually inter- and intraspecific foraging competition between predators, population dynamics, and types of mutualism and enemy predators.

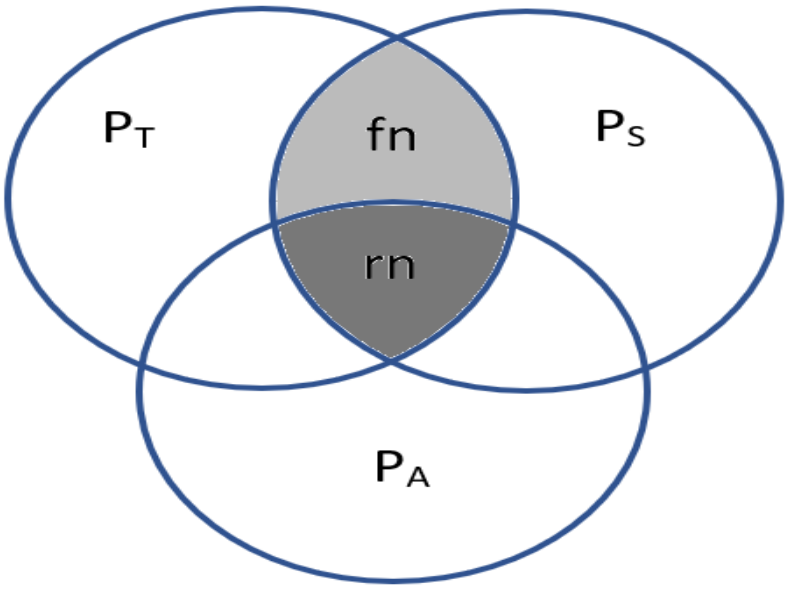

Figure 1. Venn diagram of three niche dimensions, prey type (P_t), prey size (P_S), and prey availability (P_A), yielding fundamental (fn) and realized (rn) trophic niches (taken from Pekár et. al., 2017).

1.2 Methods of diet assessment

The realized trophic niche describes the natural diet of a species and is mostly limited by the prey availability. In order to fully understand the realized trophic niche of a species, it is necessary to analyse the natural diets of populations in their prevalent areas during their whole period of activity. This minimizes bias but is often hard to obtain. Before using any methods to assess it, it is required to have some minimum

knowledge of the predator's phenology and habitat preferences to increase the effectiveness of the method. Realized trophic niches were investigated through the last few decades and several different approaches were presented: direct in-situ observation, prey-carcasses analysis, and molecular protein and DNA-based prey identification analysis (Pekár et al., 2017; King et al., 2008; Pompanon et al., 2012).

1.2.1 Direct in-situ observation

Direct in-situ observation is one of the most frequently used methods, performed by observing predators in their natural habitat. Initially, observation was performed directly by the observer, but nowadays video surveillance is used more often (Sunderland, 1988; Xue & Henderson, 2006). In the last few years, species in their natural habitat can even be observed over social media and internet servers (Skejo, 2017), whereby evidence of prey capture can be provided (Nyffeler & Knörnschild, 2013). While this method provided useful information in some cases, it was mostly limited to species which were relatively bigger in size, diurnally active, and weren't elusive. Furthermore, such research is labor intensive and the probability to find the predator actively hunting is rather low. Besides, the identification of the prey depends heavily upon the experience of the person identifying. An important remark is that while observing, the predator and prey shouldn't be disturbed in the natural habitat. All these disadvantages have led scientists to use other methods.

1.2.2 Analysis of prey carcasses and digested fragments

This method is mostly appropriate for bulk feeders or sedentary fluid feeders where the prey can still be determined by its remains. Bulk feeders can be investigated post mortem by analyzing their gut content as the gut has semi-digested prey remains (Holechek et al., 1982; Moreby, 1988; Sunderland et al. 2005). Some sedentary species, such as web building spiders, leave the prey carcasses on the web, inside or outside their retreat (Salomon, 2011; Garcia et al., 2014). Consequently, prey can simply be collected and identified to species level, ontogenetic stage and size. A substantial difference between this method and the direct in-situ observation is that the predator is not disturbed during hunting and feeding, although the method is quite invasive as it is performed post mortem and it can destroy the retreat of the individual. However, it is still labor intensive and depends on the stage of prey decomposition which leads to variable levels of identification. It can even be misleading as it won't show us if the predator consumed any soft-bodies prey (Symondson, 2002).

1.2.3 Molecular diet analysis

To overcome disadvantages of previous methods, ecologists have innovatively adapted a variety of technologies from the field of molecular biology. Initially, techniques based upon prey-specific antibodies were developed to analyze gut content and were used as target markers for specific prey species (Boreham & Ohiagu, 1978; Greenstone, 1996). Although the technique was precise, it was too expensive in terms of time and facilities and required skills in producing monoclonal antibodies. Later there were even attempts with techniques such as protein electrophoresis (Salomon et al. 1996), spectroscopy (Rothman et al., 2009), and isotopic signatures (Traugott et al., 2007). None of these methods were excessively reliable due to not distinguishing prey types good enough or being a subject of many variables.

Facilities working with DNA-based assays are, however, widespread. Sequences of conserved genome regions, which are common to a wide range of organisms, are available for free in databases. These conserved regions can be easily amplified without any knowledge of the organism's genome (Simon et al., 1994). Phylogenetic research is additionally increasing information about each specific species and

develops prey specific primers (Ortiz & Francke, 2017). All these factors enabled the possibility to investigate the natural diet through DNA-based assays. The only issue that delayed the introduction of DNA-based diet analysis is that scientist assumed that DNA breaks down to quickly during digestion (Zaidi et al., 1999). After establishing that it is necessary to amplify smaller fragments of conserved regions, as larger sequences break down more rapidly (Augusti et al., 1999), DNA-based techniques became a popular tool to investigate the natural diet. After the outset of mitochondrial primers, used to amplify genes already existing in hundreds of copies per each cell, the choice has been strengthened even more (Hoy, 1994).

1.2.4 DNA – based diet analysis

The experimental procedure of DNA-based diet studies consists of 5 steps: sampling, DNA extraction, prey-DNA PCR amplification, high-throughput sequencing, and data analysis (Clare, 2014; Pompanon et al., 2011). For the sampling step it is necessary to obtain information about predator's biology and phenology as it is defined by the type of sample. So far, the diet analysis was done on the whole gut (invertebrates), faeces (vertebrates) and even spider webs (Xu et al., 2015). DNA extraction is usually done with extraction kits for tissue samples and there is no need for additional steps. The amplification step depends on the relatedness of the predator and prey and on the previous knowledge of the predator's diet. Consequently, primers need to be constructed in such manner that they amplify all prey-DNA possible and that the amplification of predator's DNA can be avoided. Depending on the number of samples and the length of amplified sequences, an optimal high-throughput sequencing method is selected. Finally, the output data needs to be processed and filtered with algorithms, and checked through barcode databases to identify the prey.

DNA-based diet analysis is suitable to investigate diet of animals which were neglected while using previous methods. This refers mostly to cryptic species and fluid feeders. Besides, this method is less labor intensive and the observed predator (consumer) is not disturbed during hunting. Still, there are some limitations to such methods. Firstly, the useful range of barcode primers is limited to the number of species that can be retained from DNA amplification as well as by the taxonomic resolution capacity as only some barcode regions will identify taxa to species level. Secondly, prey-DNA degradation limits the period of prey-detection in diet samples and can give misleading information about the diet if the samples are taken only during one seasonal period. Finally, choosing the optimal NGS (Next Generation Sequencing) platform and performing data analysis requires skilled researchers that will obtain all informative data in the shortest time and simultaneously avoid erroneous reads. While this limit can constrain the interpretation of diet-analysis results, DNA-based diet analysis is, nevertheless, recognized as the most accurate approach to assess the natural diet and will lead ecologists to a better understanding of trophic relationships.

1.3 NGS methods used in DNA-based diet studies

Knowledge of DNA sequences has become essential in numerous biological fields. One such research field, where knowing the DNA sequence is particularly useful, is metagenomics. Metagenomics investigates the species diversity through genetic material in environmental samples. Metagenomics studies were greatly enhanced only after the introduction of high-throughput sequencing methods and subsequently, a new method named metabarcoding was developed (Valentini et al., 2009). Today, recovering many taxonomically informative sequences from environmental samples has become very fast, accurate and inexpensive. In addition, reference databases expanded massively over the last few years consequently making the metabarcoding approach even more promising. Such a development prompted scientists to investigate the diet of animals using their gut or feces rather than a usual environmental sample as well as to exploit the opportunities of next generation sequencing in full measure.

New high-throughput sequencing methods are referred to as 2nd or next-generation sequencing methods whilst automated Sanger method is considered being the "first generation". NGS methods parallelize the sequencing process, producing millions of sequence reads simultaneously at lower cost compared to the Sanger method which produces only few sequence reads in a run.

There are three main high-throughput sequencing platforms used for diet analysis and all use the sequence by synthesis approach (Pompanon et al., 2012; Glenn, 2011; Buermans & Dunnen, 2014). The first diet studies applying NGS have used the 454/Roche platform as it was the first commercially available platform producing longer read lengths. 454 uses beads on which the DNA of interest is amplified via emulsion PCR. These beads are later placed in picoliter-sized wells and pyrosequenced using luciferase for detection of added nucleotides. Another frequently used platform is Illumina, based on reversible dye-terminator technology and artificially-engineered polymerases. DNA of interest is captured on glass surface and amplified via bridge PCR forming clusters of identical sequences. These clusters are then sequenced through addition of nucleotides with dye labelled reversible terminators one at the time. The third frequently used sequencing platform is Ion Torrent. Ion Torrent's strategy is similar to 454 at the preparing stage, as it uses emulsion PCR to amplify DNA of interest, but it differs in the sequencing stage. It is equipped with a semiconductor-based detection system that detects hydrogen ions released during polymerization of DNA.

As the three mentioned sequencing platforms differ in sequencing strategies, they differ in cost, run time, error rates and data processing challenges as well (Metzker, 2010; Glenn, 2011). When comparing the cost, it should be taken into account that there are differences between the cost per Mb and purchasing cost. 454 and Ion Torrent produce longer sequencing reads (up to 400 bp), therefore their cost per Mb of data is higher compared to Illumina (over 100 bp). Cost per sample is not a significant factor as all samples can be sequenced together in one sample due to the use of sample identification tags. Regarding the minimal error rate, Illumina is the leading company as it has lower error rates (0.1%) than the other two (1%). However, the run time of Illumina is the longest (few days) in comparison to the other two (2-10 hours). Data processing challenges vary between the platforms as well. Ion Torrent and 454 give relatively little sequence output (≤5 Gb of sequence data) compared to Illumina which produces short reads in large amounts. Consequently, smaller sequence outputs take much less time to process and require a lower number of computational resources.

Overall, each platform has significant advantages and disadvantages during preparation, sequencing and data processing. It is important to estimate the costs with the gains of the results for the specific task. Sometimes it is significantly cheaper to just outsource the samples to a sequencing company rather than to purchase or use your own sequencing machine due to reagent cost. Sometimes it is easier to let a company process your data than to do it yourself. Fortunately, sequencing platforms and softwares are constantly improving and the costs are rapidly decreasing. The 3rd generation sequencing platforms, that sequence DNA molecules directly without preparation and amplification steps but still have higher error rates than 1st and 2nd generation, are already developed. These platforms could improve in the future and lower the time and cost of sequencing to such a level that the only problem during sequencing would be to choose the best sequencing platform that will give the best results for each specific research.

