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Detection of food in the gut content of *Heteromurus nitidus* (Hexapoda: Collembola) by DNA/PCR-based molecular analysis

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Abstract. The paper reports a feeding experiment showing that *Heteromurus nitidus,* a widespread and common springtail species found in a variety of habitats rich in organic matter, was capable of feeding upon nematodes in the laboratory. DNA/PCR based molecular gut analysis of *Heteromurus nitidus* for two nematode species, namely *Phasmarhabditis hermaphodita* and *Steinernema feltiae*, and one soil alga, *Chlorococcum infusionum*, was done in the laboratory using species-specific primer techniques. For detection of nematode consumption, specific primers were applied: Sf-F-1896 and Sf-R-2080 for *S. feltiae* and Ph-F-1754 and Ph-R-1887 for *P. hermaphrodita*, amplifying a 203- and 154-bp long fragment, respectively. General *Chlorococcum* primers targeting a 237-bp long fragment was designed using 18S rDNA-sequences from GenBank. Screening DNA extracts with nematode-specific primers showed nematode DNA could be detected for 54.7% of *H. nitidus* feeding on *P. hermaphrodita* and 66.5 % feeding on *S. feltiae*. Only 3.8% of *H. nitidus* was capable of feeding on soil algae. Our results clearly demonstrate that Collembola may also be facultative predators of nematodes.

Key words: *Heteromurus nitidus*, gut-content analysis, predator-prey relationship, DNA/PCR, 18S rDNA.

Introduction

Springtails are representative of soil biodiversity with numerous species and individuals occupying a wide range of trophic niches. Due to their high abundance and distribution, collembolans are a widely accepted prey group of terrestrial predators, but their feeding ecology is still a subject of many studies. Collembola appear to feed on a wide spectrum of food resources (Rusek 1998), and fungi are generally regarded as the most important diet of Collembola (Chen et al. 1995, Klironomos & Kendrick 1995). However, they have also been reported previously to feed upon animal prey. Gilmore (1970) reported that a large number of species from a wide range of collembolan families feed on nematodes and suggested that springtails may be beneficial in nematode control. Further studies, including *Isotoma* sp. (Brown 1954), *Folsomia candida*, *Sinella caeca* (Gilmore & Potter 1993) and *F. candida*, *Heteromurus nitidus* and *Protaphorura fimata* (Ruess et al. 2004) support these results.

Different approaches have been used to investigate feeding strategies of Collembola, most comprehensive are fatty acid (FA) and stable isotope analysis (Chahartaghi et al. 2005, Haubert et al. 2006, Pollierer et al. 2010, Oelbermann & Scheu 2010). The recent developments in molecular biology have made it possible to apply DNA-based technologies for gut-content analysis in a variety of animal species. Such studies help in expanding our understanding of trophic relationships between species. PCR-based approaches have been applied to study predation by Collembola on nematodes in both laboratory and field (Read et al. 2006) in order to study predation (reviewed by Sheppard & Harwood 2005; Gariepy et al. 2007). Techniques and PCR primers have been developed, both in aquatic and terrestrial ecosystems, to detect predation, including marine invertebrates (Blankenship & Yayanos 2005), Collembola (Agustı´ et al. 2003), molluscs (Harper et al. 2005), earthworms (Admassu et al. 2006), Coleoptera (Juen & Traugott 2006, Inayat et al. 2012) or algal food in the gut content of copepods (Nejstgaard et al. 2003; Troedsson et al. 2009). Recently, it was developed a PCR based assay for detection of fungal diet in the gut content of collembolan *Protaphorura armata* (Jørgensen et al. 2005). The first study about molecular detection of diet demonstrated successful extraction and PCR amplification of the prey fungal 18S ribosomal DNA from the gut content of Collembola.

The majority of the results obtained by other authors indicates that Collembola are largely unselective feeders ingesting what is available (Christiansen 1964, Walkwork 1970). Previous studies concerning the feeding strategies of *Heteromurus nitidus* were quantified by stable isotope

Table 1. Arrangement of feeding trials.

