Molecular identification and feeding ecology of arthropod generalist predators present in Mediterranean lettuce crops

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DEPARTMENT OF PLANT PRODUCTION AND FORESTRY PROTECTION

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Molecular identification and feeding ecology of arthropod generalist predators present in Mediterranean lettuce crops

by

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Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive.
Ricardo Reis

To be great, be whole;
Exclude nothing, exaggerate nothing that is not you.
Be whole in everything. Put all you are
Into the smallest thing you do.
So, in each lake the moon shines with splendor
Because it blooms up above.
Ricardo Reis

A Tata y Teresa
porque estáis conmigo siempre
siempre, siempre, siempre…
y siempre me parece poco.

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Abbreviations

BC: Biological Control.

CBC: Conservation Biological Control.

COI: Cytochrome Oxidase Subunit I.

DNA: Deoxyribonucleic acid.

FAO: Food and Agriculture Organization.

IGP: Intraguild Predation.

IGR: Insect Growth Regulators.

IPM: Integrated Pest Management.

ITS: Internal Transcriber Spacer.

LMV: Lettuce mosaic virus.

NE: natural enemy.

PCR: Polymerase Chain Reaction.

PMG: Personal Genomic Machine.

qPCR: quantitative Polymerase Chain Reaction.

SMS: Single Molecule Sequencing.

TSWV: Tomato spotted wilt virus.

Summary

The aphid *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) are two of the main pests in Mediterranean lettuce crops. Their biological control is mainly based on the use of generalist predators. Detailed knowledge about generalist predator diets is fundamental in the development of conservation biological control (CBC) programs. The general goal of this Doctoral Thesis is to study the trophic interactions present in Mediterranean lettuce crops in greater depth, in order to develop, apply and improve CBC programs. This main goal has been developed in two specific objectives. The first was to develop molecular diagnostic methods to identify the most abundant predator species (hoverflies (Diptera: Syrphidae) and *Orius* spp. (Hemiptera: Anthocoridae)) present in these crops. The second was to show the trophic interactions among the main pest species, non-pest prey and generalist arthropod predators present in these crops under field conditions. When these molecular methods were used to identify the hoverfly and the *Orius* species present in Mediterranean lettuce crops, it was shown that *Eupeodes corollae*, *Episyrphus balteatus* and *Sphaerophoria scripta*/*S. rueppellii* together with *O. laevigatus*, *O. majusculus* and *O. niger* were the most common taxa. Molecular diagnostic gut-content analysis of generalist predator of Mediterranean lettuce crops was also conducted to study predation of these generalist predators on *N. ribisnigri*, *F. occidentalis*, as well as *Entomobrya* sp. (Collembola), the most abundant non-pest prey present in the lettuce crops studied. Several molecular methods were used for this purpose; conventional PCR, real-time PCR and the next generation sequencing (NGS) platform, Ion Torrent Personal Genomic Machine (PGM). Results showed the relevance of naturally occurring predators, occupying different niches depending on the season (spring or summer). Syrphids were very abundant in spring, decreasing in summer and *Orius* spp. were only present in summer. Other common predators, like coccinellids (Coleoptera: Coccinellidae) were only present in spring, whereas spiders (Araneae: Linyphiidae, Thomosidae) were present in both seasons. Added to the trophic links already known in the literature, most of the trophic links among all these arthropod species have been demonstrated in this Thesis. This includes some trophic interactions which had never been identified before, like *Orius* and syrphid predation on Collembola, syrphid predation on *F. occidentalis* or *Orius* predation on *N. ribisnigri*. On the other hand, some prey preferences were observed. Even if hoverfly larvae were the most polyphagous predators because of their predation on *N. ribisnigri*, *F. occidentalis* and Collembola, *Orius* spp. preyed mainly on *F. occidentalis* and coccinellid larvae preyed mainly on *N. ribisnigri*. Spiders fed mainly on Collembola, the non-pest prey. NGS analyses showed evidence of a certain intraguild predation (IGP) in this agroecosystem, showing for the first time that *E. balteatus* and *O. majusculus* can prey on each other, and that both predator species can feed on spiders and coccinellids. In conclusion, molecular analysis of predation has allowed the characterization of the trophic links present in Mediterranean lettuce crops, which is of great importance in order to develop CBC programs in those crops.

