Research Article

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Molecular assessment of predation by hoverflies (Diptera: Syrphidae) in Mediterranean lettuce crops†

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Abstract

BACKGROUND: Hoverflies (Diptera: Syrphidae) are generalist predators of a great variety of pests. *Nasonovia ribisnigri* (Hemiptera: Aphididae) and *Frankliniella occidentalis* (Thysanoptera: Thripidae) are two common pests in Mediterranean lettuce crops, where they occur alongside alternative prey (e.g. Collembola). A semi-field experiment was conducted in an experimental lettuce plot where hoverfly predation on *N. ribisnigri*, *F. occidentalis* and Collembola was studied by conventional PCR and qPCR using specific primers, as well as by next-generation sequencing (NGS) in order to reveal other potential trophic interactions.

RESULTS: Trophic linkages between hoverflies and *N. ribisnigri* were the strongest both in spring and summer. *F. occidentalis* and Collembolans were also detected in both seasons, but with less frequency. qPCR detected a higher frequency of consumption than conventional PCR when both tests were run at optimal conditions. NGS analyses showed intraguild predation on other hoverfly species, as well as on anthocorids, spiders and even aphid parasitoids.

CONCLUSIONS: Conventional PCR and qPCR provided important insights into Mediterranean hoverfly species predation on target pest and non-pest prey. NGS gave a complementary approach revealing a broader diet of these predators within the studied ecosystem.

Keywords: gut content analysis; polyphagous predators; hoverflies; conventional PCR; qPCR; next-generation sequencing

1 INTRODUCTION

Understanding trophic linkages in a community can facilitate the development of conservation biological control (CBC) programmes in agroecosystems. Hoverflies (Diptera: Syrphidae) are commonly found in Mediterranean vegetable crops. Adults mainly consume nectar and pollen, but larvae of many species are polyphagous predators of a broad range of soft body insects, such as coleopteran and lepidopteran larvae,[1] as well as aphids, which are a preferred prey for most hoverfly species.[2] *Episyrphus balteatus* (De Geer) is the most abundant hoverfly species in Europe. It is commonly found in most terrestrial habitats and is even commercially available as a biological control (BC) agent. Other hoverfly species commonly found in Mediterranean vegetable crops are: *Scaeva pyrastri* (L.), *Eupeodes corollae* (F.), *Meliscaeva auricollis* (Meigen), *Sphaerophoria scripta* (L.) and *Sphaerophoria rueppellii* (Wiedemann).[3–5] Although several studies have examined predation by Mediterranean hoverfly larvae under laboratory conditions[6–8] and in lettuce fields of North America,[9–12] very little is known about hoverfly predation in Mediterranean lettuce crops. In these crops, two major pests are the aphid *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae). Hoverfly larvae may feed not only on pests but also on alternative prey species. Springtails (Collembola) are a common alternative prey in agroecosystems, which could be used for predator reproduction and maintenance of their physiological status.[13,14] These small wingless hexapods are soil and litter dwelling and are virtually ubiquitous in terrestrial systems. They are polyphagous, feeding in decomposed plants, pollen, cadavers and soil microorganisms.[15] Food webs involving generalist predators can be troublesome to construct using microscopic gut analysis or visual observation.[16] Microscopic gut analysis is a useful technique for describing insect diets based on solid food fragments, but syrphid larvae are

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exclusively fluid feeders. Visual observation can also be a valuable tool for assessing dietary diversity, but it is time consuming to generate meaningful sample sizes and can disrupt normal predator foraging decisions.

PCR-based methods can be used to detect prey DNA within the gut contents of predators. Conventional PCR visualises prey DNA products as size-specific bands on an agarose gel.[17] On the other hand, real-time or quantitative PCR (qPCR) builds upon conventional PCR by including a fluorescent dye that binds to double-stranded DNA, and thus the quantity of DNA produced in each PCR cycle is monitored using a spectrophotometer during the PCR process.[18] This technique requires a special thermocycler and specific reagents used for fluorescence, but does not require equipment associated with gel analysis used in conventional PCR. Instead, positive samples are distinguished on the basis of the strength of their fluorescent signal, leading to less subjective assignment of positive results relative to bands on an agarose gel. The qPCR-based method has been used in just a few predation studies of arthropods,[16,19–22] but its strength as a gut analysis tool relative to conventional PCR has not been well tested in studies of this kind.

Next-generation sequencing (NGS) technologies offer the opportunity for describing the dietary breadth of an arthropod, not possible using conventional PCR or qPCR. Using generalised primer sets and then sequencing the resulting molecules, NGS allows the identification of a full range of food items present in the guts of a given organism without the need for designing species-specific primers for each prey. Furthermore, identification of prey species is based on the amplification of short DNA fragments (100–350 bp), characteristic of those obtained from stomach contents or faeces, making the NGS approach very appropriate for the gut analysis of arthropods.[23,24] Indeed, some NGS technologies, such as the 454 Roche and Illumina platforms, have been used to study the diet of vertebrates and invertebrates.[23,25–29] Here, we assessed the suitability of the Ion Torrent Personal Genome Machine (PGM) NGS technology[30] to describe the diet of predatory syrphids. Because these analyses rely on general arthropod primers, a blocking primer is needed to inhibit the amplification of predator DNA.[31,32]

To summarise, the aims of this study were: (1) to analyse predation by hoverfly larvae on two major pests of Mediterranean lettuce crops (*N. ribisnigri* and *F. occidentalis*) and the most abundant non-pest prey (Collembola) in semi-field conditions using conventional and qPCR approaches; (2) to compare predation percentages obtained by conventional and qPCR; (3) to use the Ion Torrent PGM for more complete description of the diet of *E. balteatus* in Mediterranean lettuce crops.