1.4 Biology, phenology and ecology of *Zodarion rubidum* (Simon, 1914)

Spiders are among the most diverse groups of animals on earth. All of them are predators when showing their feeding behavior. This makes them exceptional compared to the other diverse groups (e.g. insects) which additionally developed other dietary strategies such as phytophagy and parasitism

(Coddington and Levi, 1991). Needless to say, all trophic strategies of predation, from euryphagy to even monophagy, exist within spiders (Nentwing, 1987; Petraková et al., 2015, Pekár & Toft 2015). It is believed that most of the species are generalist, able to hunt and consume a wide variety of prey. Fewer species are stenophagous, specialized on a single prey group. Their specialized diet consists either of spiders, flies, moths or termites (Pekar, 2004.). However, most of the stenophagous spiders are ant-eaters belonging to families Salticidae (Jackson & Pollard, 1996), Theridiidae (Porter & Eastmond, 1982), Gnaphosidae (Heller, 1974) and Zodariidae (Simon, 1864). Each family developed a specific foraging tactic to overcome the ant's defense system in addition to adaptations for obtaining all required nutrients from a single prey type.

Zodarion rubidum (Figure 2) belongs to the Zodariidae family that includes over 500 other spider species (World Spider Catalog 2018). A few decades ago *Z. rubidum* exclusively occurred in the western Mediterranean area, yet recently it is present in entire central Europe and even northern Europe (Kurka, 1981; Pekár, 2002). Several studies have been conducted on the biology, phenology and ecology of *Z. rubidum*. Like the majority of zodariid spiders, *Z. rubidum* does not spin webs but actively moves across open ground during evening and night time (Couvreur, 1980). During day-time (and over winter) it hides in igloo-shaped retreats, made of sand and small stone particles glued together by webbing, positioned on the lower surface of stones. These retreats are often located near ant nests (Pekár & Král, 2001). The spiders are active from April to September, and adult specimens can be found from May to July. Their ontogenetic development includes four to five instar stages and the adult stage when sexual maturity is reached. As all arthropods, they moult (ecdysis) between stages, gradually grow in size and develop sexual organs.

Figure 2. Zodarion rubidum (Simon, 1914), subadult male (photo: O. Machač). Image taken from website: http://www.pavouci-cz.eu/Pavouci.php?str=Zodarion_rubidum.

Z. rubidum is described as a stenophagous predator specialized in eating ants. In previous studies they were only able to subdue ant species (Pekár, 2004). Pekár and Toft (2009) showed that such specialized ant-eating spiders couldn't survive on a diet optimal for euryphagous predators. When offered fruit flies, they rejected the prey and most of them died while molting. Even severe hunger didn't motivate them to consume fruit flies. However, when they are on an ant-based diet, they accept the prey readily and develop normally. It has been shown that *Z. rubidum* spiders prefer to consume different body parts of a single ant

through one feeding (Pekár et al., 2010). The ant's forepart (head and thorax) contains a higher amount of protein, and the gaster (abdomen) contains more lipids. This way, they balance their nutritional requirements while feeding on one exclusive prey.

During the hunting process, *Z. rubidum* uses aggressive mimicry behavior. Because they hunt near ant nests, they mimic ant behavior, using their first pair of legs as antenna and the last three pairs of legs for movement. They follow the typical zigzag movement style, waving their first pair of legs. The hunting behavior involves three phases: attacking, waiting at a safe distance for the prey to cease movement, and handling the prey afterwards (Pekár, 2004). During the attack, the ant is killed by injecting venom on its rear side via the spider's fangs. While walking with a captured prey in their chelicerae, *Zodarion* mimic the ant's behavior by giving the impression of an ant carrying another dead ant. When an ant approaches the spider carrying a dead ant, the spider usually taps the antennae of the curious ant and shields behind the dead ant. Such behavior is often enough to deceive the ant and to escape with the prey unharmed (Pekár & Král, 2002). In addition, there are some indications that the spiders imitate the ants structurally, with their size and color appearance as well as with the structure of their limbs. It is believed that the structural mimicry is more a type of Batesian mimicry where the spider imitates a distasteful and harmful prey to protect himself from its own predators.

Even though the diet of *Zodarion* spiders consists exclusively of ants, there are preferences in size and type of ants, both interspecific and intraspecific. Feeding experiments with *Z. rubidum* showed that they most frequently feed on *Tetramorium* ants that belong to the Myrmicinae subfamily. Except for the above, feeding on *Formica* and *Lasius* ants (both within the Formicinae subfamily) was recorded at high frequency, as well (Pekar, 2004). There are indications that *Z. rubidum* reacts only towards chemical cues coming from Dufour's gland of *Formica* and *Lasius* ants (Cárdenas et al., 2012). Based on these indications, it is not clear why they prefer *Tetramorium* ants over *Lasius* and *Formica* ants. It is believed that this is an adaptation to the abudance of ant-prey in their locality (Pekár et al., 2012) as they have similar prey preferences as the other *Zodarion* species from Central Europe.

Looking from the intraspecific perspective, there is evidence of prey preference shift depending on the ontogenetic stage and sex of *Z. rubidum*. They change their body size greatly during ontogenetic development and it is only logical to shift to another, bigger ant species to gain as much as possible from a single hunt. A feeding experiment showed that early instars of *Z. rubidum* prefer smaller ants in comparison to adult spiders that mostly feed on larger ants (Pekár, 2004). Both the juveniles and adults refuse to attack the largest ant species, such as *Camponotus ligniperda* (Latreille, 1802) and larger forms of *Formica truncorum* (Fabricius, 1804). Nevertheless, they attack ants the same size as their own, sometimes even larger. A study conducted on a related Zodarion species showed that their venom is very potent already at the juvenile stage (Pekár et al., 2014). Another study showed there is no difference in the venom composition between ontogenetic stages (Cárdenas et al., 2014). This suggests that the prey-shift, if there is any, may solely depend on their size and the nutritional gain.

The sex is another factor that contributes to specific prey preferences. In Zodarion spiders the sexual size dimorphism is greatly female biased in chelicerae and body size (Pekár et al. 2011). Male chelicerae are the size of higher juvenile stages and their capture efficiency and paralysis latency are lower compared to females and juveniles. Consequently, adult females tend to subdue larger prey and show a higher capture efficiency than males. It is believed that this is due to higher energy requirements in females, as they strive for higher fecundity and a larger number of offspring. To the contrary, adult males tend to reduce their hunting and feeding time and focus more on mate-searching (Martišová et al., 2009). They are known to steal dead prey from female and juvenile specimens, if they get the opportunity.

1.5 Aims of research

The aim of this study is to investigate the realized trophic niche of *Zodarion rubidum* and observe differences between the diet of ontogenetic stages and sexes. All that was mentioned in the introduction points to Z. rubidum being a stenophagous predator specialized in foraging ants. However, detailed knowledge about their eating habits through ontogenetic development are still unknown. In our first hypothesis, we propose that there is a shift in prey-type during ontogenetic development of *Z. rubidum*. We assume that as spiders grow, they tend to subdue larger ants. In our second hypothesis, we expect differences between the diet of adult males and females, either in the type or size of the prey. We investigated the trophic specialization level as well, by calculating the Levin's index. The two hypotheses were tested and Levin's index was calculated after results from gut content analysis were obtained.

In addition, a feeding trial was performed to assess the potentials of gut content analysis. We investigated whether it is possible to acquire quantitative results from gut content analysis or just qualitative results (presence or absence), as mentioned in previous research (Deagle et al., 2013). Furthermore, through the feeding trial, we measured the period of prey-DNA detectability in the gut of *Z. rubidum* to estimate the amount of information we can obtain from one individual.

2. Materials and methods

2.1 Sampling

Eighty-five specimens of *Z. rubidum* (Simon, 1914) were sampled at the quarry in Hády (Brno, The Czech Republic). Sampling dates were: 3rd of May, 1st of June, 6th of June, 2nd of August and 17th of October of year 2017. *Z. rubidum* is a nocturnally-active spider. During the day, they can frequently be found in igloo-shaped retreats constructed from sand grains attached to the lower surface of a stone. Accordingly, sampling was performed during daylight by turning over stones found at the site and inspecting retreats. Immediately after collecting, spiders were placed in 99.8 % ethanol.

At the same time, all ant species found around the retreats of *Z. rubidum* at the quarry were sampled and preserved in 99.8 %ethanol. Two specimens of each colony were collected.

2.2 Determination to species level and size measuring

Adult specimens were determined to species using identification key for Spiders of Europe (https://araneae.nmbe.ch/). As the size of prosoma (cephalothorax) was the only relevant measure comparable with the ontogenetic stage of the spider, its size was measured using the stereo microscope LEICA EZ5. The size of legs and opisthosoma (abdomen) differed for each individual, even if they were at the same ontogenetic stage with the same length of prosoma (Anderson, 1974). After measuring the size, specimens were categorized into the appropriate ontogenetic stage according to earlier research of *Z. rubidum* (Pekár, 2000). Individual's sex was determined by signs of copulatory organs. They were categorized into males if the palpal bulbs were visible on the pedipalps, or into females if there were any visible patterns of the epigynum.

Likewise, every collected ant was determined to species using the identification keys (Atanassov & Dluskij, 1992; Seifert, 2007; Antwiki 2018). The size of the whole individual, from head to the end of abdomen, was measured using LEICA EZ5 stereo microscope.

2.3 DNA isolation

Collected spiders were used for the gut content analysis and, therefore, only DNA from their opisthosoma was extracted (King et al., 2008; Hernández et al., 2018). This way the amount of the predators DNA (*Z. rubidum*) was minimized and amplification of prey DNA was facilitated. In addition, the collected ants and legs of *Z. rubidum* were used for assembling a reference DNA barcoding library in case the sequences of gut content analysis were not found in the appropriate sequence database.

The extraction of the *Z. rubidum* genomic DNA was performed using the DNeasy Blood & Tissue Kit (Qiagen) with an adjusted protocol. The opisthosomas were separated from the body by a sterile needle, inserted into 180 µL of ATL (tissue lysis) buffer, and squashed by a sterile pestle. After adding 20 µL of proteinase K, samples were incubated over the night at 56 °C. The next day, 200 µL of AL (additional lysis) buffer with 200 µL of pure ethanol was added to the incubated sample. In case of gut material precipitation after adding pure ethanol, the solution was heated to 56 °C and mixed afterwards. Following steps were conducted according to the kit protocol. At the final step first 50 µL, and after a round of centrifuging, another 30 µL of elution buffer was added to elute as much DNA as possible from the column. The extracted DNA was stored at -20 °C.

The same protocol was used to extract ant DNA. Ant's leg was separated from body with a sterile needle and added to the lysis buffer without squashing it. The same was done with only the tip of the leg

(tarsus, metatarsus, and tibia) of a *Z. rubidum* spider because the leg segments closer to the body contain gut diverticula (Ruppert& Barnes, 1994; Macías-Hernández et al., 2018). This way, the possibility of contaminating the reference sequence of *Z. rubidum* with prey sequences was minimized.

