Research Article

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Understanding trophic interactions of *Orius* spp. (Hemiptera: Anthocoridae) in lettuce crops by molecular methods

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Abstract

BACKGROUND: The aphid *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) are common pests in Mediterranean lettuce crops, where *Orius* spp. are common generalist predators. Predation by *Orius* spp. was studied in a lettuce plot by conventional polymerase chain reaction (PCR) and real-time PCR analyses using specific primers of both main pests. Also, high-throughput sequencing was used to have a wider approach of the diet of these predators in natural field conditions.

RESULTS: Molecular analyses indicated a higher predation on *N. ribisnigri* in spring and on *F. occidentalis* in summer. Predation on alternative prey, like Collembola, was also found in both seasons. Real-time PCR was more sensitive than conventional PCR in showing the target trophic links, whereas high-throughput sequencing revealed predation on other natural enemies – intraguild predation (IGP), showing other trophic interactions of *Orius majusculus* within the studied ecosystem.

CONCLUSIONS: This study gives important information about the trophic relationships present in Mediterranean lettuce crops in different periods of the year. The detected predation by *Orius* spp. on alternative prey, as well as on other natural enemies, should be further investigated to clarify whether it adds or detracts to the biological control of *N. ribisnigri* and *F. occidentalis*.

Keywords: conventional PCR; gut-content analysis; high-throughput sequencing; *Orius*; real-time PCR; trophic relationships

1 INTRODUCTION

The aphid *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) are two major pests of Mediterranean lettuce crops. Their biological control in integrated pest management (IPM) systems is based on the use of polyphagous predators, like the genus *Orius* Wolff 1811 (Hemiptera: Anthocoridae). Seven *Orius* species have been reported naturally to colonise Mediterranean vegetable crops: *O. majusculus* (Reuter), *O. laevigatus* (Fieber), *O. niger* (Wolff), *O. albidipennis* (Reuter), *O. minutus* (L.), *O. horvathi* (Reuter) and *O. laticollis* (Reuter).[1–5] Even though *Orius* spp. have been mainly associated with thrips,[3] lettuce aphids may also be consumed and could be an important component of their diets.[6] Generalist predators feed not only on pests but also on non-pest food, which may be particularly important when focal pest populations are scarce. Springtails (Collembola) are commonly present in arable ecosystems and may serve as alternative prey for biological control agents of pests.[7,8] There are many laboratory predation studies of *Orius* spp. on thrips,[9–14] as well as some under field conditions in the Mediterranean basin and the Middle East.[3,15–17] However, very little is known about *Orius* spp. predation on *N. ribisnigri* and *F. occidentalis*, as well as on other pests, alternative prey or even natural enemies, i.e. intraguild predation (IGP), under natural field conditions.

Trophic relationships are difficult to observe, particularly for small or cryptic arthropods. Traditional methods of visual observation of trophic interactions can be improved using gut dissection and microscopic characterisation of the gut contents, but this is only feasible when solid remains are present.[18] This is not possible for many arthropod predators, like *Orius* spp., which are fluid feeders that practise extraoral digestion.[19] Polymerase chain reaction (PCR)-based techniques provide alternative approaches for establishing trophic links between arthropod predators and their prey.[20] Through amplification of DNA sequences unique to food species, some identifications can be achieved by conventional PCR, even within highly degraded samples such as those found in faeces, gut contents or regurgitates.[21] This approach has been applied to a wide range of vertebrate and invertebrate

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predators. Real-time PCR or qPCR can add information to the interpretation of PCR-based gut analysis based on the accumulation of the amplified product as the reaction progresses.[22–24]

Understanding food webs requires reconstructing the overall population interactions of the taxa involved, as well as the strength of trophic linkages among the interacting community members. Traditional PCR techniques can establish trophic linkages but focus only on specific consumer–food interactions based on primer sequences that amplify the prey's specific DNA. High-throughput sequencing or next-generation sequencing (NGS) technologies can be used to examine dietary breadth without the need to design species-specific primers for each prey. Instead, a particular DNA fragment from all food items in a stomach is amplified using universal primers, those amplicons are sequenced and the identities of the organisms eaten can be established by using sequence databases. The potential of NGS for characterising simultaneously many species from an environmental sample through sequencing of DNA barcodes is enormous. As NGS technology spreads and reduces costs, ecologists are turning it into a powerful tool for ecological studies, including dietary analyses.[25–27] In some of these studies, because predator DNA is typically more prevalent than ingested prey DNA, blocking primers have been used to inhibit the amplification of predator DNA.[28]

In this study, we have looked more deeply into the trophic interactions of *Orius* spp. present in Mediterranean lettuce crops. For this we have first studied the *Orius* predation on the most abundant pests (*N. ribisnigri* and *F. occidentalis*) and the most abundant non-pest prey (Collembola) by conventional and qPCR using specific primers. Then, we have analysed the presence of other trophic interactions in the same agroecosystem using NGS technologies. These non-target trophic links could have consequences for the biological control of both insect pests.