Resumen

El pulgón *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) y el trips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) son las principales plagas de lechuga en el área mediterránea. Su control biológico (CB) se basa principalmente en el uso de depredadores generalistas. Por ello, el conocimiento de la dieta de los depredadores generalistas es fundamental para el desarrollo de programas eficaces de control biológico. El objetivo general de esta Tesis Doctoral fue mejorar el conocimiento de las relaciones tróficas en los cultivos de lechuga del Mediterráneo para desarrollar, aplicar y mejorar programas de CB. Este objetivo general se desarrolló dentro de dos objetivos específicos. El primero fue poner a punto métodos moleculares para identificar las especies de depredadores más abundantes los sírfidos (Diptera: Syrphidae) y el género *Orius* (Hemiptera: Anthocoridae) en cultivos de lechuga. El segundo fue conocer las relaciones tróficas entre las principales plagas, presa alternativa y depredadores generalistas en cultivos de lechuga en condiciones de campo. Cuando estas técnicas moleculares se usaron para identificar las especies de sírfidos y *Orius* presentes en los cultivos de lechuga del Mediterráneo, los taxones más comunes identificados fueron *Eupeodes corollae*, *Episyrphus balteatus* y *Sphaerophoria scripta*/*S. rueppellii* junto con *O. laevigatus*, *O. majusculus* y *O. niger*. Además, se realizaron análisis moleculares del tracto digestivo de los depredadores generalistas recolectados en campos de lechuga del área mediterránea para estudiar la depredación sobre *N. ribisnigri, F. occidentalis*, y colémbolos del género *Entomobrya* (Collembola), la presa alternativa más abundante en los campos de lechuga estudiados. Varias técnicas moleculares fueren usadas con este propósito: la PCR convencional, la PCR a tiempo real y la plataforma Ion Torrent Personal Genomic Management (PGM) de secuenciación de nueva generación (Next Generation Sequencing [NGS]). Los resultados obtenidos muestran la importancia de los depredadores generalistas que se encuentran de forma natural en los cultivos de lechuga, ocupando diferentes nichos dependiendo de la estación del año (primavera o verano). Los sírfidos fueron abundantes en primavera, disminuyendo en verano, mientras que los *Orius* sólo se encontraron en verano. Otros depredadores comunes, como los coccinélidos (Coleoptera: Coccinellidae) estuvieron presentes en primavera, mientras que las arañas (Araneae: Linyphiidae, Thomosidae) estuvieron presentes en ambas estaciones. En esta Tesis Doctoral se han identificado la mayoría de las relaciones tróficas potenciales entre estos depredadores y sus presas, de las cuales, algunas no se habían demostrado hasta ahora, como la depredación de *Orius* y sírfidos sobre colémbolos, la depredación de sírfidos sobre *F. occidentalis* y la de *Orius* sobre *N. ribisnigri*. Por otro lado, también se han identificado algunas preferencias de estos depredadores sobre estas presas. Las larvas de sírfido fueron las más polífagas ya que se alimentaron de *N. ribisnigri*, *F. occidentalis* y colémbolos, los *Orius* spp. consumieron principalmente *F. occidentalis*, mientras que las larvas de coccinélido consumieron principalmente *N. ribisnigri*. Las arañas se alimentaron principalmente de colémbolos, la presa alternativa. Los análisis de NGS mostraron la existencia de depredación intragremial (DI) en este agroecosistema, demostrando que *E. balteatus* y *O. majusculus* se pueden depredar mutuamente, además de alimentarse de arañas y coccinélidos. En resumen, los análisis moleculares de depredación han permitido la caracterización de las relaciones tróficas presentes en cultivos de lechuga del Mediterráneo, lo cual es de gran importancia para el desarrollo de programas de CB de conservación de este cultivo en esta zona.