2.4 Assembling a reference DNA barcoding library

Ant and *Z. rubidum* DNA extracted from the leg was used to generate a reference DNA barcoding library. The cytochrome c oxidase subunit I (COI) gene fragment was amplified via PCR following procedure outlined in Table 1. Primers LCO (5'-GGTCAACAAATCATAAAGATATTGG) and HCO (5'-TAAACTTCAGGGTGACCAAAAAATCA) were used with a final concentration of 10 µM (Folmer et al., 1994). HiFi polymerase (PCR Biosystems) was used to amplify the fragments with high fidelity under the following conditions: initial denaturation at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 50.5 °C for 30s as an annealing temperature, 72 °C for 75 s; and a final extension at 72 °C for 5 min.

Table 1. Composition of one PCR reaction mixture

Composition	Volume (µL)
HiFi Polymerase	0.2
HiFi buffer 5X	4
LCO primer – forward (10 µM)	0.7
HCO primer - reverse (10 µM)	0.7
Ultraclean water	9.4
Genomic DNA	5
Final volume	20

The quality of the PCR product was verified through agarose gel electrophoresis. Agarose was mixed with 0,5XTBE (Tris-borate-EDTA) buffer to form a 2% gel. GoodView (EColi) nucleic acid stain was used for detection of DNA. After verification, amplified products were purified of their unused primers and nucleotides using the QIAquick PCR Purification Kit (Qiagen) following the protocol.

Table 2. Composition of one PCR reaction mixture for Sanger's sequencing method via capillary electrophoresis. PCR was performed with BigDye Terminator V 3.1. Cycle Sequencing Kit (Biosistems).

Composition	Volume (µL)
Forward or reverse primer	0.4
BigDye terminator V 3.1.	1
Buffer BigDye	1
Ultraclean Water	6.6
Amplified DNA	1
Final volume	10

To properly perform Sanger's sequencing method via capillary electrophoresis, the amplified fragments need to be amplified one more time, but this time with fluorescently labeled dideoxynucleotides (BigDye terminator V 3.1. Cycle Sequencing Kit) added in a mixture with normal nucleotides. Two separate PCR reactions were made, separating the forward and reverse primer to improve sequencing results (Table 2). LCO and HCO primers have been used in a final concentration of 10 µM. PCR was performed under the following conditions: initial denaturation at 96 °C for 1 min; 30 cycles of 96 °C for 30s, 50 °C for 20 s as an annealing temperature, and 60 °C for 4 min.

After the amplification with dideoxynucleotides, the PCR product was purified with BigDyeXTerminator Purification Kit (Applied Biosystems). For each reaction, forward and reverse, 22 μL of SAM solution and 5μL of XTerminatior solution were mixed and added to a 96-well plate with 5 μL of each PCR product. The 96-well plate was mixed 30 minutes on 22 °C and centrifuged at 1000 g for 2 min. Into each well with the purified PCR product 32.5 μL of ultraclean water was added and 64.5μL into the empty wells in the row. The plate was again centrifuged for 2 min at 1000 g and the plate was covered with a septum cover. Sequencing was carried out on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

2.5 Feeding experiment

False positive results occur often while using molecular methods (such as PCR) to analyze the gut content, mostly due to contamination or secondary predation (Sheppard et al., 2005; Sunderland, 1988). The aim of dietary studies is to obtain quantitative results (such as prey preferences in the diet) along with prey diversity (Witzingerode et al., 1997; Amend et al., 2010). In addition, the time limit of possible DNA detection from the gut content varies and is therefore unknown. To understand how useful the results of gut content analysis are and how far into the past can the consumed prey be detected, a controlled feeding experiment was carried out.

Thirty adult and subadult female specimens of *Z. rubidum* were sampled in April 2018 in Hády (Brno, Czech Republic). Only female specimens were used because adult males often cease hunting (Pekár et. al., 2011). The specimens were placed singly into tubes with punctured lids and were left at room temperature for 7 days to starve (Harper et al., 2006). The starvation period of 7 days was needed to stimulate the spider's hunting activity and to make the whole feeding experiment during 3 weeks from the date of collecting them in nature. This way we may find whether the prey DNA from its natural habitat can be detected 3 weeks after the start of feeding trials.

Then spiders were placed individually in small petri dishes (diameter 5 cm). A small piece of filter paper was added to the bottom of each petri dish and a drop of water was added to keep the humidity. In addition, the filter paper was introduced to avoid drowning of spiders in water droplets. The spiders were left in the petri dishes for few hours, before prey was introduced, to adjust to the new environment. The experiment was conducted at room temperature.

For the feeding experiment, specimens of three ant species were collected from the grassland around University Campus Bohunice (Brno, The Czech Republic). Ants of each species were placed together into a plastic bottle (2 dL) pierced with small holes containing a wet paper to keep humidity. The plastic bottles were placed in the refrigerator at 10 °C to reduce the metabolism of ants and to keep them alive. The three ant species used for the feeding experiment were *Lasius emarginatus* (Olivier, 1792), *Lasius niger* (Linnaeus, 1758) and *Tetramorium caespitum* (Linnaeus, 1758).

Spiders were split into seven groups, with 4 specimens in each group. The feeding schedule was carried out following Table 3. The groups 1, 2 and 3 represent spiders that were fed each week with a different ant species in a different order. The other three groups represent spiders fed with only one ant species through the whole experiment. Groups from one to six served as positive controls. Finally, the starving group consists of spiders who were left starving for three weeks. The starving group served as a negative-control group to observe if any prey DNA is still detectible after three weeks.

Table 3. Feeding schedule for the feeding experiment.

Group number	Ant species offered		
	1st week	2nd week	3rd week
1	*L. emarginatus*	*L. niger*	*T. caespitum*
2	*L. niger*	*T. caespitum*	*L. emarginatus*
3	*T. caespitum*	*L. emarginatus*	*L. niger*
4	*L. emarginatus*	*L. emarginatus*	*L. emarginatus*
5	*L. niger*	*L. niger*	*L. niger*
6	*T. caespitum*	*T. caespitum*	*T. caespitum*
7	-	-	-

The feeding was conducted during evening hours as *Z. rubidum* is nocturnally active. The ant's mandibles were cut off with small scissors before releasing them in the dish that the ant doesn't counterattack spiders. The ant was left in the petri dish with the spider for 4 hours. The feeding was monitored every 15 minutes during the 4 hours to check if the spider managed to attack the ant and started feeding. If the feeding did not happen, it was repeated the next day with a new ant specimen of the same species. After the feeding, the ant was removed from the petri dish and the spider was left for one weak to starve untill the next feeding. The filter paper in the petri dish was moisturized every 3 days and the spiders were inspected each day for mortality. Some of the feedings needed to be repeated because the spider died during experiment while molting.

Thirty minutes after the last feeding, the spiders were placed into tubes containing 99.8 % ethanol. The DNA extraction was performed the same way as outlined above by extracting DNA out of their opisthosoma using DNeasy Blood & Tissue Kit (Qiagen). Both, the spiders collected from the field, and the spiders from the feeding experiment were used in the gut content analysis method.

2.6 Gut content analysis

2.6.1 Preparation for next generation sequencing

DNA, extracted from the abdomen, was used for the gut content analysis. 1 µL of sample's extracted DNA was prepared for concentration measuring with Qubit dsDNA HS Assay Kit (Invitrogen). The concentration was measured with Qubit Fluorometer (Invitrogen). The extracted DNA was diluted to below 15 µg/mL, if needed.

The gut content analysis was carried out amplifying the COI region of the prey's DNA (186 bp long). PCR reactions were made with Multiplex PCR kit (Qiagen) according to Table 4. Universal primers MiteMiniBarF (5'-CATGCNTTYRTNATRATTTTTTTYATAG) and MiteMiniBarRmodif2 (5'-GGRTAAACWGTTCAHCCWGTHCC) were used to identify all possible arthropod prey (de Groot & al., 2016). These primers were tagged with MID identifiers (10-base-long barcoding sequences) attached to the 5' site of the primer. DNA from each spider was amplified using primers with a unique combination of the MID identifiers (Eurofins Scientific), one on the forward primer and the other on the reverse primer (Table 5.). This allowed us to assign DNA prey reads to their predator. As the DNA was extracted out of the spider's abdomen, a huge amount of the predator's DNA was still available. To amplify only the prey DNA, in addition to universal primers, a specific blocking primer BlkZodarionMiteMiniBar (5'

AGCCCCTACTCCTATCTC-C3spacer) was added to the PCR solution. The blocking primer interfered with the annealing of the universal forward primer to the predator's DNA and thus prevented the amplification of predator's COI region. The final concentration of universal primers was 10 µM, and that of the blocking primer was 20 µM. PCR amplification was performed under the following conditions: initial denaturation at 95 °C for 15 minutes; 35 cycles of 94 °C for 30s, 50 °C for 90 s as an annealing temperature, 72 °C for 90 s; and final extension at 72 °C for 10 min.

Table 4. Composition of one PCR reaction mixture

Composition	Volume (µL)
Multiplex master mix	10.6
Forward primer (10 µM)	0.8
Reverse primer (10 µM)	0.8
Q buffer	1.8
Ultraclean water	3
Blocking primer (20 µM)	3
Gut DNA (< 15µg/mL)	5
Final Volume	25

The quality of the PCR product was verified through agarose gel electrophoresis. 0,5x TBE (Tris-borate-EDTA) buffer was mixed with agarose to form a 2% gel. GoodView (EColi) nucleic acid stain was used for detection of DNA. After verification, PCR products were purified of their unused primers and nucleotides using the QIAquick PCR Purification Kit (Qiagen) following the protocol.1 µL of each PCR product was prepared for concentration measuring with Qubit dsDNA HS Assay Kit (Invitrogen). Concentration was measured with Qubit Fluorometer (Invitrogen).

After measuring the concentration, all PCR products were transferred into the same sterile Eppendorf tube, in a volume containing 30 ng of DNA to ensure equimolar concentration of amplicons. The whole PCR product was added if it contained less than 30 ng of DNA. The Eppendorf tube, containing prey amplicon of each spider individual, was sent for Ion Torrent sequencing. Enrichment with emulsion PCR (emPCR) and sequencing on the Ion Torrent with an Ion 318 chip employing 400-base read length chemistry was provided by the Centre de Recerca en Agrigenòmica.

Before conducting the gut content analysis, universal primers were tested for efficiency on DNA of the ants collected in Hády. This way we confirmed that their COI region can be amplified. *Zodarion rubidum* DNA, isolated from the tip of the leg, was tested on the universal primers as well. In addition, *Z. rubidum* DNA was tested with a mix of universal primers and several different concentrations of blocking primer. Testing different concentrations of blocking primer helped us to find the lowest effective concentration against amplifying spider's COI region.

Table 5. List of primers with molecular identifiers (MID) used for assigning DNA reads to predator individuals. Abbreviations: Tm – melting temperature.