2 MATERIALS AND METHODS

2.1 Arthropods

A colony of *N. ribisnigri* was established with specimens from the Centre for Research in Agricultural Genomics (CRAG), Bellaterra, Barcelona, Spain, on lettuce plants, and colonies of *F. occidentalis* and *O. majusculus* were established from specimens captured in vegetable crops from the El Maresme area (Barcelona, Spain) on green bean pods. These species were maintained under controlled conditions of 70 ± 10% relative humidity (RH), 16 h photoperiod and 25 ± 2 °C, except for *N. ribisnigri*, which was maintained at 19 ± 2 °C. *Orius majusculus* were fed with *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Biotop, Valbonne, France). Collembola (*Entomobrya* spp.) were collected in an experimental lettuce plot (described below) at IRTA facilities. *Orius minutus*, *O. laticollis* and *O. horvathi* were collected in La Selva del Camp (Tarragona), and *O. laevigatus*, *O. albidipennis* and *O. niger* in El Maresme (Barcelona).

2.2 Field experiments

An experimental lettuce plot (var. Maravilla) was established at IRTA facilities in Cabrils, Barcelona, Spain (41.518° N, 2.377° E). Two consecutive lettuce crops were planted from early April to late May (spring crop) and from early June to early August (summer crop) in two years (2009 and 2010).

In order to ensure the availability of sufficient target pests and *Orius* spp. to be analysed, 22 cages (40 × 90 × 60 cm) were randomly placed in the experimental plot. Each cage enclosed four lettuce plants on which additional *O. majusculus* and both target pests were added to those naturally present in the crop [15 *O. majusculus* (either females or nymphs), 25–70 *N. ribisnigri* and 30–75 *F. occidentalis* per plant]. Cages were placed in spring 2009 (14 and 21 May, $n = 3$ cages with *O. majusculus* nymphs per date), in summer 2009 (2, 6, 9 and 16 July, $n = 4$ cages with *O. majusculus* nymphs, $n = 3$ cages with *O. majusculus* nymphs, $n = 3$ cages with *O. majusculus* females and $n = 2$ cages with *O. majusculus* nymphs respectively) and in summer 2010 (23 July and 8 August, $n = 2$ cages with *O. majusculus* females). After 48 h, lettuce plants were cut and individually placed into plastic bags. All collected *Orius* spp. were frozen at −20 °C until molecular analysis. Prior to DNA extraction, they were all checked for attached prey remains under a microscope and cleaned of potential remains of other species; additionally, gender and developmental stage were determined.

Because several *Orius* species might be present in the sampled lettuces and some of them are difficult to identify by morphological methods (particularly the nymphs), molecular identification analyses were conducted individually by conventional PCR using primers (F2/R2), which amplify fragments of the internal transcribed spacer-1 (ITS-1) region of the nuclear ribosomal DNA, as described in a previous study.[5]

Once identified, all *Orius* specimens from those cages were analysed by conventional PCR using *N. ribisnigri*-, *F. occidentalis*- and Collembola-specific primers (see next section) to determine the detection percentages of each prey in females (more voracious than males) and/or nymphs of each *Orius* species.

2.3 Conventional PCR analyses

DNA was extracted from individual arthropods using the DNeasy Tissue kit (protocol for animal tissues; Qiagen, Hilden, Germany). Total DNA was eluted into 100 mL of AE buffer and stored at −20 °C. Negative controls were added to each DNA extraction set. Samples were amplified using a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR reaction volumes (25 μL) contained 4 μL of template DNA, 0.6 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 0.2 mM of dNTPs (Promega, Madison, WI, USA), 0.6 μM of each primer and 5 mM of $MgCl_2$ in 10× manufacturer's buffer. The specific primers used for the detection of *N. ribisnigri* (Nr1F/Nr2R and Nr3F/Nr3R) and *F. occidentalis* (Fo1F/Fo1R) were previously designed from the mitochondrial cytochrome oxidase I (COI) region.[27] These primers produced amplicons of 331 and 154 bp for *N. ribisnigri*, and 292 bp for *F. occidentalis*. Collembola-specific primers (Col4F/Col5R) designed from the 18S region produced an amplicon of 177 bp.[8] Samples were amplified for 35 cycles at 94 °C for 30 s, 58 °C (Fo1F/Fo1R) or 62 °C (Col4F/Col5R, Nr1F/Nr2R and Nr3F/Nr3R) for 30 s and 72 °C for 45 s. A denaturation cycle of 94 °C for 2 min initiated the PCR, and the reaction was terminated with a final extension at 72 °C for 5 min. Target DNA and water were always included as positive and negative controls respectively. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualised under UV light.

2.3.1 *Primer specificity and prey DNA decay rates*

The specificity of *Nasonovia ribisnigri*, *F. occidentalis* and Collembola primers was screened in a previous study[27] for most of the potential prey and natural enemies present in vegetable crops in the area of study (predators and parasitoids).[6,29,30] In the present study, specificity of those primers has been completed with the *Orius* spp. present in the same area (Table 1). This was done by conventional PCR and testing 2–5 individuals of each species.