2 MATERIALS AND METHODS

2.1 Arthropods

The colony of *E. balteatus* was established with specimens from Koppert (Berkel en Rodenrijs, The Netherlands). *Episyrphus balteatus* adults were reared on *Lobularia maritima* L. supplemented with commercial bee pollen, and larvae were maintained on lettuce plants infested with *N. ribisnigri*. The colony of *N. ribisnigri* was established with samples from the Centre for Research in Agricultural Genomics (CRAG) (Bellaterra, Barcelona, Spain), and the colony of *F. occidentalis* with specimens captured in vegetable crops from the El Maresme area (Barcelona, Spain). *Nasonovia ribisnigri* were reared on lettuce plants, and *F. occidentalis* on green beans. All insects were reared under controlled conditions of 70 ± 10% relative humidity (RH), 16:8 h light:dark and 25 ± 2 °C, except for *N. ribisnigri*, which was reared at 19 ± 2 °C. Collembola were obtained from an experimental lettuce plot near IRTA, where *Entomobrya* was the most abundant genus. Other aphid species tested for specificity (see Section 2.3.1) came from colonies maintained at the Institute of Agricultural Sciences – Spanish National Research Council (ICA-CSIC) (Madrid, Spain).

2.2 Prey-specific primer design

Three pairs of primers were designed from the mitochondrial cytochrome oxidase I (COI) region as described in Agustí *et al.*[33] (Table 1). Two of them were designed for the detection of *N. ribisnigri* [one pair for conventional PCR analysis (Nr1F/Nr2R) and another one, which amplifies a shorter fragment, for comparing conventional PCR and qPCR analysis (Nr3F/Nr3R)], and one for *F. occidentalis* (Fo1F/Fo1R). The following sequences from the GenBank database (www.ncbi.nlm.nih.gov) were used for primer design: EU701812.1 (*N. ribisnigri*), EU701799 [*Myzus persicae* (Sulz.)], EU701728 (*Macrosiphum euphorbiae* Thomas), FN545994 (*F. occidentalis*), FN546171 [*Thrips tabaci* (Lindeman)], EU241740 (*E. balteatus*), EU241792 (*S. scripta*), EF127328 (*S. rueppellii*), FM210189 [*Orius majusculus* (Reuter)] and FM210187 [*Orius laevigatus* (Fieber)]. Sequences were aligned using CLUSTALW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). Collembola-specific primers (Col4F/Col5R) were previously designed to target the 18S region.[34]

2.3 Conventional PCR

DNA was extracted from whole individual insects using the DNeasy Tissue kit (Qiagen, Hilden, Germany; protocol for animal tissues). Total DNA was eluted in AE buffer (100 µL) provided by the manufacturer and stored at −20 °C. Buffer-only controls were added to each DNA extraction set. Samples were amplified in a 2720 thermal cycler (Applied Biosystems, Foster City, CA). Reaction volumes (25 µL) contained resuspended DNA (4 µL), *Taq* DNA polymerase (0.6 U) (Invitrogen, Carlsbad, CA), dNTPs (0.2 mM) (Promega, Madison, WI), each primer (0.6 µM) and $MgCl_2$ (5 µM) in 10× buffer from the manufacturer. Target DNA and water were always included as positive and no-template controls respectively. 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. For all reactions, the first denaturation cycle was at 94 °C for 2 min, and the final extension cycle was at 72 °C for 5 min. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualised under UV light.

2.3.1 Species specificity and detection periods

Nasonovia ribisnigri, *F. occidentalis* and Collembola primer pairs were screened by conventional PCR against 2–5 individuals of common non-target species potentially present in vegetable crops in the area of study, as well as other natural enemies, such as other hoverfly species and parasitoids (Table 2).

Feeding trials involving *E. balteatus* larvae were performed in order to determine prey detection decay rates within the predator's gut. Individual larvae (second to third instar) were placed into 1.5 mL tubes with a moistened piece of cotton and starved for 48 h at 25 °C. Next, they were placed in small transparent plastic boxes (2.5 cm diameter) with eight individuals of *N. ribisnigri* (wingless), *F. occidentalis* (second instars) or Collembola (*Entomobrya* sp.