Primer name	MID sequence	Primer sequence	Tm (°C)
MMB-F1	ACATACGCGT	CATGCNTTYRTNATRATTTTTTTYATAG	63.2
MMB-F2	ACGCGATCGA	CATGCNTTYRTNATRATTTTTTTYATAG	65.6
MMB-F3	ATCAGACACG	CATGCNTTYRTNATRATTTTTTTYATAG	63.3
MMB-F4	CTCGCGTGTC	CATGCNTTYRTNATRATTTTTTTYATAG	65.9
MMB-F5	CGAGAGATAC	CATGCNTTYRTNATRATTTTTTTYATAG	61.4
MMB-F6	AGCACTGTAG	CATGCNTTYRTNATRATTTTTTTYATAG	61.7
MMB-F7	TCGTCGCTCG	CATGCNTTYRTNATRATTTTTTTYATAG	67.4
MMB-F8	ATATCGCGAG	CATGCNTTYRTNATRATTTTTTTYATAG	63.9
MMB-F9	AGACGCACTC	CATGCNTTYRTNATRATTTTTTTYATAG	64
MMB-F10	TCTCTATGCG	CATGCNTTYRTNATRATTTTTTTYATAG	63.4
MMB-R1	CGTGTCTCTA	GGRTAAACWGTTCAHCCWGTHCC	73.7
MMB-R2	TCTAGCGACT	GGRTAAACWGTTCAHCCWGTHCC	75.4
MMB-R3	TCGCACTAGT	GGRTAAACWGTTCAHCCWGTHCC	76.1
MMB-R4	TAGTGTAGAT	GGRTAAACWGTTCAHCCWGTHCC	70.9
MMB-R5	ATAGAGTACT	GGRTAAACWGTTCAHCCWGTHCC	65.9
MMB-R6	CGTCTAGTAC	GGRTAAACWGTTCAHCCWGTHCC	73.3
MMB-R7	TCTACGTAGC	GGRTAAACWGTTCAHCCWGTHCC	75
MMB-R8	TACGAGTATG	GGRTAAACWGTTCAHCCWGTHCC	73.5
MMB-R9	TACTCTCGTG	GGRTAAACWGTTCAHCCWGTHCC	75.3
MMB-R10	CATAGTAGTG	GGRTAAACWGTTCAHCCWGTHCC	71.8

2.6.2 Data processing

The sequencing output (FASTQ format), derived from Ion torrent sequencing, was processed using the Galaxy platform (https://usegalaxy.org/) and Bioedit 7.0.5.3 (Hall, 1999). Sequence reads were first checked for quality and filtered by quality. Sequences containing a quality cut-off value smaller than 20 in more than 10 % of bases were discarded. This way we maintained only sequences with higher quality. Filtered FASTQ file was converted into FASTA format for further processing.

Reads were split according to their molecular identifiers (MID) attached to the forward primers. The number of allowed mismatches and allowed nucleotide deletions on MID sequences was set to zero in the barcode splitter algorithm. It should be taken into consideration how sequencing was done on both strands of the prey amplicon. Because of that each of the ten forward MIDs was split by MIDs on reverse primers. This resulted in files corresponding to predator individuals containing only their prey sequences.

Split files were individually filtered by length. Sequences shorter than 200 bp were discarded because these were sequencing errors and did not contain sufficient data for barcode identification (Hajibabaei et al., 2007; Zeale et al., 2011). Afterwards, MIDs (10 base long) were removed from the forward and reverse side of each sequence. All sequences in each individual file were clustered the way that all identical sequences were merged into one and, as a result, the number of identical copies was obtained.

Merged sequences were later processed using the fastx-toolkit and the EMBOSS packages (Rice et al., 2000). Sequences were translated to amino acids according to the invertebrate mitochondrial genetic

code and those containing stop codons were excluded as they represented nonsensical sequences. Sequences with insertions and deletions causing reading frameshifts were also removed. The remaining sequences were clustered into MOTUs (molecular operational taxonomic units) using Swarm (Mahé et al.; 2014). The cut-off value of the algorithm was 6 bp or 3.2% of the sequence divergence.

Each MOTU was compared to the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using nucleotide blast, to the BOLD database (http://www.boldsystems.org/), and to the ant sequences obtained from Hády. When any sequence was more than 98 % similar to the voucher sequence obtained from the database, the prey was allocated to that species. When a sequence was more than 95 % similar to several species from a given genus, without a ≥ 98 % match to a specific species, it was assigned to that genus.

2.6.3 Statistical analysis

Because quantitative data on sequence-read counts do not correspond to the number of prey eaten (Deagle et al., 2013; Pompanon et al., 2012), the number of reads for each individual spider was transformed to qualitative data (presence/absence). Based on these data, the diet breadth of each ontogenetic group was estimated using the standardized Levin's index, B_A (Hurlbert, 1978):

$$B_a = \frac{\left(\frac{1}{\sum_{i=1}^{n} p_i^2}\right) - 1}{n - 1}$$

where p_i is the proportion of spider individuals positive for *i*-th genus of prey, and *n* is the total number of ant genera found in the gut of all specimens. The genera level was used due to the impossibility to identify the species level.

To test the hypothesis on the relationship between the spider's size and ant size, the prosoma length of each spider individual was compared to the average size of their consumed ant prey. The average ant size was calculated using the measured size of the collected ants found on Hády. The relationship between the two variables was measured using the Pearson correlation coefficient and the significance of the relation was assessed by t test (Schober et al., 2018). Average number of ant genera found in the gut was compared among groups using the Kruskal-Wallis ANOVA test (Pekár & Brabec, 2016). The Kruskal-Wallis ANOVA test is a non-parametric method for testing whether samples from different groups originate from the same distribution. As it is a non-parametric method, it does not assume a normal distribution of the sample, unlike the one-way analysis of variance.

3. Results

3.1 Sampling and determination results

Spider individuals were sorted into seven groups according to their size and sex (Table 6). Five of these groups were juvenile (J1 to J4) where J1 and J2 stages were placed together because being represented by low number of individuals. J3, J4 and adult stages were also characterized by sex. For each group 10 individuals were used for the gut content analysis. Exception is the J3 female group where only 8 were collected.

Table 6. List of *Z. rubidum* spiders categorized by ontogenetic stage and sex. The prosoma size range, together with the number of specimens used for gut content analysis is listed for each group.

Ontogenetic stage	Sex	Size of prosoma (mm)	Number of specimens
Juvenile (J1,J2)	unknown	0.72 - 0.8	10
Subadult (J3)	female	0.8 - 1.0	8
Subadult (J3)	male	0.8 - 1.0	10
Subadult (J4)	female	1.0 - 1.1	10
Subadult (J4)	male	1.0 - 1.1	10
Adult	female	1.1 - 1.4	10
Adult	male	1.0 - 1.3	10

Collected ant species were determined to species level and their size was measured (Table 7.). Seven ant species were determined belonging to three different subfamilies: Formicinae, Myrmicinae, and Dolichoderinae. Their size varied from 1.6 mm to 11.2 mm.

Table 7. List of ant species sampled at the quarry in Hády. Subfamily name and measured size range of the sampled ants are listed for each species.

Species	Subfamily	Size (mm)
Messor structor (Latreille, 1798)	Myrmicinae	5.0 - 9.0
Tetramorium caespitum (Linnaeus, 1758)	Myrmicinae	2.8 - 3.4
Plagiolepis taurica (Santchi, 1920)	Formicinae	1.6 - 2.5
Camponotus ligniperda (Latreille, 1820)	Formicinae	8.0 - 11.2
Lasius emarginatus (Olivier, 1792)	Formicinae	3.0 - 3.6
Lasius flavus (Fabricius, 1782)	Formicinae	2.0 - 3.0
Tapinoma erraticum (Latreille, 1798)	Dolichoderinae	1.9 - 3.5

3.2 Efficiency analysis of universal and blocking primers

Universal primers were tested for efficiency on DNA of the ants collected in Hády (Figure 3). *Zodarion rubidum* DNA, isolated from the tip of the leg, was tested on the universal primers as well. In addition, *Z. rubidum* DNA was tested with a mix of universal primers and several different concentrations of blocking primer.

The test confirmed that COI regions of all collected ant species can be amplified with universal primers MiteMiniBarF and MiteMiniBarRmodif2. Moreover, by using these primers only the COI region

was amplified. The optimal concentration was determined using different concentrations of blocking primer (0 µM, 10 µM, and 100 µM) together with universal primers and spider's DNA. When using 10 µM concentration of blocking primer, only a barely visible band appeared on the gel. Accordingly, concentration of 20 µM was used to block the predator's DNA amplification.

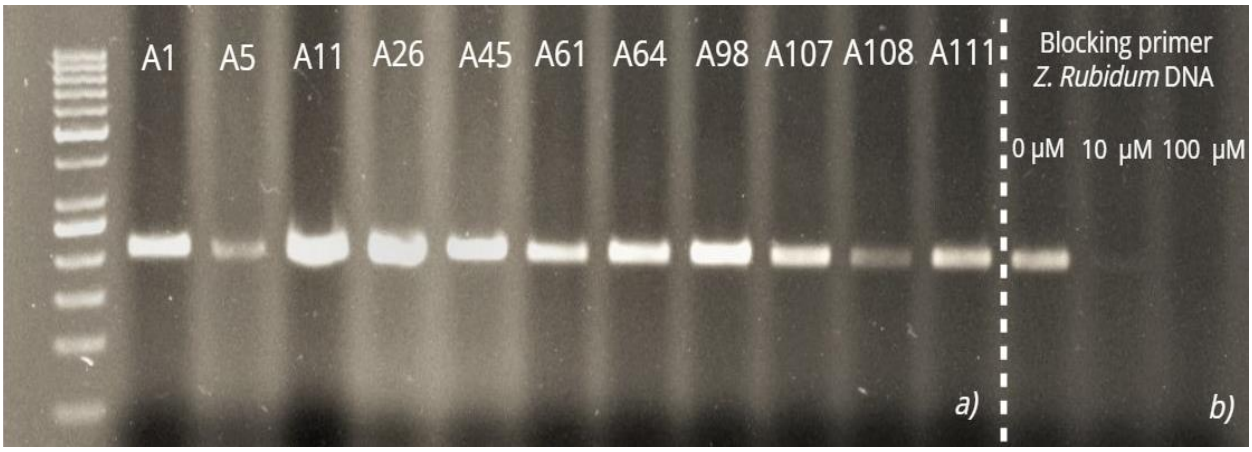

Figure 3. Testing the efficacy of universal primers to amplify COI gene fragment in prey's DNA and efficacy of blocking primer to block the *Z. rubidum's* COI gene fragment. **a)** COI gene fragment of all ant species can be amplified using universal primers. Abbreviation: A1-A111 present codes of different ant found in the field (Table 7). **b)** COI gene fragment of *Z. rubidum* can also be amplified with universal primers when there is no blocking primer present. The blocking primer is effective in concentrations above 10 µM.

3.3 Gut content analysis

3.3.1 Overview of sequence data

Ion torrent sequencing produced altogether 1 801 889 reads. The sequence-lenght range was from 25 bp to 522 bp with an average of 225 bp (Figure 4). Average phred-quality score per read was 30, which reveals that the probability that each base is sequenced incorrectly is 1/1000. In other words, the average sequencing accuracy per base is 99.9% (Figure 5). After filtering out the sequences with a quality value lower than 20, 1 490 582 reads remained (82.73%).

After assigning sequences to their specific forward and reverse molecular identifiers (MIDs), 1 287 765 reads remained (71.5%) with 202 817 sequences unassigned to MID's. The total number of asssigned sequences varied considerably between combinations of MID's used for each spider individual (mean = 12 531, range = 192 – 69 569). The starving group from the feeding experiment had the least number of reads per individual, while the J3 and J4 instars of subadult males had the largest number of reads.