Table 1. Species tested in the present study for cross-reactivity using the specific primers for *Nasonovia ribisnigri* (Nr1F/Nr2R, 331 bp, and Nr3F/Nr3R, 154 bp), *Frankliniella occidentalis* (Fo1F/Fo1R, 292 bp) and Collembola (Col4F/Col5R, 177 bp)[27]

Order	Family	Species[a]	Nr1F/Nr2RNr3F/Nr3R	Fo1F/Fo1R	Col4F/Col5R
Hemiptera	Anthocoridae	*Orius majusculus*	–	–	–
		Orius laevigatus	–	–	–
		Orius albidipennis	–	–	–
		Orius horvathi	–	–	–
		Orius laticollis	–	–	–
		Orius minutus	–	–	–
		Orius niger	–	–	–
Hemiptera	Aphididae	***Nasonovia ribisnigri***	+	–	–
Thysanoptera	Thripidae	***Frankliniella occidentalis***	–	+	–
Collembola	Entomobryidae	***Entomobrya* sp.**	–	–	+

[a] In bold, the target species detected with their respective specific primers.

Orius majusculus feeding trials were performed to establish prey decay rates within the predator guts. Ten females of *O. majusculus* were analysed for each time period and each prey species (*N. ribisnigri*, *F. occidentalis* and Collembola). Individual females were placed into 1.5 mL tubes and starved for 24 h with a moistened piece of cotton at 25 °C. After that, they were placed in transparent plastic boxes (2.5 cm diameter) with four individuals of *N. ribisnigri* (immatures), *F. occidentalis* (second instars) or Collembola (*Entomobrya* spp. adults). Predators were allowed to consume them for up to 2.5h at room temperature. Individuals that had consumed 2–4 items were immediately frozen ($t = 0$ h) or maintained individually without prey at 25 °C for 2, 4 or 8 h and frozen at −20 °C until PCR analysis. Each predator was tested up to 3 times and considered positive if prey DNA was detected in one of them. The number of positive *O. majusculus* was recorded and the percentage of positives was calculated for each post-ingestion period. The time interval associated with 50% positive responses (i.e. detectability half-life) was calculated by reverse prediction from best-fitted equations.

2.4 Real-time PCR analyses

Real-time and conventional PCR-based methods were compared for sensitivity toward *N. ribisnigri* detection. Because qPCR optimally amplifies PCR products with short (<200 bp) amplicons, the predation comparison between conventional PCR and qPCR was conducted on some *Orius* spp. specimens using the *N. ribisnigri*-specific pair of primers (Nr3F/Nr3R), which amplified the shortest amplicon (154 bp). These specimens were *O. majusculus* from the *N. ribisnigri* feeding trials (0, 2, 4 and 8 h after feeding, $n = 10$ for each time period), together with some selected *Orius* spp. specimens from the 2009 field cages (16 nymphs from 14 May, 28 nymphs from 21 May, 29 nymphs from 2July and 30 females from 9 July). qPCR reactions (25 µL) contained 12.5 µL of 2× Brilliant SYBR Green qPCR master mix (Qiagen), 0.3 µM of each primer, 1 µL of template DNA and 9.5 µL of PCR-grade water. Reactions were run on an MX3000P qPCR thermocycler (Stratagene, La Jolla, CA) using the following conditions: 95 °C for 15 min, followed by 50 cycles of 94 °C for 15 s, 53 °C for 30 s and 72 °C for 30 s.

2.5 High-throughput sequencing analyses

A few *O. majusculus* specimens collected in spring inside the field cages were tested by NGS. They were analysed using the Ion Torrent personal genome machine (PGM) platform with the general arthropod primers ZBJ-ArtF1c and ZBJ-ArtR2c,[31,32] which amplify a fragment of 157 bp located within the COI barcode region. Previous PCR analyses conducted with these general primers showed that some arthropod species were not amplified, including the two target prey species of the present study, *N. ribisnigri* and *F. occidentalis*.[27,31,32] Nevertheless, we decided to use them because we were able to amplify a curtailed range of other arthropods potentially present in the studied and other agroecosystems, including: *Forficula auricularia* L. (Dermaptera: Forficulidae); *Theridion* sp. Walckenaer (Araneae: Theridiidae); *Philodromus cespitum* (Walckenaer) (Araneae: Philodromidae); *Xysticus* sp. Koch (Araneae: Thomisidae); *Centromerita bicolor* (Blackwall), *Clubiona* sp. Latreille, *Pachygnatha degeeri* Sundevall and *Pachygnatha clercki* Sundevall (Araneae: Linyphiidae); *Adalia decempunctata* (L.) (Coleoptera: Coccinellidae); *O. majusculus*; *O. laevigatus; Macrolophus pygmaeus* Rambur (Hemiptera: Miridae); *Trichopsocus clarus* (Banks) and *Ectopsocus briggsi* McLachlan (Psocoptera: Ectopsocidae); *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae); *Aphis gossypii* Glover and *Aphis spiraecola* Patch (Hemiptera: Aphididae); *Entomobrya* sp. Rondani (Arthropoda: Collembola); and *Aphidius colemani* Dalman (Hymenoptera: Braconidae).