Table 1. Prey-specific primers used: target species, sequence (5′–3′), amplified fragment length, region targeted and study where they are described

Target species	Primer name	Sequence	Length (bp)	Region	Reference
N. ribisnigri	Nr1F	TATTAGATTTTGATTATTACCTCCATCT	331	COI	Present study
	Nr2R	TAATATTGTAATAGCACCG			
	Nr3F	TCAAATTCCTTTATTCCCT	154	COI	Present study
	Nr3R	TAGGATAGGATCTCCTCCT			
F. occidentalis	Fo1F	AGTTTACCCACCTTTGTCAACT	292	COI	Present study
	Fo1R	ACCTCCTCTCGGATCAAAGAAGGAT			
Collembola	Col4F	GCTACAGCCTGAACAWTWG	177	18S	34
	Col5R	TCTTGGCAAATGCTTTCGCAGTA			

adults). Predators were allowed to consume them for up to 2.5 h at room temperature. Only those that had consumed 5–6 items were frozen after the exposure period ($t = 0$ h) or maintained individually without prey at 25 °C for 2, 4 or 8 h and frozen at −20 °C until PCR analysis. Ten individuals of *E. balteatus* were analysed for each time period and food. Each predator was tested up to 3 times and considered to be positive if prey DNA was detected in one of them. The time interval associated with 50% positive responses (i.e. median detection time) was estimated by reverse prediction from best-fitted (linear or exponential) equations.

2.3.2 Field experiment

Conventional PCR analyses were conducted for studying predation by *E. balteatus* of *N. ribisnigri*, *F. occidentalis* and Collembola. Two consecutive lettuce plots (var. Maravilla) located at IRTA facilities (Cabrils, Barcelona, Spain; 41.518° N, 2.377° E) were planted per year from early April to late May (spring) and from middle June to early August (summer) in 2009 and 2010. In order to estimate *N. ribisnigri*, *F. occidentalis* and Collembola natural abundances in the plot, 17–30 lettuce plants were collected in spring 2009 (18 and 20 May), summer 2009 (7 and 14 July), spring 2010 (11, 18 and 25 May and 1 June) and summer 2010 (13, 20 and 27 July and 3 August). All lettuces were brought individually in plastic bags to the laboratory where the three target prey were counted per plant.

In order to increase the number of hoverfly larvae to be analysed, a total of 17 cages (40 × 90 × 60 cm) were randomly placed in the experimental plot in spring 2009 (14 May, $n = 3$ cages), summer 2009 (24 June, $n = 5$; 2 July, $n = 1$; 23 July, $n = 3$), spring 2010 (13 May, $n = 1$; 20 May, $n = 1$; 28 May, $n = 1$) and summer 2010 (3 July, $n = 2$). Each cage enclosed four lettuce plants (which were not cleaned of endemic arthropod community), onto which were introduced: 2–3 *E. balteatus* larvae, 25–70 *N. ribisnigri* larvae and 30–75 *F. occidentalis* larvae per plant. After 48 h, lettuces were cut, individualised in plastic bags and screened for predators in the lab, which were frozen until gut analysis. Conventional PCR analyses of all hoverfly larvae were conducted to obtain predation percentages with *N. ribisnigri*, *F. occidentalis* and Collembola-specific primers as previously described. Prior to DNA extraction, all predators were checked and cleaned to avoid attached remains under a microscope. All hoverfly larvae found inside the cages were first identified on the basis of their COI gene sequence,[5] and they were then considered in subsequent analyses.

2.4 qPCR analysis

In order to compare the sensitivity of qPCR and conventional PCR gut analyses, some *E. balteatus* previously analysed by conventional PCR for the presence of *N. ribisnigri*, *F. occidentalis* and Collembola in their guts were also analysed using qPCR at the North Central Agricultural Research Laboratory (USDA-ARS, Brookings, SD) facilities. These specimens were 40 *E. balteatus* from the *N. ribisnigri* feeding trials at different post-digestion times (0, 2, 4 and 8 h), together with 23 *E. balteatus* from the field experiment (24 June 2009, $n = 14$; 3 July 2010, $n = 9$).

Because qPCR optimally amplifies PCR products with short (<200 bp) amplicons, the predation comparison between conventional PCR and qPCR was conducted using the pair of *N. ribisnigri*-specific primers that amplified the shortest amplicon (Nr3F/Nr3R, 154 bp). PCR reactions (25 µL) contained 2× Brilliant SYBR Green qPCR master mix (12.5 µL) (Qiagen), each primer (300 nM), template DNA (1 µL) and PCR water (9.5 µL). Reactions were run on an MX3000P qPCR thermocycler (Stratagene, La Jolla, CA) using the following qPCR optimal 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. On each 96-well plate, a series of five positive controls of DNA from five pooled extractions of *N. ribisnigri* and three no-template controls were included.

2.5 NGS analysis

Massive DNA sequencing of *E. balteatus* gut contents following PCR amplification with a universal primer of arthropods was conducted using Ion Torrent PGM technology. The output of the massive sequencing process was treated bioinformatically. Below we detail all steps involved in NGS sequencing.