Afterwards, MID-assigned seqeuences were filtered by lenght and assigned to species or genera using local blast in NCBI and BOLD databases. Of the starting 1 801 889 reads, only 344 715 (19.22%) were assigned to species or genera level and out of these sequences, 204 034 sequences belonged to species of ants (11.21%). Remaining sequences (140 468; 8%) belonged to the predator *Z. rubidum.*

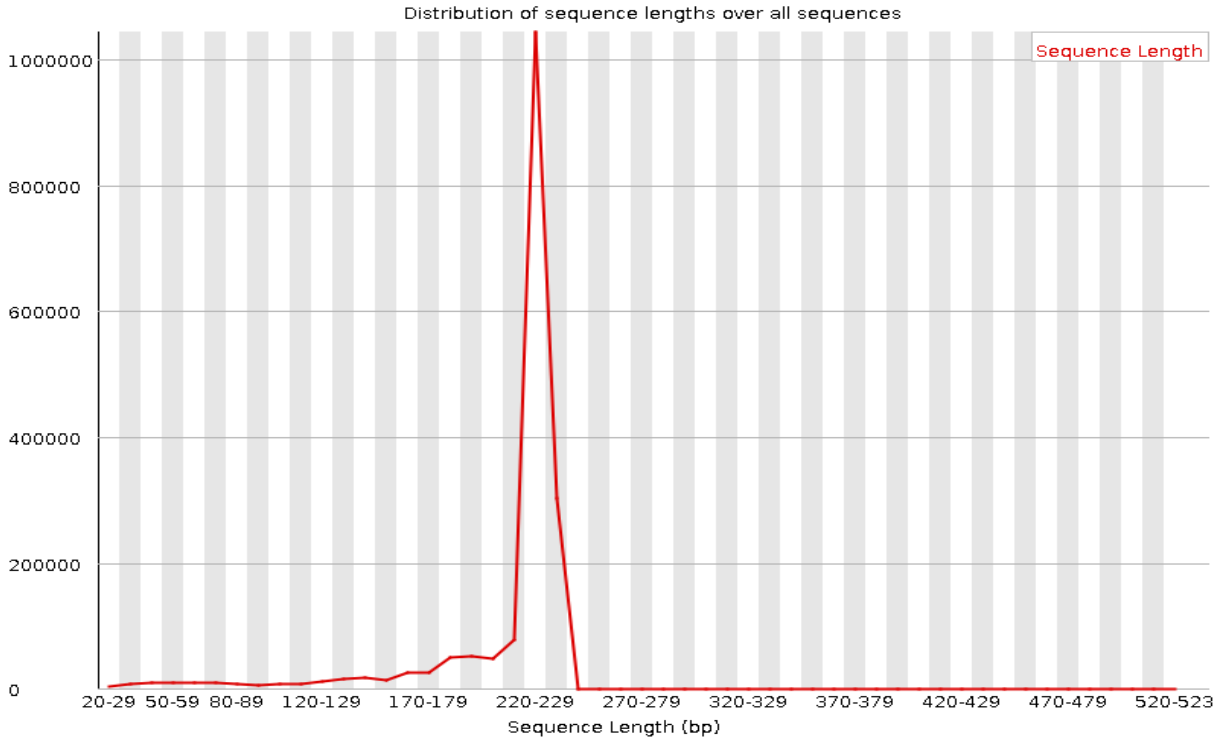

Figure 4. Sequence length distribution results obtained after data analysis of Ion Torrent sequencing output. The data was analysed over the Galaxy platform (usegalaxy.org).

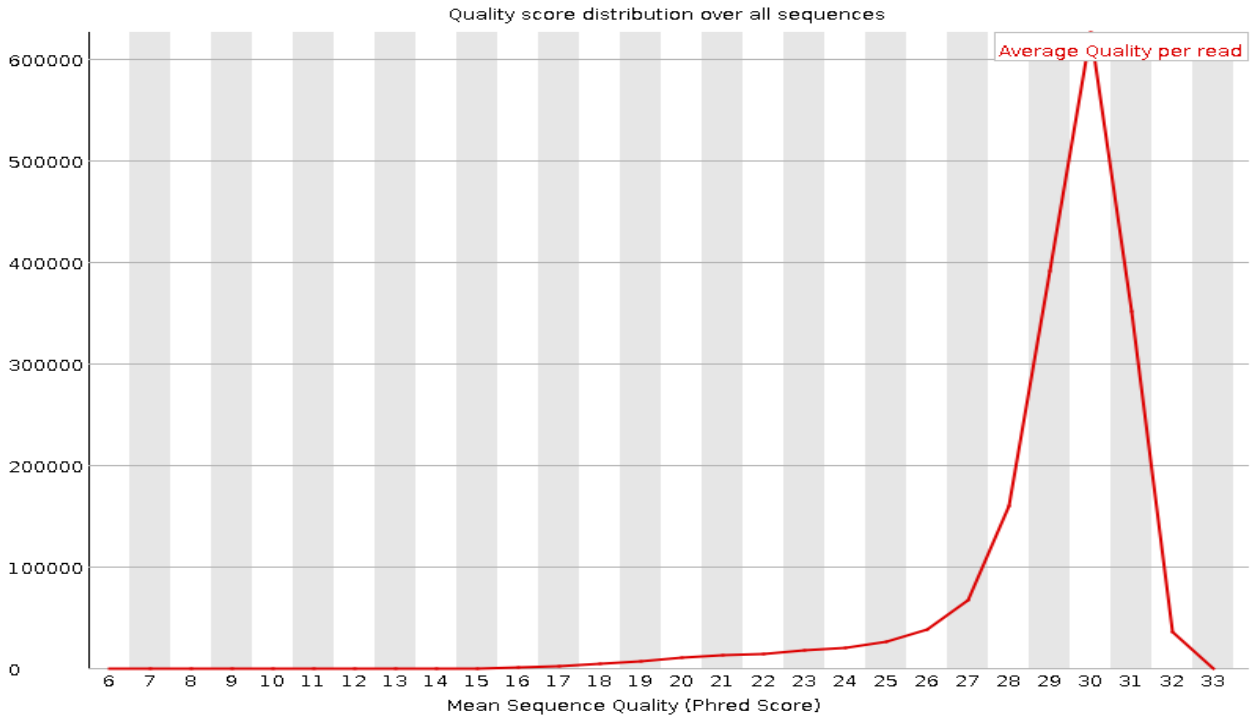

Figure 5. Quality score distribution results obtained after data analysis of Ion Torrent sequencing output. The data was analysed over the Galaxy platform (usegalaxy.org)

Altogether 213 sequences (0.0001%) were considered contaminations as these were assigned to improbable prey, namely *Homo sapiens* (Linnaeus, 1758), domestic cat *Felis catus* (Linnaeus, 1758), wild boar *Sus scrofa* (Linnaeus, 1758), and the spider *Anyphaena sp.* (Sundevall, 1833). The origin of sequences

belonging to *Felis catus* and *Sus scrofa* is unknown. Small contaminations of human DNA occur often during gut-content analysis. *Anyphaena sp.* reads ocurred probably due to insufficently-clean lab equipment as research was done on this species in our lab before.The number of sequence contaminations per spider individual did not exceed 6 reads. Therefore, a threshold number of 6 reads for prey results was selected to exclude possible contaminations.

3.3.2 Feeding experiment

The feeding experiment provided results needed to assess the accuracy of our sequencing outputs (Appendix: Table 1). There were seven different feeding groups, each fed acording to Table 3. Unfortunately, ants *L. niger* and *L. emarginatus* could not be distinguished as two different ant species after data processing. When their sequences were clustered into MOTUs (cut-off value = 6 bp) and local blast was performed, both species were found in the results with a identitiy around 98 %. Even with a lower cut-off value in MOTU-clustering, their sequences were too similar to each other to distinguish them. However, even without the possibility to separate them, some findings were obtained from the results of data processing.

In all individuals from the feeding experiment a simillar contamination pattern was found and the same threshold (6 reads) as for the sampled individuals was used to exclude possible false positive results.

One of the aims of the feeding experiment was to establish if the prey proportions found in each spider individual corresponds to the conducted feeding schedule. When comparing feeding schedule in Table 3 with the results of gut-content analysis (Appendix: Table 1), it is evident that the number of prey reads does not correspond with the order of feeding trials. *Tetramorium caespitum* reads were always in lower numbers than reads from the *Lasius* group, regardless the feeding schedule in the first three feeding groups. Feeding groups 4, 5, and 6 showed prey reads only of the single prey species they ate 3 weeks in a row. The starving group (group 7) was without any prey results.

Considering the results of the feeding trial, a time limit of possible DNA detection can be assessed. Six out of seven specimens from feeding groups 1 and 2 had prey DNA in their gut after one week of feeding on the specific ant. In group 3, the only two speciems which survived the whole feeding trial, had DNA of the specific ant fed on 2 weeks ago. Three weeks after collecting from the field, none specific prey DNA was visible. Groups 4, 5, and 6 did not have any prey DNA other than the one we fed them with over the first 2 weeks. This was also proved through the starving group which had no prey results after 3 week starvation.

3.3.3 Realized trophic niche results

The results of data processing were presented through a table containing all prey species for each spider individual with the number of sequence counts (Appendix: Table 2). The total number of sequences assigned to ant species varied considerably between each ontogenetic group (mean = 23 583, range = 436 – 74 496). Adult males had the lowest number of prey reads, while J3 and J4 instars of subadult males had the highest number of prey reads.

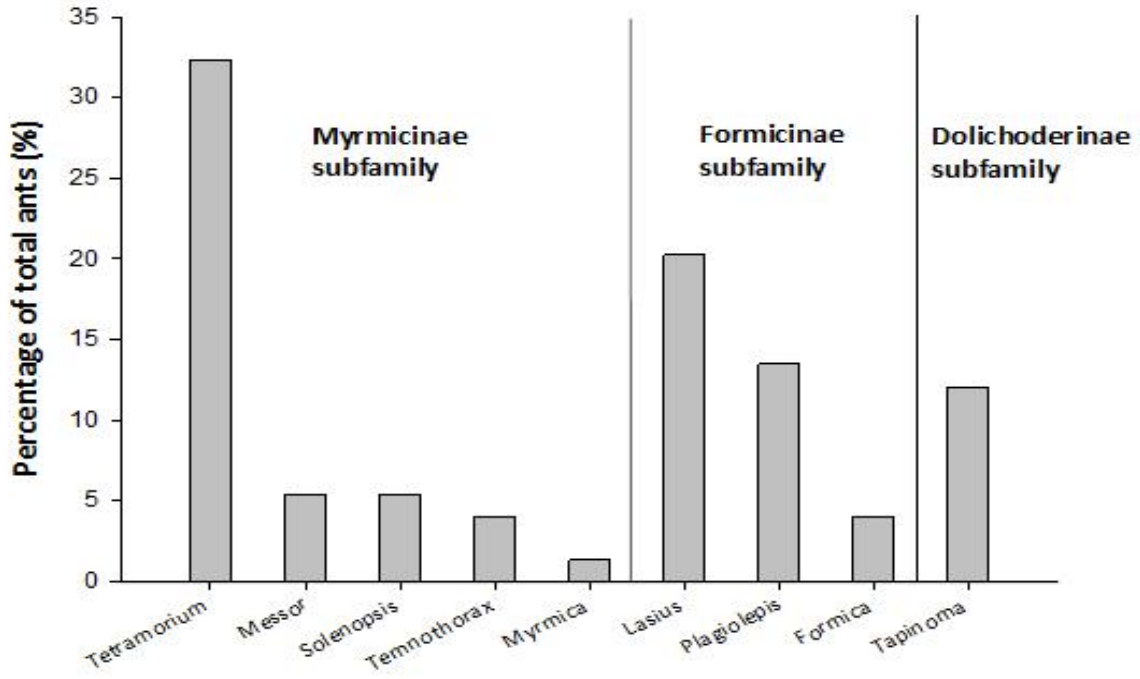

Figure 6. Percentage of ant genera retrieved from gut-content analysis of sampled spiders (N = 68).