Because the predator used in this study, *O. majusculus*, was one of the amplified species, we developed an *O. majusculus*-specific blocking probe to inhibit its DNA amplification as described in Vestheim and Jarman[28] and Deagle *et al.*[33] This blocking primer (BloOm2 5′-TATATTTTATTTTTGGGATATGAGCAGGAATAC-3′-C3) was modified with a C3 spacer at the 3′-end of the forward universal arthropod primer (ZBJ-ArtF1c), preventing elongation during the PCR without noticeably influencing its annealing properties. To evaluate the efficiency of the blocking primer, conventional PCR amplifications were performed on *O. majusculus* DNA with primers ZBJ-ArtF1c and ZBJ-ArtR2c, and adding different concentrations of the blocking primer BloOm2. PCR reactions (10 µL) were conducted using 0.2 µM of each of the primers ZBJ-ArtF1c and ZBJ-ArtR2c, 9 µL of Platinum® PCR SuperMix High Fidelity (Invitrogen) and 0.6 µL of template DNA. The blocking primer was included at 1–6 times the concentration of PCR primers during amplification. Samples were amplified for 40 cycles at 94 °C for 30 s, 45 °C for 45 s and 68 °C for 45 s. A first denaturation cycle of 94 °C for 5 min and a final extension at 68 °C for 10 min were carried out. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualised under UV light.

Table 2. Fusion primers used for sequencing *Orius majusculus* field-collected specimens in the Ion Torrent PGM

Primer name[a]	Sequence[b]
AkT1 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*CTAAGGTAACAGATATTGGAACWTTATATTTTATTTTTGG
AkT2 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*TAAGGAGAACAGATATTGGAACWTTATATTTTATTTTTGG
trP1-ZBJ (R)	CCTCTCTATGGGCAGTCGGTGATWACTAATCAATTWCCAAATCCTCC

[a] F = forward; R = reverse.
[b] In bold, 'A' sequence; in italics, 'key' sequence; underlined, barcodes (tags) to identify bulks; double underlined, 'trP1' sequence (Ion Torrent, Life Technologies, 2011); dotted underlined are ZBJ-ArtF1c and ZBJ-ArtR2c primers.[32]

Fusion primers needed for the NGS analyses were prepared following the Ion Torrent recommendations (Life Technologies Corporation, Carlsbad, CA, USA, 2011), consisting of (i) the Ion Torrent primer A linked to the specific forward primer (ZBJ-ArtF1c), and (ii) the Ion Torrent primer trP1 linked to the specific reverse primer (ZBJ-ArtR2c). Two fusion forward primers were prepared, each one having a different 10 bp barcode (Tag) before the forward primer (ZBJ-ArtF1c) to allow the multiplexing of two bulk samples in a single sequencing run (Table 2).

Samples were analysed in two bulks as follows: 22 *O. majusculus* from spring 2009 field cages (14 May, $n = 14$ nymphs; 21 May, $n = 8$ nymphs), which were positive for any of the three prey tested (*N. ribisnigri*, *F. occidentalis* and/or Collembola) in conventional and/or qPCR analyses (bulk 1); 18 *O. majusculus* from spring 2009 field cages (14 May, $n = 8$ nymphs; 21 May, $n = 10$ nymphs), which were negatives for all three prey tested in conventional and qPCR analyses (bulk 2). Each bulk was amplified in 40 µL PCR reactions that contained 2.4 µL of template DNA, 36 µL of Platinum® PCR Supermix High Fidelity (Invitrogen), 0.2 µM of each fusion primer and 2.5 times the concentration of fusion primers of blocking primer (i.e. 2 µL). Samples were amplified for 40 cycles at 94 °C for 30 s, 45 °C for 45 s and 68 °C for 45 s following an initial denaturation step at 94 °C for 5 min and before a final extension step at 68 °C for 10 min. PCR products were purified with the QIA-quick PCR Purification kit (Qiagen). Fragments obtained (157 bp) were purified (E-Gel® SizeSelect 2% agarose gel, Invitrogen) and quantified (DNA High Sensitivity kit, Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA, USA), and each bulk was prepared as an equimolar pool. Then, each pool was sequenced on a Ion Torrent PGM platform as described by the manufacturer (Ion Torrent, Life Technologies) using a single 314 chip with the sequencing chemistry for a 200 bp read length and version 2.2 of the Torrent Suite software for base calling (Ion Torrent, Life Technologies).

The output of the massive sequencing process was treated bioinfomatically to discard any remaining *O. majusculus* reads. All reads obtained from each of the two bulks were separated by the Ion Torrent software itself in two different FASTQ files, taking advantage of the sequence barcodes (tags) included in the forward fusion primers (Table 2). Subsequent analyses were carried out in parallel with the two files corresponding to the two experimental situations (bulks), following the methodology explained in Gomez-Polo *et al.*,[27] based on the use of BLAST (NCBI website) and MEGAN (MEtaGenomics Analyzer[34]) to compute and explore the taxonomical content of the dataset.