Resum

El pugó *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) i el trips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) són les principals plagues d'enciam a l'àrea mediterrània. El seu control biològic (CB) es basa principalment en l'ús de depredadors generalistes. Per això, el coneixement de la dieta dels depredadors generalistes és fonamental per al desenvolupament de programes eficaços de control biològic. L'objectiu general d'aquesta Tesi Doctoral va ser conèixer les relacions tròfiques en els cultius d'enciam de l'àrea Mediterrània per desenvolupar, aplicar i millorar programes de CB. L'objectiu general es va desenvolupar dins de dos objectius específics. El primer va ser posar a punt mètodes moleculars per identificar les espècies de depredadors més abundants les sírfids (Diptera: Syrphidae) i del gènere *Orius* (Hemiptera: Anthocoridae) en cultiu d'enciam. El segon va ser conèixer les relacions tròfiques entre les principals plagues, presa alternativa i depredadors generalistes en cultiu d'enciam en condicions de camp. Quan aquestes tècniques moleculars es van utilitzar per identificar les espècies dels sírfids i d'*Orius* presents en els cultius d'enciam de la Mediterrània, els tàxons més comuns van ser *Eupeodes corollae*, *Episyrphus balteatus* i *Sphaerophoria scripta* / *S. rueppellii* juntament amb *O. laevigatus*, *O. majusculus* i *O. niger*. Seguidament, es van realitzar anàlisis moleculars del tracte digestiu dels depredadors generalistes en camps d'enciam de la Mediterrània per conèixer la depredació sobre *N. ribisnigri*, *F. occidentalis*, i colèmbols del gènere *Entomobrya* (Collembola), la presa alternativa més abundant en els camps d'enciam estudiats. Diverses tècniques moleculars van ser emprades amb aquest propòsit: la PCR convencional, la PCR a temps real i la plataforma Ion Torrent Personal Genomic Management (PGM) de seqüenciació de nova generació (Next Generation Sequencing [NGS]). Els resultats obtinguts van mostrar la importància dels depredadors generalistes que es troben de forma natural en els cultius d'enciam, ocupant diferents nínxols depenent de l'estació de l'any (primavera o estiu). Els sírfids van ser abundants a la primavera, disminuint a l'estiu mentre que els *Orius* només es van trobar a l'estiu. Altres depredadors comuns, com els coccinèlids (Coleoptera : Coccinellidae) van ser presents a la primavera, mentre que les aranyes (Araneae: Linyphiidae, Thomosidae) van ser presents en les dues estacions. En aquesta Tesi Doctoral s'ha identificat la majoria de les relacions tròfiques potencials entre aquests depredadors i les seves preses, de les quals, algunes no s'havien demostrat fins ara, com la depredació d'*Orius* i sírfids sobre colèmbols, la depredació de sírfids sobre *F. occidentalis*, i la d'*Orius* sobre *N. ribisnigri*. D'altra banda, també s'ha identificat algunes preferències dels depredadors sobre les preses. Les larves dels sírfids van ser les més polífagues ja que es van alimentar de *N. ribisnigri*, *F. occidentalis* i collèmbols, els *Orius* spp. van consumir principalment *F. occidentalis*, mentre que les larves de coccinèlids van consumir principalment *N. ribisnigri*. Les aranyes es van alimentar principalment de colèmbols, la presa alternativa. Les anàlisis de NGS van mostrar una certa evidència de depredació intragremial (DI) en aquest agroecosistema, mostrant que *E. balteatus* i *O. majusculus* es poden depredar mútuament, a més d'alimentar-se d'aranyes i coccinèlids. En resum, les anàlisis moleculars de depredació han permès la caracterització de relacions tròfiques presents en cultius d'enciam de la Mediterrània la qual cosa és de gran importància per al desenvolupament de programes de CB de conservació d'aquest cultiu a la zona estudiada.