The genus level was used to present results due to the impossibility to determine the species level of some ant sequences. Nine different ant genera were found in total (Figure 6), associated to three distinct subfamilies: Formicinae, Myrmicinae, and Dolichoderinae. The genus *Tetramorium* was present in one third of the spider individuals, followed by genus *Lasius* with the second highest percentage (20.3 %), and *Plagiolepis* and *Tapinoma* at the third highest percentage (13.5 % and 12.1 %). Other ant genera were found only in a small number of spider individuals (≤5 %).

Gut-content analysis indicated that there were three further ant genera in the diet of *Z. rubidum* which were not found during sampling (*Solenopsis*, *Temnothorax* and *Myrmica*). Genus *Camponotus*, found during sampling, was the largest in size of all ant genera but was not present in the gut of *Z. rubidum*.

The prosoma length of each spider individual was compared to the average size of their consumed ant-prey. There was no correlation between the spider's size and the size of the prey (Pearsons correlation, $r = 0.08$, $p = 0.469$, $N=77$, Figure 7). There was no significant difference in average prey size between the groups either (Kruskal-Wallis ANOVA , $H = 6.961$, $df = 6$, $P = 0.324$; Figure 8).

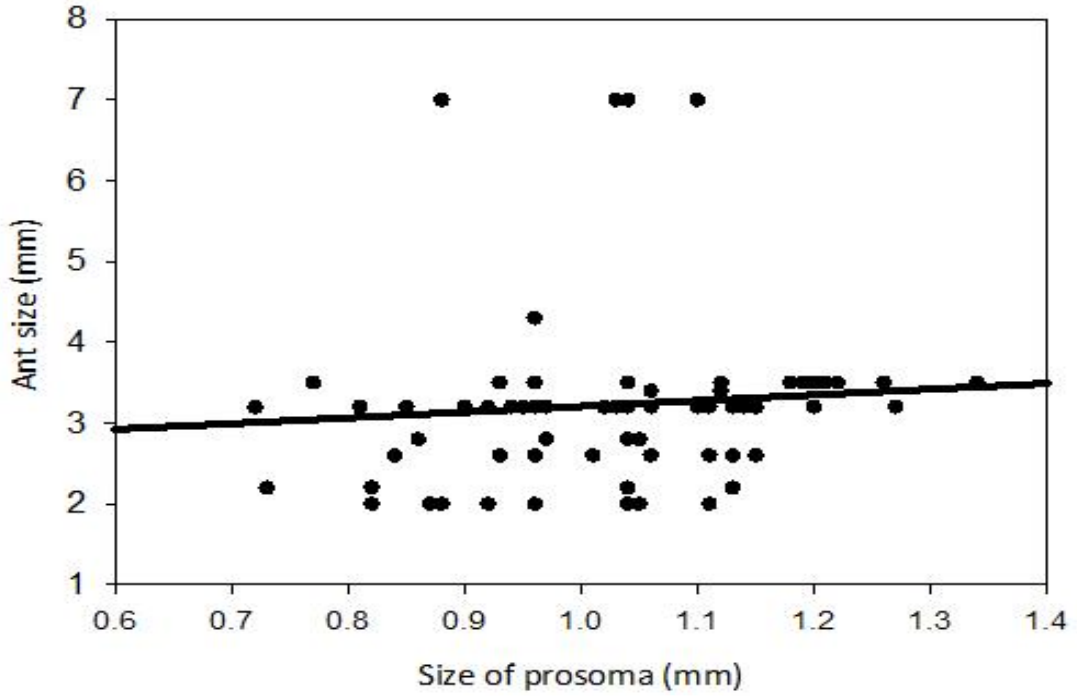

Figure 7. Relationship between the predator's size and the size of their consumed ant prey.

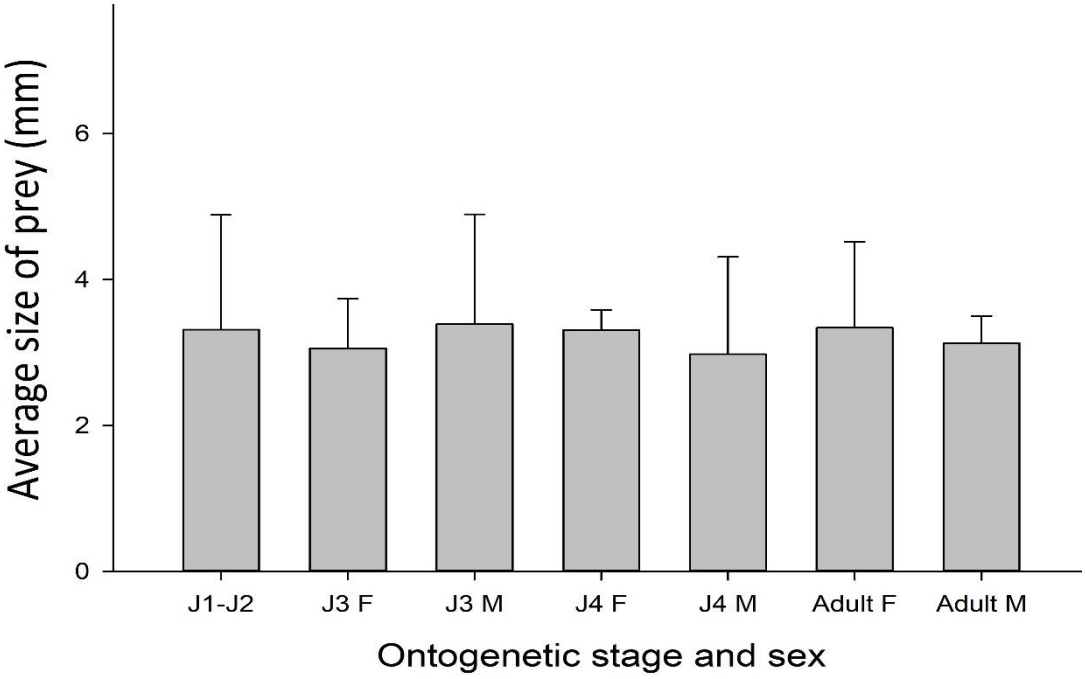

Figure 8. Comparison of prey size among ontogenetic stages and sex. **Abbreviations:** J1-J4 - represent juvenile stages. F – female; M- male.

Number of consumed ant genera was similar among juvenile groups (Figure 9). Adult males and females were the exception as they had a smaller number of prey genera. Only three out of ten adult males had any prey results inside their gut (Figure 10). J1-J2 group had only six out of ten spider individuals with any prey. In other groups all individuals had at least one prey.

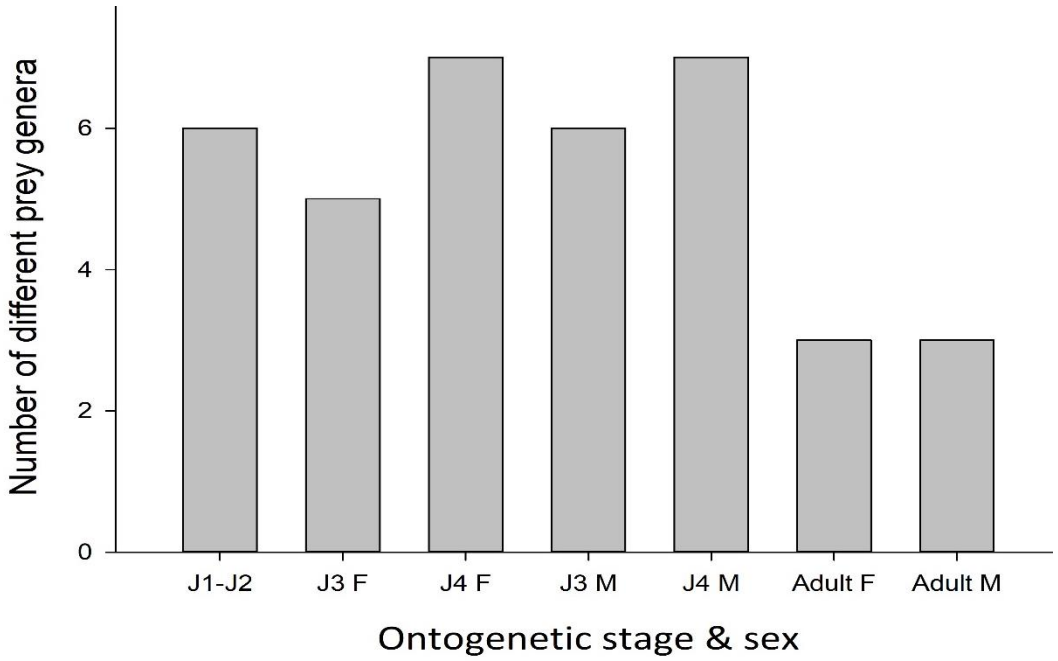

Figure 9. Comparison of the number of consumed ant genera among groups ontogenetic stages and sex. **Abbreviations:** J1-J4 - represent juvenile stages. F – female; M- male.

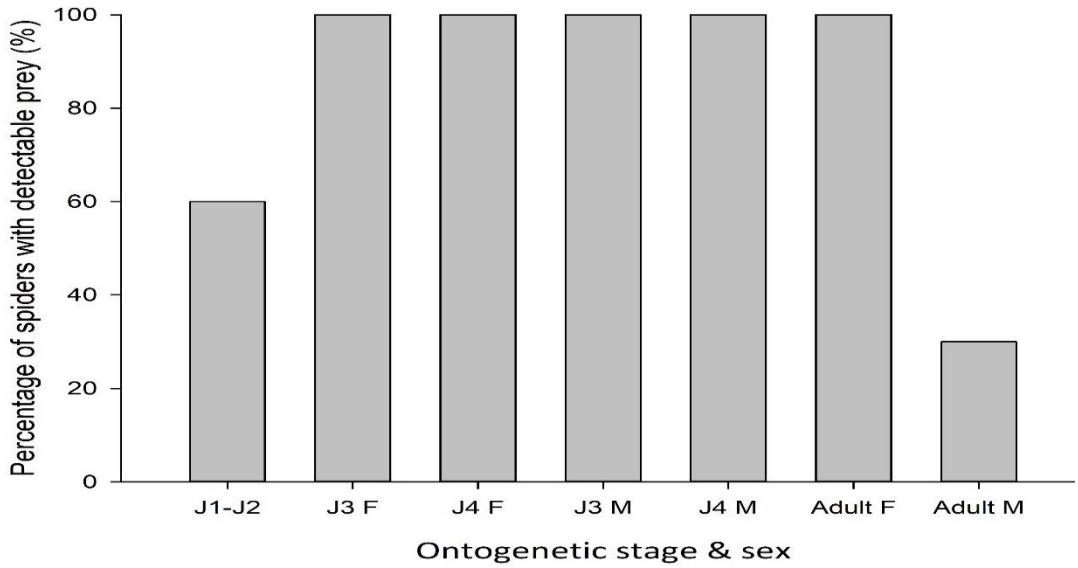

Figure 10. Comparison of the percentage of spiders with detectable prey among ontogenetic stages and sex. **Abbreviations:** J1-J4 - represent juvenile stages. F – female; M- male.