3 RESULTS

3.1 Primer specificity and prey DNA decay rates

Specific primers of *N. ribisnigri* (Nr1F/Nr2R and Nr3F/Nr3R), *F. occidentalis* (Fo1F/Fo1R) and Collembola (Col4F7/Col5R) showed successful amplifications of the three target prey. When the four pairs of primers were tested for cross-amplification against the most common *Orius* species found in the studied area, only the target species were detected, showing high specificity (Table 1).

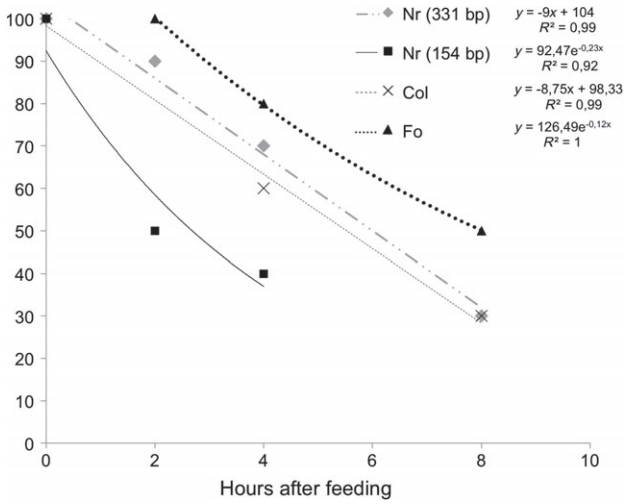

Figure 1. Detection curves of ingested *Nasonovia ribisnigri* (primers Nr1F/Nr2R, 331 bp, and Nr3F/Nr3R, 154 bp), *Frankliniella occidentalis* (primers Fo1F/Fo1R, 292 bp) and *Entomobrya* spp. (primers Col4F/Col5R, 177 bp) by *Orius majusculus* at different times after ingestion. Nr: *Nasonovia ribisnigri*; Fo: *Frankliniella occidentalis*; Col: Collembola.

When these primers were used to analyse *O. majusculus* females fed with *N. ribisnigri*, *F. occidentalis* or *Entomobrya* spp., all predators tested positive immediately after ingestion for the three target prey. Detection after being maintained for 4 h at 25 °C was variable, but never lower than 40% (Fig. 1). The detection of these three prey within *O. majusculus* gut was better fitted to an exponential decay for *N. ribisnigri*-specific primers (Nr3F/Nr3R; 154 bp) and *F. occidentalis*-specific primers, and a linear decay for *N. ribisnigri*-specific primers (Nr1F/Nr2R; 331 bp) and Collembola-specific primers. Half-lives calculated from these equations were 2.7 h for *N. ribisnigri* (Nr3F/Nr3R; 154 bp), 5.5 h for Collembola, 6 h for *N. ribisnigri* (Nr1F/Nr2R; 331 bp) and 8.6 h for *F. occidentalis*.

3.2 Conventional PCR analyses of field-collected *Orius* spp.

When field cages were opened, 346 *Orius* spp. specimens ($n = 80$ nymphs in spring 2009, $n = 166$ nymphs in summer 2009 and $n = 100$ females in summer 2009 + 2010) were found, which were all molecularly identified as described in Gomez-Polo *et al.*[5] As expected, because of being the species introduced into the cages, the most abundant *Orius* species was *O. majusculus* (84, 65 and 95% in spring 2009, summer 2009 and summer 2009 + 2010

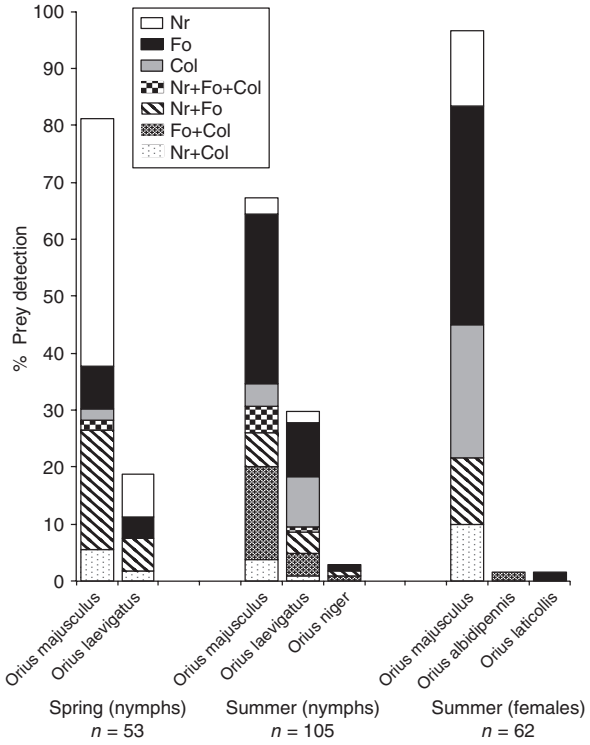

Figure 2. Molecular prey percentages obtained within the positive *Orius* nymphs and females collected in the field cages of the experimental lettuce plot in three different periods: spring 2009 (nymphs), summer 2009 (nymphs) and summer 2009 + 2010 (females). Nr: *Nasonovia ribisnigri*; Fo: *Frankliniella occidentalis*; Col: Collembola.