2.5.1 Universal and blocking primers

We amplified arthropod DNA from *E. balteatus* DNA extracts using the general invertebrate primers ZBJ-ArtF1c and ZBJ-ArtR2c.[35] These primers yielded a 157 bp amplicon located within the COI barcode region, which amplified a wide range of insect and spider orders.[26,35] When preliminary PCR analyses were conducted using these general invertebrate primers with 21 arthropod species tested, five of them were not amplified [the whiteflies *Trialeurodes vaporariorum* (Westwood) and *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae), the earwig *Forficula pubescens* Serville (Dermaptera: Forficulidae) and the target prey species of the present study, *N. ribisnigri* and *F. occidentalis*]. Nevertheless, we decided to use them because we were able to amplify at least a curtailed range of other arthropods potentially present in

Table 2. Prey and predator species tested for cross-reactivity using *Nasonovia ribisnigri* (Nr1F/Nr2R and Nr3F/Nr3R), *Frankliniella occidentalis* (Fo1F/Fo1R) and Collembola (Col4F/Col5R) specific primers (in bold, the target species)

			Primers		
Order	Family	Species	*N. ribisnigri*	*F. occidentalis*	Collembola
Predators					
Diptera	Syrphidae	*Dasysyrphus albotriatus*	–	–	–
		Epistrophe nitidicollis	–	–	–
		Episyrphus balteatus	–	–	–
		Eupeodes corollae	–	–	–
		Eupeodes lucasi	–	–	–
		Eupeodes luniger	–	–	–
		Melanostoma mellium	–	–	–
		Melangyna cincta	–	–	–
		Meliscaeva auricollis	–	–	–
		Meliscaeva cinctella	–	–	–
		Paragus tibialis	–	–	–
		Platycheirus albimatus	–	–	–
		Platycheirus clypeatus	–	–	–
		Scaeva albomaculata	–	–	–
		Scaeva pyrastri	–	–	–
		Scaeva selenitica	–	–	–
		Sphaerophoria rueppellii	–	–	–
		Sphaerophoria scripta	–	–	–
		Syrphus ribesii	–	–	–
		Xanthandrus comptus	–	–	–
	Cecidomyiidae	*Aphidoletes aphidimyza*	–	–	–
Preys					
Hemiptera	Aphididae	*Aphis gossypii*	–	–	–
		Aulacorthum solani	–	–	–
		Hyperomyzus lactucae	–	–	–
		Macrosiphum euphorbiae	–	–	–
		Myzus persicae	–	–	–
		Nasonovia ribisnigri	**+ (331/154 bp)**	–	–
Thysanoptera	Thripidae	***Frankliniella occidentalis***	–	**+ (292 bp)**	–
		Thrips tabaci	–	–	–
Collembola	Entomobrydae	***Entomobrya* sp.**	–	–	**+ (177 bp)**
Parasitoids					
Hymenoptera	Aphelinidae	*Aphelinus abdominalis*	–	–	–
	Braconidae	*Aphidius colemani*	–	–	–

the studied agroecosystems. One of these species was *E. balteatus*, and because predator DNA is typically more prevalent than prey DNA, a blocking primer was designed to inhibit *E. balteatus* DNA amplification as described in previous studies.[25,31] A modified non-extendable primer was used that binded to predator mtDNA, but not to the target species. This blocking primer (BloEb2 5′-TATATTTTCTATTCGGAGCTTGAGCTGGAATAG-3′-C3) was modified with a C3 spacer at the 3′ end of the forward universal primer (ZBJ-ArtF1c), preventing elongation during the PCR without noticeably influencing its annealing properties. To evaluate the efficiency of the blocking primer, PCR analysis was performed on *E. balteatus* DNA using primers ZBJ-ArtF1c and ZBJ-ArtR2c and adding different concentrations of the blocking primer BloEb2. Total reactions (volume 10 µL) were conducted with primers ZBJ-ArtF1c and ZBJ-ArtR2c (0.2 µL each, 10 µM), Platinum® PCR SuperMix High Fidelity (9 µL) (Invitrogen) and template DNA (0.6 µL). 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 single initial denaturation cycle of 94 °C for 5 min and a final extension at 68 °C for 10 min was carried out. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualised under UV light.

2.5.2 *Analysis of field samples*

Fusion primers were also designed following the Ion Torrent recommendations[36] (Table 3). Briefly, each pair of primers consisted 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 designed, each having a different 10 bp barcode (tag) before the ZBJ-ArtF1c primer to allow the multiplexing of two bulks of samples in a single sequencing run.