All prey results from the gut content analysis belonged to ants. The diet breadth of each group was estimated using the standardized Levin's index on the level of only one prey type (ant). Levin's index differed between all groups (Figure 11) but all groups were in the range below 0.4, indicating a narrow trophic niche. J1 and J2 instars had the biggest value for the Levin's index ($B_a = 0.3$), unlike J3 instars of subadult females which had the smallest value ($B_a = 0.06$). Adult males were excluded from measuring the Levin's index due to having a sample of only three spider individuals with any prey in their gut, which was not enough to compare it with other groups

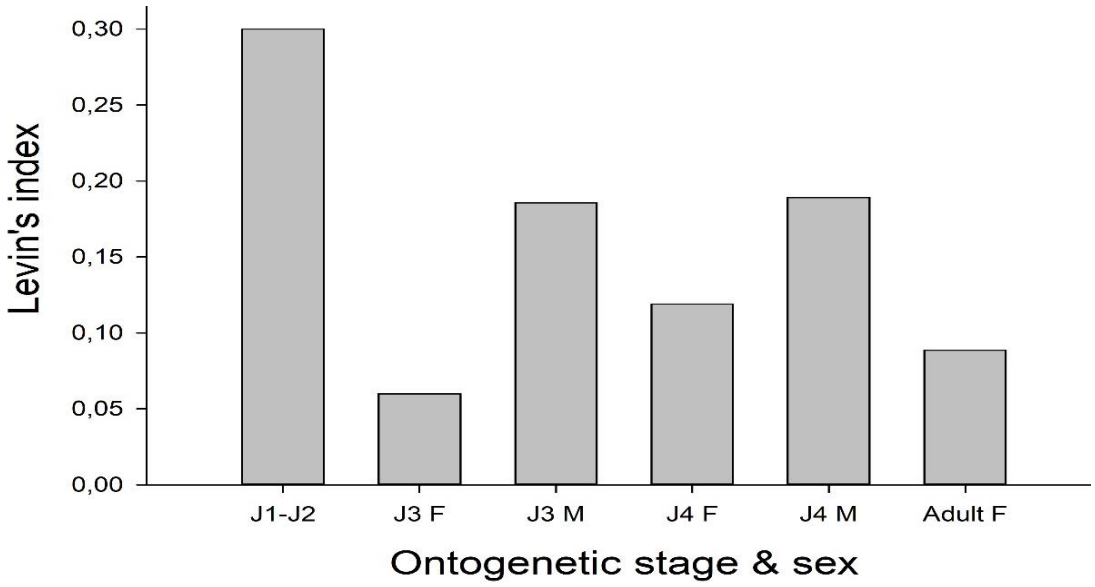

Figure 11. Comparison of the diet breadth among groups estimated using standardized Levin's index on the level of ant prey type. The adult male group is not included due to a small number of individuals having any prey. **Abbreviations:** J1-J4 - represent juvenile stages. F – female.

4. Discussion

4.1 Potentials of using the Ion Torrent platform in DNA-based diet studies

DNA-based diet analysis is nowadays the most frequently used method of natural diet assessment as it can give accurate results based on barcode sequences. The experimental design of DNA-based diet studies consists of few steps and each step depends on the quality of the previous. There is considerable optimism about the use of high-throughput sequencing methods in DNA-based diet studies, but biases associated with the approach are still being examined (Deagle et al., 2013). The Ion Torrent sequencing platform produced a high number of sequences but only 82.73% had a phred-quality score over 20 meaning there are sequenced with 99% accuracy. After assigning the sequences over MIDs to their spider individuals, the total number of asssigned sequences varied considerably between combinations of MID's. However, they varied according to our assumptions that the spiders with the most prey DNA would have the highest number of sequences retrieved.

Only 19.22% of sequences were assigned to species or genera level and out of these sequences, 204 034 sequences belonged to species of ants (11.21%) and the rest (8.01%) belonged to the predator *Z. rubidum.* Although the concentration of the designed blocking primer was almost 8 times higher than that of the amplification primer, a high number of predator DNA was still detecable after sequencing, thus sugesting to either increase the concentration of blocking primer during the amplification step or change the primer binding site and design new primers in addition with a new blocking primer.

Altogether 213 sequences (0.0001% of the total number) were considered contaminations as these were assigned to improbable prey. Such contaminations occur almost always when approaching the assessment of natural diet via high-throughput sequencing. Contaminating sequences often occur because of carelessness during the DNA isolation and barcode amplification step. For this reason, human COI gene fragments appeared in the results as the gut content analysis was performed with human action. Likewise, DNA of other spiders was found in small amounts *(Anyphaena sp.)* as the research was performed in a lab where this spider was previously handled. Even when cleaning and sterilizing all the equipment used in DNA-based diet studies, contaminations are often unavoidable. However, such contaminating sequences are usually in relatively small numbers compared to the number of sequences gained from the real prey. Therefore, a threshold for the number of sequences belonging to a potential prey can be selected to exclude possible contaminations and to reduce the possibility of biased results.

One specific goal DNA-based diet studies want to accomlish is to obtain quantitative information based on sequence read proportions generated by high-throughput sequencing to assess the chronology of feeding on different taxa. Our feeding experiment provided the same information as studies before, although in smaller amounts than we expected due to the impossibility to distinguish sequences of two ant-species that were fed to the spiders. When comparing feeding schedule with the results of gut-content analysis, it is evident that the number of prey reads does not correspond with the order of feeding trials. Regardless of which order the spiders were fed by the three ant species, one ant species had always a fairly smaller number of sequence reads than the combination of the other two. The spiders that were fed only on one ant type during two weeks had a small amount of sequence reads, although we predicted a large number of sequence reads.

Such discrepancy can be caused by many biological factors such as taxon specific variation in DNA copy number per cell, variation in tissue cell density and differrences in enviromental persistance of their DNA (Deagle et al., 2013). The last factor was investigated through our feeding trial as well. The results

are not as informative as we hopped for due to the problem with differentiating two ant species through sequences gained from sequencing. Still, the results indicate that prey-DNA can be detected in the gut of *Z. rubidum* even after a week of the feeding on the specific prey and there is a possibility that it can be detected even after two weeks. The control groups (4, 5, 6 and 7) show that no prey is detectable after 3 weeks. Additional research should be carried out to fully assess this problem and to determine the half-life of DNA in spider gut.

The biggest technical factor that affects the results of DNA-based diet studies is the selection of the proper DNA barcode marker. When studying the diet of carnivores, the cytochrome c oxidase I (COI) gene is frequently used as the DNA barcode marker because no other marker can be found in barcode-databases in such a large number of taxa (Deagle et al., 2014). Its sequence variation usally allows high taxonomic resolution. However, the accuracy of the results is highly dependend on the used amplification primers. If we assume that the studied species has a broad diet niche, the primers should be designed so that they bind to the same genomic region in a large variety of different species. We used such "universal" primers to detect all the ant prey inside the gut, although the primers were used to identify mites (Acari) in first studies (de Groot et al., 2016). These primers were proven to be good markers for detecting almost all arthropods, but we still tested the primers on the ants we sampled on the field. Neverthless, although these "universal" primers are effective to detect a large range of prey, their taxonomic resolution is consequently lower and thus our diet analysis gave prey results only to the genus level. That is why our feeding trial was less succesful as we were not able to distinguish between two *Lasisus* species. In future studies, it should be considered to sequence several barcode markers from each sample to overcome taxonomic breadth and resolution .

Also, there are other technical factors affecting the results gained by investigating the diet through high-throughput sequencing platforms. For a given sample, forward and reverse sequences come from opposing strands of the same set of amplicons, so although the sequences are reverse complements, they should be present in equal numbers after the PCR step. During Ion Torrent sequencing there is an additional amplification step called emulsion PCR that could preferentially amplify certain DNA molecules over others (Quail et al., 2012). Alternatively, the sequencing process could sequence certain sequences more efficiently, resulting in giving biased results. In addition, some primer tags (or MIDs in our case) can be favoured in PCR and sequencing reactions, which again can lead to biased results. Although this problem was not investigated in our study, there are indications that some MIDs are sequenced more often than others because all samples had the same concentration of amplified DNA before the sequencing run. It would be prudent to design studies so that the same MIDs are used across samples in different sequencing runs. With this type of experimental design, it could be possible to evaluate biases introduced by MIDs and calculate corrections of the results (Deagle et al. 2018).

Another problem the Ion Torrent sequencing platform is facing, is that the sequence quality is particularly affected by homopolymer repeats found in the prey-sequences. During sequencing these repeats are sequenced simultaneously as they are signalized by hydrogen ions being released during nucleotide addition, and thus causing a problem when estimating the number of nucleotides added. If a species may have a larger number of homopolymer repeats in their barcode sequence, it may lead to strong divergences in quality scores between sequences and such prey could be discarded during data processing. Such problems highlight the potential benefit of averaging the results through multiple sequencing runs to minimize biases gained from the sequencing platform. The only issue with this solution is the cost of resequencing the sample multiple times.

All mentioned above may become less of an issue as platforms stabilize. However, a new generation of sequencing technologies is emerging, where no amplification step will be needed, and single sequences will be sequenced fast and cheap. Thus, stabilization of second-generation sequencing platforms is less likely to occur and in the future new methods could be used to assess the natural diet.

4.2 Realized trophic niche of *Zodarion rubidum*

Several studies already exist regarding the ecology of the genus *Zodarion* (Cardenas et al., 2014; Pekár et al., 2011; Pekár et al., 2010). So far, the realized trophic niche was assessed in six Zodarion species from the Iberian Peninsula (Pekár et al., 2018). However, to our knowledge, for the first time the realized trophic niche was characterized in a spider to determine whether there is an ontogenetic shift in their diet.

The natural diet of *Zodarion rubidum* was assessed using the modern method of gut content analysis. Based on the given results, we have proven that their diet consists only of ants during their entire life cycle. Such diet habits have been predicted in previous studies which have shown that they have preferences for ants and can't survive on diets based on different prey taxa (Pekár & Toft, 2009). Earlier studies indicated that *Zodarion rubidum* possesses capture and venom adaptations more effective on Formicinae ants. Further, *Z. rubidum* was able to detect chemical signals only from species belonging to Formicinae subfamily (Cardenas et al., 2012). Although our results showed that *Z. rubidum* feeds on ants belonging to Formicinae, 1/3 of all prey found in the gut was the ant *Tetramorium caespitum* from the Myrmicinae subfamily. In addition, 13 % of all ant-prey belonged to the Dolichoderinae subfamily (genus *Tapinoma*). Based on the results, we assume that this is an adaptation to the abundance of ant-prey in their locality after migrating from southern Europe as they have similar prey preferences as the other *Zodarion* species from Central Europe.