No of treatment	Name of feeding trials	No. of Collembola specimens used for molecular detection
1.	*H. nitidus* + *C. infusionum*	15 - algal detection
2.	*H. nitidus* + *S. feltiae*	15 - nematode detection
3.	*H. nitidus* +*P. hermaphrodita*	14 - nematode detection
4.	*H. nitidus* + *P. hermaphrodita* + *S. feltiae*	14 - nematode detection
5.	*H. nitidus* + *S. feltiae* + *C. infusionum*	15 - preference for nematode or algae
6.	*H. nitidus* + *P. hermaphrodita* + *C. infusionum*	12 - preference for nematode or algae
7.	*H. nitidus* + *S. feltiae* + *P. hermaphrodita* + *C. infusionum*	13 - preference for nematode or algae

methods and fatty acid analysis (Ruess et al. 2004, Scheu & Folger 2004, Haubert et al. 2006, 2008). Here we report, the use of PCR to detect feeding type of *Heteromurus nitidus* in laboratory conditions using two parasitic nematode species (*Phasmarhabditis hermaphodita* and *Steinernema feltiae)* and one species of soil algae [*Chlorococcum infusionum* (Schrank) Menegh]. The objectives of the present study were to determine: (i) if *Heteromurus nitidus* feeds on nematodes and soil algae; (ii) if this species preferentially feeds on certain nematode species; (iii) if the collembolan favors animal prey over algal food.

Materials and Methods

Animal and algal cultures

Algal food consisted of *Chlorococcum infusionum* (Schrank) Meneghi (Chlorophyceae). Species of the genus *Chlorococcum* are ubiquitous in soils. Algal cultures grow on a soil-agar medium and were obtained from SAG- Culture Collection of Algae (University of Göttingen, Germany). Two nematode species, *Phasmarhabditis hermaphrodita* (Schneider) and *Steinernema feltiae* (Filipjev) (supplied by prime factory, Hennstedt, Germany) were used as animal prey species. These two nematode species are subject of many studies due to their entomopathogenic qualities (Wilson et al. 1993, Fairbairn et al. 2000). Top consumer *Heteromurus nitidus* (Templeton, 1835) was taken from laboratory cultures (University of Darmstadt, Germany) fed with baker's yeast. Only adult specimens were used in the experiments.

Collembola feeding trials

For the experiments collembolan specimens were put into plastic vessels (diameter 5 cm; height 3,5 cm) with a 1-cm-thick layer of plaster of Paris mixed with activated charcoal (2:1) at the bottom. The feeding trials were kept moist with distilled water. Each plastic vessel contained five individuals of the collembolan species. This low density was chosen to avoid cannibalism due to crowding. The specimens of *H. nitidus* were starved for a period of 72h and then allowed to feed on the nematodes and soil algae for 96 h at 17° C in the dark. Algal food source was offered as pieces (1 cm diameter) cut out of the agar cultures aseptically, whereas nematodes were pipetted directly onto the plaster layer in the middle of the plastic vessels. *H. nitidus,* algal and nematode species were arranged in six different combinations (Table 1) that spanned one or two trophic levels. Three replicates per treatment were performed for each combination. In total, 98 individuals of *H. nitidus* were used for molecular detection of algal food and nematode prey in springtails guts (Table 1).

Extraction of algal and animal DNA

Algal DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad CA, USA) following the manufacturers instructions. DNA of nematodes and springtails was extracted using the DNeasy blood & tissue kit (QIAGEN, Hilden, Germany) following the manufacturers guidelines.

Primer design and gut content analysis

General *Chlorococcum* primers CLO-GEN-S3 (5'-GCATGGAATMRCACGATAGGACTC-3') and CLO-GEN-A4 (5'-CGGCATCGTTTATGGTTGAGACTAC-3') targeting a 237 bp long fragment were designed in this study, using 18S rDNA gene sequences from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). A database of 21 aligned complete and nearby complete 18S rDNA gene sequences from available *Chlorococcum* species, nematodes and springtails species were edited and aligned using Bioedit™ package (Hall 1999). All representative organisms including Genbank sequence accession numbers are provided in Table 2. Primers were designed following the guidelines of Apte & Daniel (2003) and King et al. (2008). Primer design tools available in the Primer Premiere 5 software package (Premier Biosoft International) were used to facilitate optimal primer design.