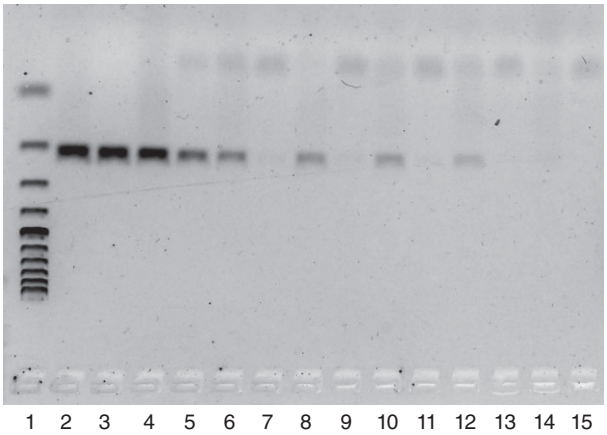

Figure 3. Conventional PCR amplifications using different concentrations of the *Orius majusculus* blocking primer (BloOm2). Lane 1: DNA size marker (100 bp ladder); even-numbered lanes correspond to an *O. majusculus* specimen fed on *Entomobrya* spp. (Collembola) and frozen immediately after consuming three individuals; odd lanes correspond to a starved (24 h, 25 °C) *O. majusculus*. Lanes 2 and 3: without BloOm2; lanes 4 and 5: with 1× BloOm2; lanes 6 and 7: with 2×; lanes 8 and 9: with 3×; lanes 10 and 11: with 4×; lanes 12 and 13: with 5×; lanes 14 and 15: with 6×.

respectively). However, owing to the natural colonisation prior to the placement of the cages, other *Orius* species were found in the plot: *O. laevigatus* (16, 30 and 1% in spring 2009, summer 2009 and summer 2009 + 2010 respectively), *O. niger* (4 and 1% in summer 2009 and summer 2009 + 2010 respectively), *O. albidipennis* (1 and 2% in summer 2009 and summer 2009 + 2010 respectively) and *O. laticollis* (1% in summer 2009 + 2010).

Prey DNA was detected in 64% of all sampled *Orius* ($n = 346$) by conventional PCR using the primers Nr1F/Nr2R (331 bp), Fo1F/Fo1R (292 bp) and Col4F/Col5R (177 bp). Taking only those positive predators, 65% of them had consumed only one prey species, 32% had consumed two and 3% had consumed all three. From those in which only one prey species was detected, 52, 28 and 20% were positive for *F. occidentalis*, *N. ribisnigri* and Collembola respectively. From those that consumed two prey species, 46, 33 and 21% were positive for *N. ribisnigri* + *F. occidentalis*, *F. occidentalis* + Collembola and *N. ribisnigri* + Collembola respectively. Figure 2 shows prey detection rates of those *Orius* individuals that gave a positive detection according to the detected prey, *Orius* species and period (spring 2009, $n = 53$ nymphs; summer 2009, $n = 105$ nymphs; summer 2009 + 2010, $n = 62$ females). A higher predation was recorded for *N. ribisnigri* than for *F. occidentalis* or Collembola in spring, whereas in summer the detection rate for *F. occidentalis* was higher than for *N. ribisnigri* or Collembola.

When *Orius* nymphs and females were analysed separately, the positive nymphs ($n = 157$; spring 2009 and summer 2009 together) were 60% positive for one prey (30, 20 and 10% of *F. occidentalis*, *N. ribisnigri* and Collembola respectively), 36% positive for two prey (16, 14 and 6% for *N. ribisnigri* + *F. occidentalis*, *F. occidentalis* + Collembola and *N. ribisnigri* + Collembola respectively) and 4% positive for all three prey. The percentages of positive *Orius* females ($n = 60$; summer 2009 and 2010 together) were 77% positive for one prey (40, 14 and 23% for *F. occidentalis*, *N. ribisnigri* and Collembola respectively) and 23% positive for two prey (12, 1 and 10% for *N. ribisnigri* + *F. occidentalis*, *F. occidentalis* + Collembola and *N. ribisnigri* + Collembola respectively). None of the *Orius* females was positive for all three prey.

3.3 Real-time PCR analyses of laboratory and field-collected *Orius* spp.

When *N. ribisnigri* feeding trials by *O. majusculus* were analysed by qPCR, 100% detection was obtained at $t = 0$, 2 and 4 h post-ingestion, and 90% detection was obtained after 8 h. These results revealed a much higher frequency of detection in the same specimens and primer sets than that obtained using conventional PCR (shown in Fig. 1).