Resumo

O pulgón *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) e o trips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) son as principais pragas de leituga na área mediterránea. O seu control biolóxico (CB) baséase principalmente no uso de depredadores xeralistas. Por iso, o coñecemento da dieta dos depredadores xeralistas é fundamental para o desenvolvemento de programas eficaces de control biolóxico (CB). O obxectivo xeral desta tese foi estudar as relacións tróficas nos cultivos de leituga do Mediterráneo para desenvolver, aplicar e mellorar programas de CB. O obxectivo xeral desenvolveuse dentro de dous obxectivos específicos. O primeiro foi poñer a punto métodos moleculares para identificar as especies de depredadores máis abundantes (sírfidos (Diptera: Syrphidae) e *Orius* (Hemiptera: Anthocoridae)) en cultivos de leituga. O segundo foi estudar as relacións tróficas entre as principais pragas, presa alternativa e depredadores xeralistas en cultivos de leituga en condicións de campo. Cando estas técnicas moleculares se usaron para identificar as especies de sírfidos e *Orius* presentes nos cultivos de leituga do Mediterráneo, os taxóns máis comúns identificados foron *Eupeodes corollae*, *Episyrphus balteatus* e *Sphaerophoria scripta*/*S. rueppellii* xunto con *Orius laevigatus*, *O. majusculus* e *O.niger*. Ademais, realizáronse análises moleculares do tracto dixestivo dos depredadores xeralistas en campos de leituga do Mediterráneo para estudar a depredación sobre *N. ribisnigri*, *F. occidentalis*, e colémbolos do xénero *Entomobrya* (Collembola), a presa alternativa máis abundante nos campos de leituga estudados. Varias técnicas moleculares foron usadas con este propósito: a PCR convencional, a PCR a tempo real e a plataforma Ion Torrent Personal Genomic Management (PGM) de secuenciación de nova xeración (next generation sequencing [NGS]). Os resultados obtidos mostran a importancia dos depredadores xeralistas que se encontran de forma natural nos cultivos de leituga, ocupando diferentes nichos dependendo da estación do ano (primavera ou verán). Os sírfidos foron abundantes en primavera, diminuíndo en verán mentres que os *Orius* só se encontraron en verán. Outros depredadores comúns, como os coccinélidos (Coleoptera: Coccidellidae) estiveron presentes en primavera, mentres que as arañas estiveron presentes nas dúas estacións. Nesta Tese Doutoral estudáronse a maioría das relacións tróficas potenciais entre estes depredadores e as súas presas, das cales, algunhas non se demostraran ata agora, como a depredación de *Orius* e sírfidos sobre colémbolos, a depredación de sírfidos sobre *F. occidentalis* e de *Orius* sobre *N. ribisnigri*. Por outro lado, tamén se observaron algunhas preferencias dos depredadores sobre as presas. As larvas dos sírfidos foron as máis polífagas xa que se alimentaron de *N.ribisnigri*, *F. occidentalis* e colémbolos e os *Orius* spp. consumiron principalmente *F. occidentalis*, mentres que as larvas de coccinélidos consumiron principalmente *N. ribisnigri*. As arañas alimentáronse principalmente de colémbolos, a presa alternativa. As análises de NGS mostraron a evidencia de depredación intragremial (DI) neste agroecosistema, mostrando por primeira vez que *E. balteatus* e *O.majusculus* pódense depredar os uns aos outros, e ambalas dúas especies aliméntanse de arañas e coccinélidos. En resumo, as análises moleculares de depredación permitiron a caracterización de relacións tróficas presentes en cultivos de leituga do Mediterráneo o cal é de grande importancia para o desenvolvemento de programas de CB deste cultivo na zona mediterránea.

General introduction

1. Biological Control and Conservation

The protection of crops in intensive agriculture has often been understood as a synonym of chemical pest control. This strategy has some drawbacks that should be taken into account, like pest resistance to pesticides, the toxicological risk for the applicator, the toxic residues in the crops and persistence in the environment. Currently, European Regulation 1107/2009 regulates pesticide in the EU and establishes the framework for Community action to achieve a sustainable use of pesticides encouraging the development of less-harmful substances and promoting low pesticide-input pest management.

Integrated Pest Management (IPM) is an ecosystem approach to crop production and protection that combines different management strategies and practices to grow healthy crops and to minimize the use of pesticides. Its aim is to keep pest populations below the economic threshold of tolerance. Integrated Pest Management involves the use of several techniques to control pests, such as Biological Control (BC), biotechnology, chemical control, pheromones, Insect Growth Regulators (IGR) and appropriate cultural practices. According to Eilenberg et al (2001), there are four BC strategies : (1) Classical, based on the intentional introduction of exotic agent control; (2) Inoculative, involving the introduction of a BC agent to increase its population and to control the pest for an extended period of time, but not permanently; (3) Inundative, based on the release of a BC agent that allows a pest to be controlled by the released organisms themselves; and (4) Conservative, which involves the modification of the environment or existing practices to protect and enhance the presence of naturally present Natural Enemies (NE) to reduce the effect of a pest. The effectiveness of BC programs depends on the biological characteristics of the NE used, as well as their proper use. Conservation Biological Control (CBC) requires an in-depth knowledge of the ecology of the NE involved, as well as of the ecological communities where they belong (Jonsson et al., 2008). It also requires integration of the scientific discovery process and its application by growers (Cullen et al., 2008). The use of CBC programs has the main advantage that NE are already adapted to the habitat and to the target pest, which reduces time and costs and increases effectiveness of the BC of insect pests. Cropping systems can be modified to favor NE, providing a suitable habitat where they can live and reproduce, helping to ensure the survival of NE populations and providing a higher biodiversity in farm landscapes (Boller et

al., 2004).