For detection of nematode consumption species- specific primers were applied: Sf-F-1896 and Sf-R-2080 for *S. feltiae* and Ph-F-1754 and Ph-R-1887 for *P. hermaphrodita* (Read et al. 2006), amplifying a 203 and 154 bp long fragment respectively. To ensure specificity, all primers were tested against all organisms in this system, amplifying none but the very prey group (Table 3).

Each 10 µl of the screening-PCR-reaction mix contained 1 µl PCR water, 5 µl multiplex-PCR reaction mix (Qiagen), 1 µl primer mix and 3 µl of DNA extract. PCR cycling conditions were 95°C for 15 min followed by 35 cycles of 94°C for 30 s, 63°C (*Chlorococcum*)/56°C (*S. feltiae*)/58°C (*P. hermaphrodita*) for 90 s, 72°C for 90 s and a

Table 2. 18S rRNA gene sequences used in this study for the design of Chlorococcum specific PCR primers.

Species	GenBank accession nr.	Taxonomy
Chlorococcum dorsiventrale	AB058303	Chlorococcales;Chlorococcaceae
Chlorococcum minutum	GQ122365	Chlorococcales;Chlorococcaceae
Chlorococcum diplobionticum	U70587	Chlorococcales;Chlorococcaceae
Chlorococcum sp.	AB490286	Chlorococcales;Chlorococcaceae
Chlorococcum sp.	AB490287	Chlorococcales;Chlorococcaceae
Chlorococcum sp.	AB183580	Chlorococcales;Chlorococcaceae
Chlorococcum sp.	AB058335	Chlorococcales;Chlorococcaceae
Phasmarhabditis hermaphrodita	FJ516755	Nematoda; Rhabditidae
Phasmarhabditis hermaphrodita	DQ639980	Nematoda; Rhabditidae
Phasmarhabditis hermaphrodita	DQ639981	Nematoda; Rhabditidae
Steinernema feltiae	DQ310470	Nematoda; Steinernematidae
Steinernema feltiae	DQ310469	Nematoda; Steinernematidae
Steinernema feltiae	AY170336	Nematoda; Steinernematidae
Steinernema feltiae	GQ377418	Nematoda; Steinernematidae
Steinernema feltiae	EU200355	Nematoda; Steinernematidae
Steinernema feltiae	EU200354	Nematoda; Steinernematidae
Steinernema feltiae	EF595635	Nematoda; Steinernematidae
Steinernema feltiae	EF595634	Nematoda; Steinernematidae
Steinernema feltiae	EF595633	Nematoda; Steinernematidae
Heteromurus nitidus	AJ605710	Collembola; Entomobryidae
Heteromurus tenuicornis	DQ016564	Collembola; Entomobryidae

Table 3. PCR primers and PCR reaction conditions used in this study.

Specificity	Forward primer (5′→3)	Reverse primer (5′→3)	Product size
Chlorococcum infusionum	CLO-GEN-S3 (GCATGGAATMRCACGATAGGACTC)	CLO-GEN-A4 (CGGCATCGTTTATGGTTGAGACTAC)	237 bp
Steinernema feltiae	Sf-F-1896 (TTTAGGCCATCCTGGAAACAGG)	Sf-R-2080 (GCTAAAACCGGTAAAGAAAG)	203 bp
Phasmarhabditis hermaphrodita	Ph-F-1754 (TGGGTGCCCCTGATATAAGAT)	Ph-R-1887 (CGGATGACCAAGGGTACTTAAT)	154 bp

final elongation at 72°C for 10 min. PCR products were electrophoresed in 3% ethidium bromide-stained agarose gels at 90 V and visualized by ultraviolet transillumination.

Statistical analyses

The differences between the results of the single, double and triple trials were tested using logistic regresion. All calculations were performed using SAS version 9.2 (Cary, NC, USA).

Results

Detection of nematode and algal DNA within *H. nitidus*

In separate feeding trials, the collembolan *H. nitidus* was screened for the presence of target nematode DNA within its gut after feeding *ad libitum* for 96 h on nematode *P. hermaphrodita* (trial numbers 3, 4, 6, 7) and *S. feltiae* (trial numbers 2, 4, 5, 7) respectively (Fig. 1). Screening DNA extracts with nematode-specific primers showed nematode DNA (Table 3) could be detected for 54.7% of *H. nitidus* feeding on *P. hermaphrodita* and 66.5 % of Collembola feeding on *S. feltiae* (Fig. 2).