Samples were analysed at CRAG facilities in two bulks: 12 *E. balteatus* from the cages of 25 June 2009 and 3 July 2010, which were positive for any of the three prey (*N. ribisnigri*, *F. occidentalis* and/or Collembola) tested by conventional PCR and qPCR (bulk 1), and the same 12 *E. balteatus* without using blocking primer (bulk 2). Each bulk was amplified in 40 µL reaction volumes containing template DNA (2.4 µL), Platinum® PCR Supermix High Fidelity (Invitrogen) (36 µL), each fusion primer (0.8 µL at 10 µM) and 2.5

Table 3. Fusion primers used for sequencing *E. balteatus* specimens in the Ion Torrent PGM

Primer name[a]	Sequence[b]
AkT5 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*CAGAAGGAACAGATATTGGAACWTTATATTTTATTTTTGG
AkT6 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*CTGCAAGTTCAGATATTGGAACWTTATATTTTATTTTTGG
trP1-ZBJ (R)	CCTCTCTATGGGCAGTCGGTGATWACTAATCAATTWCCAAATCCTCC

[a] F = forward; R = reverse.
[b] In bold, the 'A' sequence; in italics, the 'key' sequence; underlined, barcodes (tags) to identify bulks; double underlined, the 'trP1' sequence (Ion Torrent, Life Technologies, 2011); dotted underlined, ZBJ-ArtF1c and ZBJ-ArtR2c primers.[35]

times the concentration of fusion primers of blocking primer (except the bulk without blocking primer). 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 QIAquick PCR Purification kit (Qiagen). Fragments of the expected size (157 bp) were selected (E-Gel® Size Select 2% agarose gel; Invitrogen) and quantified (DNA High Sensitivity kit, Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA), and each bulk was prepared as an equimolar pool. Then, we amplified (emulsion PCR) the samples, and each pool was sequenced in the PGM as described by the manufacturer (Ion Torrent, Life Technologies, Carlsbad, CA). A single 314 chip was used with the sequencing chemistry for a 200 bp read length, as well as v.2.2 of the Torrent Suite software for base calling (Ion Torrent, Life Technologies).

2.5.3 *Processing and analysis of data*

The output of the massive sequencing process was treated bioinformatically to discard any remaining *E. balteatus* reads as follows. 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 3). The primer sequence from the 5′ end of each read was eliminated using TagCleaner.[37] Sequences shorter than 150 bp were discarded, the remaining sequences were then trimmed to 150 bp, and finally those with a mean quality score lower than 25 were discarded (all using PRINSEQ[38]). To make the downstream computation simpler, the FASTA files generated by PRINSEQ were visually inspected for common sequences. A purpose-made perl script counted the number of occurrences of a given common sequence and generated another FASTA file with the rest. The common sequence was identified using BLAST, and usually corresponded to the predator *E. balteatus*. The process was iterated several times until the number of unidentified remaining sequences in the rest file was small enough (less than 2000 sequences) to be BLASTed at the NCBI website. The output from BLAST was imported into MEGAN (MEtaGenomics ANalyzer[39]) to explore the taxonomical content of the dataset.

3 RESULTS

3.1 Species specificity and detection periods

The designed primers for *N. ribisnigri* (Nr1F/Nr2R and Nr3F/Nr3R) and *F. occidentalis* (Fo1F/Fo1R), as well as the previously designed primers for Collembola (Col4F/Col5R), showed successful amplifications of the target prey. When they were tested for cross-amplification against other potential prey, only the target prey was detected, showing high specificity (Table 2).

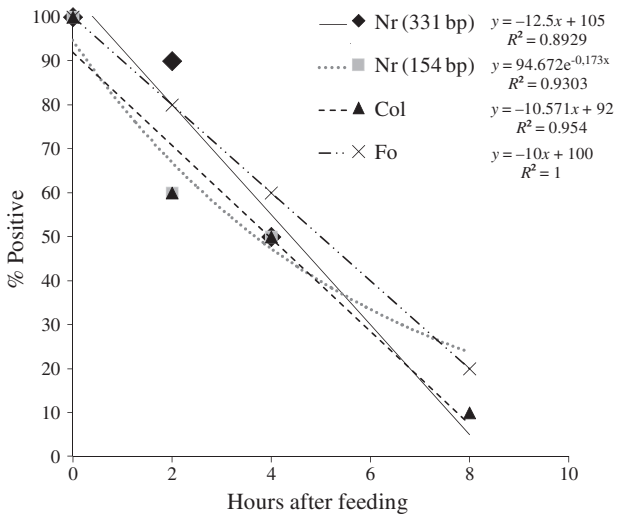

Figure 1. Detection curves of ingested *Nasonovia ribisnigri* [with primers Nr1F/Nr2R (331 bp) and Nr3F/Nr3R (154 bp)], *Frankliniella occidentalis* [with primers Fo1F/Fo1R (292 bp)] and *Entomobrya* sp. [with primers Col4F/Col5R (177 bp)] at different times after ingestion within *Episyrphus balteatus*. Best-fitted equations and R^2 values for each detection curve are also shown.

When these primers were used to analyse *E. balteatus* larvae fed on *N. ribisnigri*, *F. occidentalis* and *Entomobrya* sp., all predators tested positive at $t = 0$ h (immediately after ingestion). Detection 4 h later was variable, but never lower than 50% (Fig. 1). Prey DNA detection times within *E. balteatus* was better fitted to a linear decay in all cases, except for primer Nr3F/Nr3R (154 bp), which was better fitted to an exponential decay. Equations and R^2 values are shown in Fig. 1. Median detection times calculated by reverse prediction from these equations were 5, 4, 4.4 and 3.7 h for *F. occidentalis*, Collembola, *N. ribinigri* (Nr1F/Nr2R) and *N. ribisnigri* (Nr3F/Nr3R) respectively.