In our first hypothesis, we proposed that there would be a shift in size or prey type during ontogenetic development of *Z. rubidum*. Our study showed that there was no correlation between the size of spiders and the size of their prey. Furthermore, there was no significant difference in the average size of the prey between ontogenetic stages. However, when comparing the diet by the number of different prey types consumed in each ontogenetic stage, there are indications of a reduction in the number of preferred genera they consumed at the adult stage. Only three genera were found in the diet of adult specimens (*Lasius*, *Tapinoma* and *Tetramorium)* while there was a higher number of different genera in the gut of juvenile stages. All three genera found in the gut of adult specimens were medium-sized ants represented as the three most common consumed ant-genera. Therefore, adult specimens of *Z. rubidum* seem to forage only on the most preferable genera. However, the abundance of the prey in the quarry was not investigated and we are not sure if this is a real preference on the three mentioned genera or they are just the three most occurring genera in the spider's habitat.

In our second hypothesis, we expected differences between the diet of adult males and females, either in the type or size of the prey due to their sexual dimorphism (Pekár et al. 2011). The results showed that adult males experienced a decline in foraging activity. Only three out of ten adult males had detectable prey inside their gut. These three males fed on the same type of prey as the female spiders did. Such behavior was described in another *Zodarion* species as well (Martišova et al., 2009) and was associated with maximizing mating opportunities. Males preferred to cease active hunting and started kleptoparasiting on prey left by other *Zodarion spiders*, while searching for female foraging sites. Although our results cannot confirm if the males solely fed on the prey-remains left by female spiders, they indicate that the diet of adult males can be affected by the diet of female individuals. Further diet studies on male *Zodarion* spiders need to be conducted to analyze this topic in more detail.

The trophic specialization level was assessed by calculating the Levin's index. Usually, the Levin's index is calculated using a high taxonomic level (order or family) to describe all the prey types in the diet, but as the diet of *Zodarion* consists of only one type of prey (ants), it was calculated on the genus level. The J1 and J2 instars had the highest value due to a broad variety of ant genera found and each spider specimen having only one prey in their gut. The J3 female instars had the lowest value due to having a small variety of ant genera and almost each spider having the same prey. Levin's index differed between all groups, but all groups were in the range below 0.4, indicating a narrow trophic niche. Although Levin's index is a useful standardized test to calculate the diet breadth of a species in their locality, it is strongly biased by the sample size and the meal frequency. The Levin's index should be used only on calculating the diet breadth of populations and not on groups inside the population.

Although there were nine different ant-genera found inside the gut of spider specimens, our study showed that only four were occurring in larger percentages. All these preferable ants are about the same size as the spider and the spiders attacking larger ants is more an exception than rule. Taking all the facts into consideration, the diet breadth of *Zodarion rubidum* is narrow and they can be categorized into specialized, stenophagous predators foraging only on ants, probably preferring small to medium sized ants.

As mentioned before, this study was conducted on a single population sampled throughout one year. Further studies on the diet of *Zodarion rubidum* in other localities through central and southern Europe could demonstrate slight to significant changes in the diet depending on the geographical distribution and prey availability. Additionally, further research could confirm that there is no shift in the diet through ontogenetic development and that adult males tend to cease hunting activities. Only after investigating a fair number of populations in different geographical areas, we could understand the natural diet to some extent and predict all the factors that affect it.

5. Conclusion

- This study reveals the realized trophic niche of a *Zodarion rubidum* population from quarry Hády, Brno (Czech Republic).
- Their diet consisted only of ants, in particular of nine different ant genera that were classified into three different subfamilies, thus confirming them as specialized stenophagous predators.
- During ontogenetic development there was no shift in prey size or prey type. Adult female specimens of *Z. rubidum* seem to forage only on the most preferred ant-genera while adult males tend to cease foraging activity.
- The feeding trial showed that prey DNA can be detected inside the spider's gut even after a week of feeding.

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7. Appendix

Table 1 Results of gut content analysis of the feeding experiment. Feeding schedule can be found in Table 3. Most of the sequences were only determined to genus level. Prey species with a number of reads $N \leq 6$ were excluded from final results due to the possibility that they are contaminant sequences.

Group individual	Number of reads	Species
1.1	12734	*Lasius* sp.
	376	*Tetramorium* sp.
1.2	1455	*Lasius* sp.
	45	*Tetramorium caespitum*
1.3	2210	*Lasius* sp.
2.1	46	*Lasius* sp.
	13	*Tetramorium caespitum*
2.2	1442	*Lasius* sp.
	30	*Tetramorium caespitum*
2.4	1177	*Lasius* sp.
	196	*Tetramorium caespitum*
3.1	7716	*Lasius* sp.
	212	*Tetramorium caespitum*
3.2	9601	*Lasius* sp.
	34	*Tetramorium caespitum*
4.1	132	*Lasius* sp.
5.1	503	*Lasius* sp.
6.1	243	*Tetramorium caespitum*
6.2	13	*Tetramorium caespitum*
6.3	761	*Tetramorium caespitum*
7.1	-	-
7.2	-	-
7.3	-	-

Table 2. Results of gut content analysis for each spider individual categorized by ontogenetic stage and sex. Results show only species-specific sequences having a threshold value of the number of reads higher than 6. Most of the sequences were determined only to genus level. **Abbreviations:** J1-J4 - represent instar stages. F – female; M- male.

Ontogenetic stage and sex	Number of reads	Species
J1-J2	89	*Lasius* sp.
J1-J2	41	*Solenopsis* sp.
J1-J2	2940	*Temnothorax* sp.
	47	*Tetramorium caespitum*
J1-J2	402	*Tapinoma* sp.
	112	*Plagiolepis* sp.
J1-J2	2016	*Messor structor*
J1-J2	13	*Tetramorium caespitum*
F J3	67	*Tetramorium caespitum*
	21	*Plagiolepis* sp.
F J3	141	*Tetramorium caespitum*
F J3	23	*Tetramorium caespitum*
F J3	28	*Myrmica* sp.
F J3	57	*Lasius* sp.
F J3	35	*Tetramorium caespitum*
F J3	41	*Tetramorium caespitum*
F J3	23	*Tetramorium caespitum*
	18	*Temnothorax* sp.
F J4	7192	*Plagiolepis* sp.
	363	*Messor structor*
F J4	752	*Messor structor*
	643	*Tetramorium caespitum*
F J4	1144	*Tapinoma* sp.
	23	*Tetramorium caespitum*
F J4	1692	*Plagiolepis* sp.
	13	*Tapinoma* sp.
F J4	1328	*Tetramorium caespitum*
	10	*Solenopsis* sp.
F J4	2855	*Lasius* sp.
F J4	2078	*Lasius* sp.
F J4	703	*Tetramorium caespitum*
	20	*Solenopsis* sp.
F J4	165	*Formica* sp.
M J3	8300	*Tapinoma* sp.
	324	*Plagiolepis* sp.
M J3	1018	*Lasius* sp.
	214	*Tapinoma* sp.

M J3	1005	*Plagiolepis* sp.
M J3	2808	*Lasius alienus*
M J3	5687	*Tetramorium caespitum*
M J3	4391	*Plagiolepis* sp.
M J3	264	*Tetramorium caespitum*
M J3	642	*Solenopsis* sp.
	22	*Plagiolepis* sp.
M J3	125	*Tetramorium caespitum*
M J3	25060	*Messor structor*
M J4	3417	*Tetramorium caespitum*
	2043	*Messor structor*
M J4	903	*Plagiolepis* sp.
M J4	1809	*Tetramorium caespitum*
M J4	16100	*Lasius* sp.
	62	*Lasius alienus*
	5385	*Temnothorax* sp.
M J4	27227	*Formica* sp.
M J4	5773	*Lasius* sp.
M J4	2647	*Tetramorium caespitum*
M J4	4655	*Tapinoma* sp.
M J4	4087	*Tetramorium caespitum*
	355	*Tapinoma* sp.
Adult F	637	*Tetramorium caespitum*
Adult F	8	*Tetramorium caespitum*
Adult F	11	*Tapinoma* sp.
	8	*Tetramorium caespitum*
Adult F	10	*Tetramorium caespitum*
Adult F	7547	*Lasius* sp.
Adult F	5827	*Lasius* sp.
Adult F	205	*Lasius* sp.
Adult F	796	*Lasius* sp.
Adult F	24	*Lasius* sp.
Adult F	46	*Lasius* sp.
Adult M	107	*Tapinoma* sp.
	16	*Tetramorium caespitum*
Adult M	14	*Tetramorium caespitum*
Adult M	270	*Lasius* sp.

8. Curriculum vitae

NAME AND SURRNAME: Domagoj Gajski

EDUCATION:
2016 – 2019: **Master studies in molecular biology**; Faculty of Science, University of Zagreb, Croatia; Graduation thesis: Trophic niche of an ant-eating predator during its ontogenetic development
2013 – 2016: **Bachelor studies in molecular biology**; Faculty of Science, University of Zagreb, Croatia
2008 – 2012: **High school education**; Sesvete Gymnasium, Zagreb, Croatia

RESEARCH EXPERIENCE:
3/2018 – 08/2018: **Internship in molecular gut content analysis**; Masaryk University, Brno, Czech Republic; Prof. Dr. Mgr. Stanislav Pekár
12/2015 – 10/2017: **Internship in molecular phylogeny**; Ruđer Bošković Institute, Zagreb; Croatia Evolution genetics lab (LEG); Dr. sc. Branka Bruvo Mađarić
10/2016 – 02/2017: **Demonstrator in Molecular Genetics**; Faculty of Science, University of Zagreb, Croatia
10/2014 – 02/2015: **Demonstrator in Zoology**; Faculty of Science, University of Zagreb, Croatia

WORK EXPERIENCE:
8/2018 – 2/2019: **Pharmacovigilance Associate**; PrimeVigilance; Zagreb, Croatia

LANGUAGES:
Croatian (mother tongue), English (independent user; Eureka certificate; B2), German (proficient user, DAAD certificate, C1)

HONOURS, AWARDS, GRANTS, FELLOWSHIPS:
2018: **ERASMUS + Student Mobility of Placement (SMP)**; Department of Botany and Zoology, Faculty of Science, Masaryk University; Brno, Czech Republic
2018: **Rector's award 2018**; University of Zagreb, Croatia
2017: **Fellowship for outstanding Master students**; Rotary club Sesvete; Zagreb, Croatia
2015: **Rector's award 2015**; University of Zagreb, Croatia

PROJECTS:
2018: International Youth Naturalist Tournament (IYNT) in Georgia
2017: International Youth Naturalist Tournament (IYNT) in China
2017: Summer School of Science in Požega
2015: 7th Biology Night
2014: 6th Biology Night

MEMBERSHIPS:
2015 – present: Croatian society of Arachnology "Narcis Damin"
2016 – present: Youth Research Center (ICM)
2015 – 2018: Folkdance Group Ivan Goran Kovačić
2013 – 2015: Biology Student Association (BIUS)