In separate feeding trials (trial numbers 1, 5, 6, 7), the collembolan *H. nitidus* was screened for the presence of target algal DNA within its gut on *C. infusionum* (Fig. 1). Screening DNA extracts with algal- specific primers could be detected only for 3.8% of *H. nitidus* feeding on soil algae (Fig. 2).

H. nitidus feeding on nematode and algae

Single-food approach. H. nitidus feeding on algae (*C. infusionum*) differed significantly compared to those feeding on nematode (*P. hermaphrodita* and *S. feltiae*):

(*H. nitidus* vs. *C. infusionum*) vs. (*H. nitidus* vs. *P. hermaphrodita*): χ^2=11.84; df=1; P=0.0006 (95% level); (*H. nitidus* vs. *C. infusionum*) vs. (*H. nitidus* vs. *S. feltiae*): χ^2=20.00; df=1; P<0.0001 (99% level). *H. nitidus* feeding on both nematode prey species

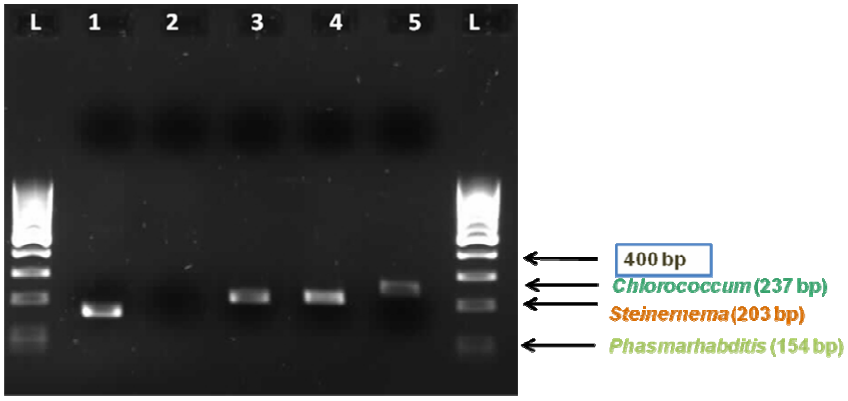

Figure 1. Detection of food in the gut content of *Heteromurus nitidus* after feeding on nematode (*P. hermaphrodita* and *S. feltiae*) and algal soil *C infusionum:* L - Ladder, Lane 1, 3, 4, 5 - positive control, Lane 2 -'No DNA'negative control. The size of the PCR–amplified product is shown on the right. PCR products were visualized and sized electrophoretically on a 2% agarose gel.

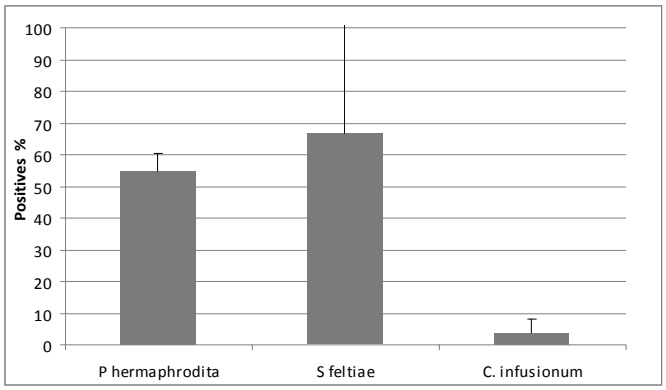

Figure 2. Percentage of Collembola (*Heteromurus nitidus)* tested positive for predation on both nematode species *P. hermaphrodita* using the primer pair Ph-F-1754 + Ph-R-1887 and *S. feltiae* using the primer pair Sf-F-1896 + Sf-R-2080 and algal food *C infusionum,* using the primer pair CLO-GEN-S3 + CLO-GEN-A4.

did not differ significantly (*H. nitidus* vs. *P. hermaphrodita*) vs. (*H. nitidus* vs. *S. feltiae*): χ^2=1.77; df=1; P=0.18).