3.2 Field experiment

The sampled lettuce plot was naturally colonised (outside the cages) by predators and pests. *Nasonovia ribisnigri* abundances were overall much higher in spring (31.07 ± 36.31 individuals lettuce^{-1}) than in summer (0.16 ± 0.18 individuals lettuce^{-1}). This pattern was reversed with *F. occidentalis*, which had substantially higher populations in summer (2.13 ± 0.93 individuals lettuce^{-1}) than in spring (0.05 ± 0.07 individuals lettuce^{-1}). Regarding Collembola, their abundance was only measured in 2009, with 19.5 ± 6.36 and 21.1 ± 0.45 individuals lettuce^{-1} in spring and summer respectively.

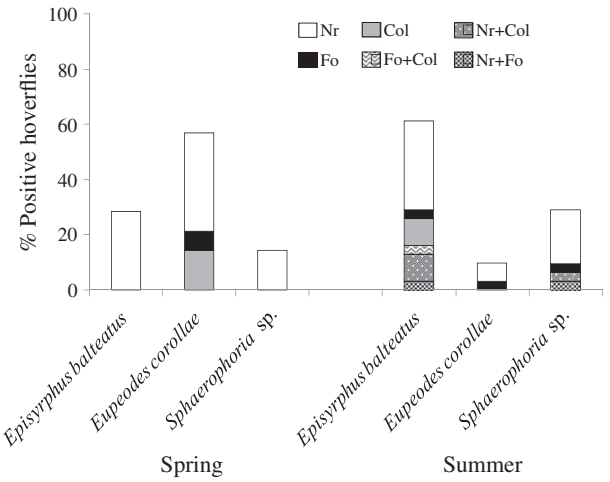

Figure 2. Percentages of PCR detection of *Nasonovia ribisnigri* (Nr), *Frankliniella occidentalis* (Fo) and *Collembola* (Col) within positive *Episyrphus balteatus*, *Eupeodes corollae* and *Sphaerophoria* spp. larvae from the cages placed in the experimental lettuce plot in spring and summer 2009 and 2010.

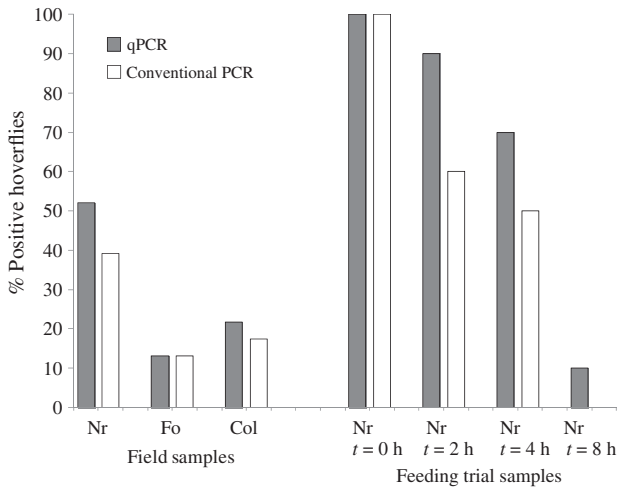

Figure 3. Percentages of qPCR and conventional PCR detection of *Nasonovia ribisnigri* (Nr), *Frankliniella occidentalis* (Fo) and *Collembola* (Col) within *Episyrphus balteatus* larvae from the field cages placed in the experimental lettuce plot, as well as from the feeding trials conducted in the laboratory.

After 48 h of being placed, cages were opened and 125 hoverfly larvae ($n = 73$ and 52 in spring and summer respectively) were found, which were identified by molecular analyses as described in Gomez-Polo *et al.*[5] As expected, because it was the species introduced into the cages, the most abundant syrphid inside the cages was *E. balteatus* ($n = 37$ and $n = 34$ in spring and summer respectively). However, because lettuces inside the cages were not previously cleaned of other endemic arthropods, other syrphid species such as *E. corollae* ($n = 32$ and $n = 2$ in spring and summer respectively), *Sphaerophoria* spp. ($n = 3$ and $n = 16$ in spring and summer respectively) and *M. auricollis* ($n = 1$ in spring) were also found. Therefore, the hoverfly community inside the cages was composed of *E. balteatus*, *E. corollae*, *Sphaerophoria* spp. and *M. auricollis*, with 51, 44, 4 and 1% in spring and 65, 4, 31 and 0% in summer respectively.

Prey DNA was detected in 36% of all sampled hoverfly larvae ($n = 125$) by conventional PCR using the primers Nr1F/Nr2R (331 bp), Fo1F/Fo1R (292 bp) and Col4F/Col5R (177 bp). Considering only those positive predators, 84% of them had consumed only one prey species and 16% had consumed two. From those fed on one prey species, 64, 9 and 11% were positive exclusively for *N. ribisnigri*, *F. occidentalis* and Collembola respectively. From those that consumed two prey species, 9, 5 and 2% were positive for *N. ribisnigri* + Collembola, *N. ribisnigri* + *F. occidentalis* and *F. occidentalis* + Collembola respectively. Prey detection rates for each hoverfly species in spring ($n = 14$) and summer ($n = 31$) are presented in Fig. 2, showing a higher predation of *N. ribisnigri* than *F. occidentalis* or Collembola in both seasons for all hoverfly species. In spring, *E. balteatus* and *Sphaerophoria* spp. consumed only one species (*N. ribisnigri*), whereas in summer the rate of detection of multiple prey increased (Fig. 2).