2. Lettuce crop

Lettuce (*Lactuca sativa* L.) is an annual plant of the family Asteraceae. It is most often grown as a leaf vegetable, but sometimes also for its stem and seeds. The Food and Agriculture Organization (FAO) of the United Nations reports that world production of lettuce and chicory (both crops are combined for reporting purposes) in 2012 was 24 million tons (FAOSTAT, 2012). This comes primarily from China (59%), the US (16%), India (5%) and Spain (2%). Although China is the top world producer of lettuce, the majority of the crop is consumed domestically with an annual production in 2012 of 14 million tons (FAOSTAT, 2012). The lettuce-cultivated area in Spain was 33,179 ha in 2012, being after the tomato, the biggest vegetable crop cultivated area (MAPA, 2012).

2.1. Main pests in lettuce crops

Several pest species are present on lettuces, mainly aphids, thrips, lepidopterans and leafminers. Damage is both direct by sucking the sap and taking the plant vigor, and indirect by being effective vectors of viruses. Aphids also excrete honeydew, on which sooty molds are installed. Among all the aphid species found on lettuce in the Mediterranean basin, the most damaging is *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) (Morales et al., 2013; Pascual-Villalobos et al., 2004), together with *Aphis gossyppii* Glover, *Aulacorthum* solani (Kaltenbach), *Macrosiphum euphorbiae* Tomas, *Hyperomyzus lactucae* (Linnaeus) and *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) (Lacasa et al., 2003; Nebreda et al., 2005; Nebreda et al., 2004). *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is the main thrips affecting lettuce and is a vector of the *Tomato spotted wilt virus* (TSWV) (Medeiros et al., 2004; Peters, 2008; Reitz and Funderburk, 2012). Among the pests that cause defoliation, lepidopteran caterpillars, such as *Autographa gamma* (Linnaeus), *Helicoverpa armigera* (*Hübner)*, *Spodoptera littoralis* (Boisduval) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) are important pests (Brødsgaard and Albajes, 2000; Gengotti and Censi, 2004; Gengotti and Tisselli, 2002). Lettuces can also be attacked by generic soil pests, such as *Agrotis* spp. (Lepidoptera: Noctuidae), causing considerable damage to seedlings (Lossbroek and Theunissen, 1985). The vegetable leafminers, *Liriomyza*

trifolii (Burgess) and *Liriomyza huidobrensis* (Blanchard) (Diptera: Agromyzidae) excavate galleries inside the leaves and when the attacks are severe the plant becomes weaker, decreasing plant development (Burgio et al., 2005; Hernández-García et al., 1999).

Other general insect pests, like *Agriotes* spp. (Coleoptera: Elateridae), can destroy the root system of the plant (Chillemi and Lazzarin, 1998). Also, some nematodes, like *Meloidogyne* spp., produce galls on the roots and can transmit viruses and bacteria (Castillo et al., 2006). Snails and slugs may also become a problem in lettuce, causing significant damage to the crop, particularly in high humidity regions (Castillejo et al., 1996).

2.1.1. Nasonovia ribisnigri and Frankliniella occidentalis

The main pests in lettuce crops of European temperate regions are *N. ribisnigri* and *F. occidentalis* (Diaz and Fereres, 2005; Martin et al., 1996; Moreno and Fereres, 2012; Satar et al., 2012) (Fig. 1). Both are vectors of pathogenic viruses and are considered cosmetic pests because their presence in harvested lettuces reduces the market value of the products (Kift et al., 2004; Palumbo, 2000). Historically, farmers and growers have controlled them through the application of pesticides. Further limitations to the effective suppression of both pests with chemical pesticides, the resistance to pesticides, the feeding preferences of *N. ribisnigri* for heart leaves, and the fact that *F. occidentalis* pupates in the soil have led to the adoption of IPM and BC practices (Alomar et al., 2008; Hopper et al., 2011; Nelson et al., 2012; Parker et al., 2002; Satar et al., 2012; Smith and Chaney, 2007; Smith et al., 2008).