Double-food approach. *H. nitidus* feeding on algae differed significantly compared to those feeding on *P. hermaphrodita* when offered both prey at once (*H. nitidus* vs. *C. infusionum* and *P. hermaphrodita*): χ^2=5.0; df=1; P=0.0247 (95% level). Also, feeding on *P. hermaphrodita* differed significantly compared to those feeding on *S. feltiae* when offered both *H. nitidus* vs. *P. hermaphrodita* and *S. feltiae*: χ^2=6.30; df=1; P=0.0121 (95% level). There was no significant difference between feeding results of algae and *S. feltiae* (*H. nitidus* vs. *C. infusionum* and *S. feltiae*): χ^2=0.37; df=1; P=0.5428.

Triple-food approach. *H. nitidus* feeding on three prey types at once was highly significant: *H. nitidus* vs. *C. infusionum, S. feltiae* and *P. hermaphrodita*: χ^2=20.13; df=2; P<0.0001 (99% level); *H. nitidus* vs. *C. infusionum* and *P. hermaphrodita*: χ^2=10.53; df=1; P=0.0012 (99% level); *H. nitidus* vs. *S. feltiae* and *P. hermaphrodita*: χ^2=11.56; df=1; P=0.0007 (99% level).

Our results clearly demonstrate the predation of Collembola on nematodes in the laboratory. Both species of nematodes were consumed by the springtails. Preference was particularly evident for *Steinernema feltiae,* the nematode species most frequently found in springtails guts, followed by *Phasmarhabditis hermaphrodita* and soil algae.

Discussion

PCR-based techniques, using species-specific primers, were used to detect type of prey (algae or nematodes) in the Collembola guts in the laboratory. *Heteromurus nitidus* has been shown to feed upon nematodes in the laboratory and preferentially choose nematodes over the soil alga *Chlorococcum infusionum.*

The use of PCR-based assays for detection of prey species consumed by predatory species is becoming increasingly common (Chen et al. 2000, Agustí et al. 2003, Sheppard et al. 2005, Ma et al. 2005, Hoogendoorn & Heimpel 2001, Juen & Traugott 2006, Read et al. 2006, King et al. 2011, Heidemann et al. 2011, Eitzinger et al., 2013). The effects of predators on prey populations can be modified by a number of abiotic factors: temperature (von Berg et al. 2008a, Hosseini et al. 2008), rain (von Berg et al. 2008b) or others such as time since feeding, subsequent food intake, sex, weight, and species of predator on prey detectability (Hosseini et al. 2008). However, direct quantification of target prey species presents a unique set of methodological challenges, and to our knowledge only two studies have attempted such quantification in Collembola; one investigated the diversity of fungi in soils and in the guts of *Protaphorura armata* (Jørgensen et al. 2005); the second demonstrated the nematode predation by springtails in both the lab and the field (Read et al. 2006). The feeding experiment done by these authors under field conditions provided valuable ecological information, identifying two species of Collembola (*Isotoma viridis* and *Isotomurus palustris)* as significant predators of *Phasmarhabditis hermaphrodita* (Read et al. 2006). Another species, *Lepidocyrtus cyaneus*, did not feed on this nematode, demonstrating strong evidence of differences in prey choice. A previous PCR-based study had shown that *L. cyaneus* and two other Collembola species are themselves prey of linyphiid spiders (Agustí et al. 2003). These techniques, particularly gut-content analysis using PCR and prey-specific primers, may allow unprecedented progress in quantifying who is feeding on who without disturbing the system under study prior to predator collection (Symondson 2002, Sheppard & Harwood 2005, King et al. 2008).