When some individuals of *Orius* spp. from spring field cages ($n = 44$ nymphs) and summer field cages ($n = 59$ nymphs and adults) were analysed by qPCR, *N. ribisnigri* detection percentages were 44 and 73% respectively. The same *Orius* specimens had a much less frequent detection level when the specimens were analysed using conventional PCR (15% in spring and 44% in summer), showing again a higher sensitivity using qPCR compared with conventional PCR.

3.4 High-throughput sequencing analyses of field-collected *O. majusculus*

When different concentrations of the designed blocking primer (BloOm2) were evaluated by conventional PCR to determine the optimal concentration for blocking *O. majusculus* DNA amplification, it was shown that predator DNA was sufficiently blocked when the concentrations of the blocking primer doubled (2×) the concentration of the fusion primers (Fig. 3). To be conservative, 2.5 times the amount of blocking primer was added to the Ion Torrent reactions.

Table 3. Number of reads (sequences) and percentages of prey obtained by Ion Torrent PGM after analysing two bulks of *Orius majusculus* collected in the spring field cages of the experimental lettuce plot

	Bulk 1 (+ for prey) $n = 22$	Bulk 2 (− for prey) $n = 18$
Number of reads (raw)	75 401	92 915
Number of reads (good)	36 648	47 319
Number of reads (non-*O. majusculus*)	309	174
Detected prey	Percentages (%)	
Orius laevigatus (Hemiptera: Anthocoridae)	0	6.2
Syrphini	1.6	1.2
Episyrphus balteatus (Diptera: Syrphidae)	97.8	90.2
Diptera	0.3	0.6
Lepidoptera	0	1.2
Adalia decempunctata (Coleoptera: Coccinelidae)	0	0.6
Oedothorax fuscus (Araneae: Linyphiidae)	0.3	0

The Ion Torrent PGM platform run produced two FASTQ files, and the quality control process reduced the number of reads, but in spite of the use of a specific *O. majusculus* blocking probe, most of the reads (>99%) still belonged to the predator (Table 3). The Ion Torrent sequencing provided 483 prey sequences (taking both bulk samples) useful to describe the diet of *O. majusculus* (Table 3). None of these prey sequences corresponded to *N. ribisnigri* and *F. occidentalis* because, as previously mentioned, the general invertebrate primers used (ZBJ-ArtF1c and ZBJ-ArtR2c) did not amplify these species. The obtained prey sequences were clearly dominated in both bulks by hoverflies of the tribe Syrphini and the species *Episyrphus balteatus* (De Geer) in particular (Table 3). The other prey sequences obtained were all from species known to be present in the studied area, being detected in much lower percentages. They corresponded to another *Orius* species (*O. laevigatus*), the lyniphid spider *Oedothorax fuscus*, some Lepidoptera, the coccinellid *A. decempunctata* and other unidentified Diptera.

4 DISCUSSION

In this study, we show the molecular detection of *N. ribisnigri*, *F. occidentalis* and Collembola within several *Orius* species, which are common polyphagous predators in Mediterranean lettuce crops. The use of conventional PCR-qPCR and Ion Torrent in parallel has given complementary information about their diet. This study has demonstrated that *Orius* spp. preyed on pest as well as on non-pest prey, and that other interactions, like IGP, are also present in this agroecosystem. Considering these results, we advocate understanding the constraints and benefits of each form of molecular analysis, and using multiple approaches for describing trophic interactions. As discussed in Pompanon *et al.*,[26] NGS provides an excellent tool for initial screening of predators, supplying an invaluable guide to the composition and range of species consumed. Then, it can be followed by complementary PCR analyses based upon species- and group-specific primers directed at those prey groups of main interest.

When conventional PCR was used to assess the digestion rates of *O. majusculus* fed on *N. ribisnigri*, *F. occidentalis* and Collembola, it was demonstrated that prey DNA was rapidly digested, as shown by the half-lives obtained (between 5.5 and 8.6 h). These half-lives were all within the same order of magnitude, as were those obtained with the hoverfly predator *E. balteatus* in a companion study[27] that used the same *N. ribisnigri*-, *F. occidentalis*- and Collembola-specific primers. Therefore, the interpretation of the obtained predation percentages is not expected to be strongly biased, depending on the predator species. Half-life values obtained in both studies were quite short, as were those obtained with other hemipteran predators, like *M. pygmaeus*,[35] probably owing to their small size, the physiology of their digestive tract and their feeding habits. On the contrary, other predators, like the earwig *F. auricularia*, fed on aphids have shown a half-life of 24 h,[36] and the spider *Pardosa cribata* Simon (Araneae: Lycosidae) fed on *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) showed a half-life of 78 h.[37]