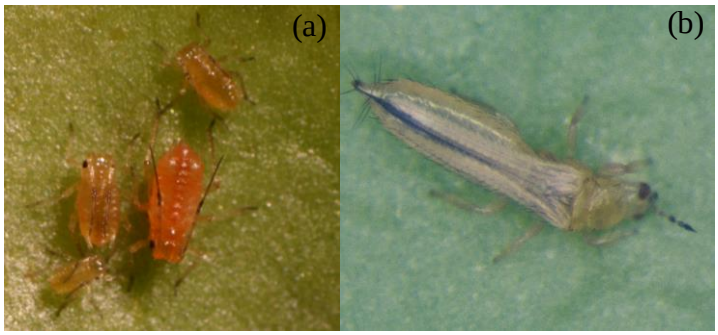

Fig. 1. *Nasonovia ribisnigri* nymphs (a) and *Frankliniella occidentalis* adult (b).

Immature *N. ribisnigri* aphids are orange-green and wingless. Adults can be winged or wingless greenish to yellow-green, with irregular narrow dark bands on the abdomen. Their feeding on the young lettuce leaves causes leaf distortion and reduces seedling vigor, and can

transmit virus diseases, like the *Lettuce mosaic virus* (LMV) (Morales et al., 2013; Nebreda et al., 2004). They colonize hearts and rosettes, making them unsaleable. This is a pest of vegetable crops such as lettuce, chicory, endive and radicchio (La Rossa et al., 2005).

The life cycle of the lettuce aphid, *N. ribisnigri,* is holocyclic heteroecious between its primary host, the shrub *Ribes* sp. L. (Saxifragales: Grossulariaceae) and secondary hosts (Asteraceae, Scrofulariaceae and Solanaceae) (Vam Emden, 2013). Females lay eggs on the primary hosts where the overwintering eggs hatch. When they grow these females, called "fundatrix" or "stem mother", infest the young shoots. They are asexual and also produce "viviparous females", which also reproduce asexually for several generations. When populations are abundant or the quality or quantity of food is scarce, winged individuals appear and migrate to other more favorable plants or crops. In unfavorable conditions, parthenogenetic viviparous females can produce a sexual generation with males and oviparous females, which will produce the initial overwintering eggs (Ogawa and Miura, 2014).

Thrips are present season long in leafy vegetables, but are usually most abundant during the summer, when temperatures start to increase (McDonald et al., 1998). They can build up in weedy areas and other surrounding crops, moving to lettuce in large numbers when host plants begin to dry down (Atakan et al., 2013; Atakan and Uygur, 2005). They damage the plant in several ways. The female oviposition in the plant tissue causes the major damage, but the plant is also injured by feeding, leaving areas of silvery discoloration when it reacts to the insect's saliva.

Western flower thrips (*F. occidentalis*) have a broad host range of more than 500 species in 50 plant families and are associated with many cultivated crops and ornamentals. Crops attacked by this pest include plants like beans, cucumber, eggplant, lettuce, onion, tomatoes, watermelon and ornamental crops include carnation, chrysanthemum, orchids and rose (Yudin et al., 1986). It is native to the Southwestern United States, but has spread to other continents, including Europe (Tipping, 2008). They reproduce by arrhenotokous parthenogenesis, with females arising only from fertilized eggs and males from unfertilized eggs. Eggs are laid in the plant tissues then they have four nymph instars. During instars I and II, they are white or nearly transparent changing to light yellow and they feed on the plant. In instar II, they crawl down into the soil to instars III and IV, which are non-feeding stages and show buds. Adults have four wings fringed with long hairs and folded over the back

lengthwise. Females have three color forms (pale, intermediate and dark), all of which can mate with the pale males. Development from egg to adult takes 8 to 20 days depending on temperature (Jackman and Drees, 1998; Leigh and Goodell, 1996).

2.2. Natural enemies of N. ribisnigri and F. occidentalis

There is a large scientific literature describing predation on aphids and thrips (see following sections). In particular, predation on *N. ribisnigri* and / or *F. occidentalis* has been recorded on several families of hoverflies (Diptera: Syrphidae), anthocorids (Hemiptera: Anthocoridae), coccinelids (Coleoptera: Coccinellidae), spiders (Araneae) and other less common predators like mites (Acari), lacewings (Neuroptera: Chrysopidae) and even some thrips (Thysanoptera) (Alomar et al., 2008; Heinz et al., 1996; Nelson et al., 2012; Riudavets and Castañé, 1998; Sabelis and Van Rijn, 1997; Semyanov, 1996; Shrestha and Enkegaard, 2013; Zrubecz et al., 2008).