3.3 qPCR analysis

In both field and laboratory *E. balteatus* specimens, qPCR was more sensitive in detecting prey DNA than conventional PCR at their optimal conditions (Fig. 3). When *E. balteatus* larvae fed on *N. ribisnigri* and frozen at different times were analysed by qPCR, *N. ribisnigri* detection percentages were higher than those obtained by conventional PCR using the same *N. ribisnigri*-specific primers (154 bp). Also, qPCR revealed a higher percentage of *E. balteatus* larvae positive for *N. ribisnigri* in field cages than conventional PCR. The percentage of field-collected *E. balteatus* that tested positive for *F. occidentalis* was again higher when the samples were analysed using qPCR, but detection frequency was the same for both methods (conventional and qPCR) on field-collected specimens. Collembola were also more frequently detected in *E. balteatus* guts using qPCR than conventional PCR in field-collected specimens.

3.4 NGS analysis

When the efficiency of the blocking primer (BloEb2) was evaluated at different concentrations by conventional PCR, *E. balteatus* started to be blocked at concentrations higher than twice those used with the generalist primers (i.e. 0.4 µL of blocking primer added in the 40 µL PCR reaction). Based on this, 0.5 µL of blocking primer was added in the Ion Torrent reactions.

The Ion Torrent PGM produced two FASTQ files (Table 4). The quality control process reduced the number of obtained reads, but, in spite of the use of a specific *E. balteatus* blocking probe, most of them still belonged to the predator *E. balteatus* itself. Therefore, the Ion Torrent sequencing provided a total (considering the two bulk samples) of 895 prey sequences useful to describe the diet of *E. balteatus* larvae (Table 4). When the number of sequences obtained for the same bulk of samples was compared with or without blocking primer, a very similar number of sequences was observed (471 and 424 reads respectively). Detected prey included some potential pest species (Lepidoptera and Diptera), as well as non-pest species (Collembola). Also, potential BC agents (predators) of insect pests, such as spiders, the lady beetle *Adalia decempunctata* L. (Coleoptera: Coccinellidae), some *Orius* species (Hemiptera: Anthocoridae), the aphid parasitoid *Aphidius colemani* Dalman (Hymenoptera: Braconidae) and even another hoverfly genus (*Sphaerophoria* spp.), were detected, showing a certain intraguild predation (IGP), even between hoverfly species.

Table 4. Number of reads (sequences) and percentages of prey obtained by Ion Torrent PGM after analysing two bulks of *E. balteatus*

	Bulk 1[a]	Bulk 2[b]
Number of reads (raw)	10 8470	12 8072
Number of reads (good)	38 976	35 912
Number of reads (non-*E. balteatus*)	471	424
Detected prey	Percentages (%)	
Sphaerophoria spp. (Diptera: Syrphidae)	54	55.9
Cyclorrhapha	0.2	0
Cecidomyiidae	0.2	0
Diptera	2.5	0.2
Oedothorax fuscus (Araneae: Linyphiidae)	32	25.7
Cheiracanthium mildei (Araneae: Miturgidae)	0	2.4
Philodromus (Araneae: Philodromidae)	0	2.1
Entomobryoidea (Collembola)	6	6.1
Plodia interpunctella (Lepidoptera: Pyralidae)	0.4	0.2
Adalia decempunctata (Coleoptera: Coccinellidae)	2.3	2.6
Orius majusculus (Hemiptera: Anthocoridae)	0.2	0.7
Orius laevigatus (Hemiptera: Anthocoridae)	0.2	0.2
Orius spp. (Hemiptera: Anthocoridae)	0.2	2.1
Cimicoidea	0.4	0.5
Aphidius colemani (Hymenoptera: Braconidae)	1.5	1.3

[a] Bulk 1 = 12 specimens positive for *N. ribisnigri*, *F. occidentalis* and/or *Entomobrya* sp. by conventional and qPCR with blocking primer BloEb2.
[b] Bulk 2 = the same as bulk 1 without adding blocking primer BloEb2.

4 DISCUSSION

The molecular detection of *N. ribisnigri*, *F. occidentalis* and Collembola within several hoverfly species, common polyphagous predators in Mediterranean vegetable crops, has been demonstrated in this study. The four pairs of primers used were highly specific, not showing cross-reactivity with other prey and predator species potentially present in the crop (Table 2). They did not amplify other syrphid species present in the system either, making them a useful tool for studying predation by these predators, as well as other predators such as *Orius* spp. Feeding trials performed showed 100% detection at $t = 0$ for the three prey species, and a loss of detection was observed with time because of the degradation of prey DNA through digestion.