When *Orius* spp. collected from the field cages were analysed, a high proportion of specimens (64%) screened positive for *N. ribisnigri*, *F. occidentalis* or Collembola. This percentage was much higher than that observed in hoverfly larvae (36%) in the same study system,[27] showing a higher level of detection for *Orius* fed these prey species. In this experimental plot (outside the cages), *N. ribisnigri* was naturally more abundant in spring than in summer, whereas *F. occidentalis* had low natural abundance in spring, becoming more abundant in summer.[27] Inside the cages, *N. ribisnigri* and *F. occidentalis* abundances were modified to ensure prey availability (similar numbers of both pests were introduced in both seasons), and under this situation predators consumed all three prey (*N. ribisnigri*, *F. occidentalis* and Collembola) either alone or in different combinations (Fig. 2). Consumption of non-pest prey, like Collembola, may contribute to the maintenance of predator populations,[7] but further studies should be conducted to investigate whether this is the case in this agroecosystem. On the other hand, even having similar prey abundances inside the cages, different predation percentages were observed in spring and summer (higher predation of *N. ribisnigri* in spring and higher predation of *F. occidentalis* in summer) (Fig. 2), which seems to be related to the natural abundances found outside the cages. This may reflect the adaptation of these two insect pests released in the cages to the different temperatures present in the plot in both seasons. As shown in a previous study conducted in several Mediterranean lettuce plots,[38] the presence of *Orius* spp. in natural conditions was only observed in summer, when *F. occidentalis* was the main pest present in those crops. Therefore, even if *Orius* spp. can prey on *N. ribisnigri*, as shown in the present study, it is expected that these predators, which are mainly present in summer, feed on the main pest present at that moment, exerting a more significant control on this pest species than on *N. ribisnigri*.

Generalist predators need to diversify food intake to balance nutritional needs.[39,40] In this study, multiprey detection was observed because two or even three prey species were detected within the same *Orius* specimen. This multiprey detection was higher in *Orius* nymphs, showing higher percentages than adults. This agrees with data obtained by Harwood *et al.*,[41] where predation of *Orius insidiosus* (Say) nymphs and adults was studied and the proportion of nymphs containing more than one prey species was also higher than the adults. As stated by the authors, it is possible that dietary diversification was necessary, or at least more likely to promote growth and development of immature stages of this generalist predator. The nutrition of different food types may complement one another to provide an optimal diet to a predator.[42]

In this study, *N. ribisnigri* percentages were higher using qPCR than conventional PCR under optimal conditions of each technique. These results echo those observed with predation of *N. ribisnigri* by the hoverfly *E. balteatus*.[27] Other studies have obtained similar results using both techniques.[43–49] qPCR represents a significant advance in gut content analysis, with a number of technical advantages such as speed, sensitivity, reduced risk of contamination and less subjective conclusions (e.g. there are no bands on gels to interpret). However, conventional PCR could be more convenient, depending on the aim of the study, particularly considering the high number of PCRs to be run for a gut content analysis and the relative costs of reagents and equipment of these two approaches.

Ion Torrent NGS was used in this study to investigate further the dietary breadth of *O. majusculus* collected in spring field cages placed at the experimental lettuce plot. As mentioned in the Section 3, even if previous analyses revealed that these primers did not amplify the two focal prey (*N. ribisnigri* and *F. occidentalis*), we decided to go ahead with the NGS analyses for a more in-depth study of the diet of *O. majusculus*. This lack of amplification could be the consequence of a poor performance of the universal primers used, which on the other hand seems to be something common to all universal primers.[50] A possible solution to improve the chances of having all present species amplified might be the use of several universal primers in combination, like the primers used here together with those proposed in other studies.[51,52]

In the present study, prey sequences belonging to the tribe Syrphini were found in *O. majusculus* guts. This tribe includes many Mediterranean hoverfly genera, like *Episyrphus* Matsumura & Adachi, *Eupeodes* Osten Sacken, *Meliscaeva* Frey, *Scaeva* Fabricius and *Sphaerophoria* Lepeletier & Serville, which are common predators in Mediterranean lettuce crops.[27,53] *Episyrphus balteatus* was the most detected species (with more than 90% detection in each bulk), described as one of the most abundant hoverfly species at that experimental plot in spring.[27,53] The present study is the first to show hoverfly predation by *O. majusculus*, a form of IGP that merits further attention given its implications for the biological control of target pests. Other prey sequences obtained corresponded to species potentially present in the study system, like linyphiids spiders, lepidopterans, coccinellids, other dipterans and even another *Orius* species (*O. laevigatus*), highlighting the high suitability of NGS for identifying unknown trophic links between species. Previous studies conducted under laboratory conditions revealed that *Orius* spp. were able to prey on other natural enemies, like phytoseiids, coccinellids, spiders, other hemipterans and even parasitoids.[54–56] Some were even conducted under field and greenhouse conditions.[14,41,42,57,58] The species detected within *O. majusculus* in the present field study were common predators present in lettuce crops of the studied area, like hoverflies, coccinellids and spiders.[27,38] The information obtained by NGS in the present study about *O. majusculus* predation reveals unknown trophic interactions not only with pest species present in the crop but also with other biological control agents, which could have either positive or negative effects on the biological control of these target pests in this agroecosystem. This should be further investigated in order to develop suitable biological control programmes in lettuce for the main pest species.

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