Some other works report the feeding of springtails upon nematodes. Lee & Widden (1996) found that the 'fungivorous' collembolan, *Folsomia candida*, will preferentially feed upon the nematode *Caenorhabditis elegans* rather than fungi. Two species of Collembola, *F. candida* and *S. caeca*, have been observed consuming the entomopathogenic nematodes *Steinernema carpocapsae*, *Steinernema feltiae* and *Steinernema glaseri* (Gilmore & Potter 1993), which are biocontrol agents of wax moth larvae (*Galleria mellonella*) and Japanese beetle (*Popillia japonica*). Fatty acid and stable carbon isotope ($\delta^{13}C$) analysis have shown a strong feeding preference of *F. candida* and *Proisotoma minuta* for the nematodes over the offered fungi (Chamberlain et al. 2004, 2006). When given single diets *P. minuta* has been observed to consume nematodes in the laboratory (Chamberlain et al. 2005). The diet of the collembolan *Gomphiocephalus hodysoni* was investigated by microscopic examination of the contents of 197 faecal pellets and 32 guts. Detritus, filamentous cyanobacteria, eukaryotic microalgae and fungal hyphae were the most frequent contents found (Davidson & Broady 1996). Other studies reporting algal food consumed by Collembola were done by Wolters (1985), Verhoef et al. (1988), and Worland & Lukesova (2000). However, for many springtails species food organisms are not known, and some species have a much broader food range than others.

There is relatively little information concerning the diets of Collembola, especially *H. nitidus.* Previous work showed that this species was attracted to earthworms, particularly to their excretions of mucus and urine which were directly absorbed by Collembola (Salmon & Ponge 1999; Salmon 2001). This attraction may be responsible for the spatial distribution of *H. nitidus,* which is restricted to mull humus at pH > 5 (Salmon 2001). According to Arpin et al. (1980), *H. nitidus* is a coprophagous species; their intestinal contents were composed of excrements of other animals, but not of earthworms. Recently, the applicability of FAs as biomarkers for different diets-bacteria (Haubert et al. 2006), fungi (*Chaetomium globosum*) (Haubert et al. 2008) and three species of nematodes [*Aphelenchoides* sp., *Aphelenchoides saprophilus* Franklin, 1957, and *Acrobeloides buetschlii* (Man, 1884)] (Ruess et al. 2004)--has been verified for *H. nitidus*, reflecting the different diet of the consumer. Therefore, Pollierer et al. (2010) investigated the transfer of FAs from different basal resources [fungi (*Chaetomium globosum*), plant leaf litter (*Tilia europaea)*, Gram-positive (*Bacillus amyloliquefaciens*) and Gram-negative bacteria (*Stenotrophomonas maltophilia*)] via Collembola (*Heteromurus nitidus*) as first order consumers into predators

[*Lithobius forficatus* (Chilopoda) and *Pardosa lugubris* (Arachnida)]. Marker FAs of basal resources were clearly detectable in predators, suggesting that FA analysis allows separating trophic channels of soil food webs. Stable isotope analysis indicated that *Heteromurus nitidus* generally benefit from feeding on mixed diets (Scheu & Folger 2004). In single and mixed diet experiments, the author has investigated the reproduction of *Heteromurus nitidus* (Collembola) feeding on conidial fungi, ectomycorrhizal fungi and soil algae (*Chlorococcum infusorium)*. Feeding on mixed diets generally increased Collembola reproduction even in combinations of fungi/algae of high food quality with those of low food quality. Fractionation of ^{13}C and ^{15}N in *H. nitidus* feeding on single and mixed diets varied between diets and differed between juveniles and adults, being more pronounced in adult (-2.7‰) than in juvenile (-0.91‰) (Scheu & Folger 2004).

It may be easy to demonstrate that a particular species will prefer a specific type of food in the laboratory (Thiele & Larink 1990, Van Amelsvoort & Usher 1989), but more difficult to prove that it will be chosen under field conditions (Matic & Koledin 1985). It is difficult to avoid the conclusion that Collembola feed unselectively and that their gut contents represent a random selection of the components of their environment. Indeed the opportunistic nature of the feeding behaviour of many species of Collembola may be one reason for their success (Hopkin 1997).

Collembola are significant predators of nematodes in the field and hence may be capable of limiting nematode numbers in the soil. Our work has provided some baseline information about feeding habits of Collembola. This work can be expanded and dietary breadth of *Heteromurus nitidus* and other springtails species can be further carried out.

Molecular gut content analysis enables identification of species-specific trophic relationships among soil invertebrates, opening new directions to study soil food webs in Romania. DNA/PCR-based molecular analysis is a valuable complementary tool future which helps in generating knowledge about the feeding strategies of the predator species that would have been difficult with "non molecular" approaches.

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