A substantial percentage of the field-collected syrphid larvae screened positive for at least one of these three prey (36%), particularly considering the relatively short median detection times obtained. In the sampled plot (outside the cages), *N. ribisnigri* was more abundant in spring (31.07 individuals lettuce^{-1}) than in summer (0.16 individuals lettuce^{-1}), whereas *F. occidentalis* had lower abundances in spring (0.05 individuals lettuce^{-1}), becoming more abundant in summer (2.13 individuals lettuce^{-1}). *N. ribisnigri* was the most detected prey in spring as well as in summer, when *N. ribisnigri* was much less present outside the cages. This was not surprising given that syrphids are known BC agents of this pest.[2] In spring, only one prey species was detected in their guts, but in summer two species were detected within some *E. balteatus* and *Sphaerophoria* spp. The diminishing natural infestation of *N. ribisnigri* during summer might cause this diet diversification of hoverfly larvae. Although syrphid predation rates on *F. occidentalis* and Collembola were not as high as on *N. ribisnigri*, they were quite important in both seasons. In a companion predation study[40] conducted with *Orius* spp. using the same specific primers, a higher *N. ribisnigri* detection rate was observed in spring than in summer. In that study, *Orius* spp. consumed more thrips than aphids during summertime, reflecting both the relative prey abundance during summer and this species' affinity for thrips as prey. Predation on Collembola was notably higher than on *F. occidentalis*, probably owing to their higher abundance in both seasons.

When syrphid larvae found inside the cages were molecularly analysed for species identification,[5] several hoverfly species were found (*E. balteatus*, *E. corollae*, *M. auricollis* and *Sphaerophoria* spp.). These are all very common species in Mediterraean vegetables,[41] particularly in Spanish lettuce and pepper crops.[3–5,42] Even if the most abundant species inside the cages in spring and summer was *E. balteatus* because it was the species introduced, *E. corollae* and *Sphaerophoria* spp. were also found. *Eupeodes corollae* was more abundant in spring, and *Sphaerophoria* spp. in summer. *Meliscaeva auricollis* abundance was trivial. The present study has demonstrated that *E. balteatus*, *E. corollae* and *Sphaerophoria* spp. fed on *N. ribisnigri*, *F. occidentalis* and Collembola; this is the first record of a syrphid larvae feeding on Collembola. Future research should investigate whether this alternative prey adds or detracts from predation on focal pests.

This work shows that qPCR is more efficient at detecting hoverfly larva predation than conventional PCR at their optimal conditions and in both laboratory-fed and field-collected predators. This conclusion is supported in other insect studies,[40] as well as in other disciplines.[43–48] qPCR represents a significant advance in PCR-based gut analysis, with a number of undisputable technical advantages, such as speed, sensitivity and reduction of contamination risk.[48] However, depending on the aim of the study, conventional PCR is still a powerful tool that can effectively answer a number of ecological questions, such as the qualitative evaluation of predation, which can help narrow down which predators may be important targets for BC programmes of a pest species.

As discussed in Pompanon *et al.*,[24] NGS provides an excellent tool for initial screening of predators or herbivores, providing an invaluable guide to the composition and range of species consumed. After that, NGS can be followed by complementary PCR analyses based upon species- and group-specific primers directed at prey groups of interest. When PCR analyses were conducted with the general invertebrate COI primers ZBJ-ArtF1c and ZBJ-ArtR2c,[35] it was found that they did not amplify *N. ribisnigri* and *F. occidentalis*. Even so, a wider range of other arthropods were amplified, giving a wider picture of the dietary breadth for this species. Other species amplified with those primers can be found in other diet assessment studies.[26,35,49] Something to consider in future studies would be the use of two or more sets of universal arthropod primers, which combined should amplify a wider range of arthropods.

When Ion Torrent PGM was used to analyse *E. balteatus* gut contents, some prey species were detected, but also some predators, such as the hoverfly genus *Sphaerophoria*, some spiders, the coccinellid *A. decempunctata* and some *Orius* species. Previous studies showed that *Sphaerophoria* is a common genus in summer in the studied area, in fact the most abundant.[5] Other

BC agents, such as mirid bugs, earwigs, lacewings, coccinellids, hymenopteran parasitoids and even entomopathogenic fungal-infected aphids, have been cited to be consumed by hoverfly larvae.[7,50–55] After these results, further studies should be conducted in order to determine whether or not these IGP interactions might weaken the trophic interactions with the target pest. The fact that other natural enemies, particularly some parasitoids, such as cecidomyiids and the braconid *A. colemani*, were detected through NGS analyses within *E. balteatus* could also have a consequence on the BC of *N. ribisnigri* and *F. occidentalis*.

Considering these results, different experimental goals will be differentially accommodated using the various molecular methods explored in this research. The use of both methods (conventional/qPCR and Ion Torrent NGS) in parallel has given complementary information about the diet of *E. balteatus*. This study has demonstrated predation by Mediterranean syrphid larvae on pest and non-pest prey. It has also provided important insights into *E. balteatus* predation, showing other interactions such as intraguild trophic links in this agroecosystem that should be considered in order to develop, apply or improve new CBC programmes.

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