Other NE also used for the BC of aphids and thrips are parasitic species. Some tiny wasps oviposit into juvenile aphids, like: *Aphidius hieraciorum* (Stary), *Aphidius ervi* Haliday and *Aphidius colemani* Viereck (Hymenoptera: Braconidae), which is commercially available (Nebreda et al., 2005). Another parasitoid commercially available is *Aphelinus abdominalis* (Hymenoptera: Aphididae), particularly recommended to control *M. euphorbiae* populations in greenhouses (Alomar et al., 1997). Other species, like *Ceranisus* menes (Walker) and *Ceranisus americensis* (Girault) (Hymenoptera: Eulophidae) (Fourez et al., 1995; Galazzi et al., 1992), have been described as playing a very minor role in *F. occidentalis* control (Loomans and Lenteren, 1996; Loomans et al., 2006). Apart from these two species nothing else is known about a potential BC of *N. ribisnigri* and *F. occidentalis* using parasitoids.

2.2.1. Hoverflies

Hoverflies are sometimes called flower flies or syrphid flies. Adults mainly consume nectar and pollen, but larvae of many species (Fig. 2) are polyphagous predators of a broad range of soft-bodied arthropods. They have been described as being important aphid predators (Lakhanpal and Desh, 1998; Michaud and Belliure, 2001; Rojo et al., 2003; Rojo and Marcos-García, 1998). Some studies have examined predation by hoverfly larvae on *N. ribisnigri* in North American lettuce fields (Hopper et al., 2011; Nelson et al., 2012; Smith and Chaney, 2007; Smith et al., 2008), but very little is known about hoverflies as predators on *N. ribisnigri* in Mediterranean lettuce crops. Some authors have suggested that syrphids could

also prey on thrips (Sabelis and Van Rijn, 1997; Tawfik et al., 1974; Thompson, 1977), although nothing is known about their predation on *F. occidentalis* in particular.

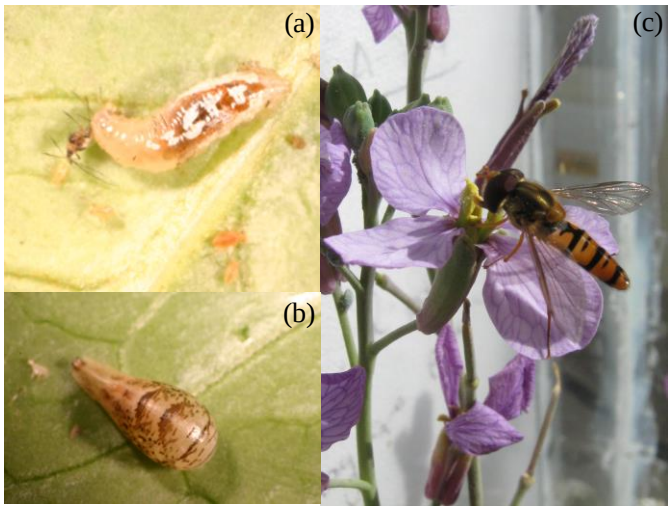

Fig. 2- Larva (a), pupa (b) and adult (c) of the syrphid *Episyrphus balteatu*

Emerging from the egg, hoverfly larvae have three instars. Larvae do not possess segmented legs or a head capsule, and they are known to be important sucking predators (Tinkeu and Hance, 1998). The first two stages last just for a few days, but the third stage lasts from several days to months, depending on the species. Larvae of this stage have a fused breathing tube projecting from the end of the body (Rotheray, 1993), and they turn into a teardrop-shaped puparium (Fig. 2) on the host plant or in the soil (Stubbs and Falk, 1983). Unless the pupal stage overwinters, adults emerge in one or two weeks. It is very difficult to obtain an accurate identification of the preimaginal stages using morphological identification (Bastian, 1986; Láska et al., 2006), but adults are usually easier to identify based on morphological features and the male genitalia (Láska et al., 2013; Speight and Sarthou, 2011). They have spots, bands or stripes of yellow, brown against a dark-colored background, and the ability to hover whilst they are flying, suspended in the air, keeping the head absolutely still.

Hoverfly species commonly found in Mediterranean vegetable crops are: *Episyrphus balteatus* (De Geer), *Scaeva pyrastri* (L.), *Eupeodes corollae* (F.), *Meliscaeva auricollis* (Meigen), *Sphaerophoria scripta* (L.) and *Sphaerophoria rueppellii* (Wiedemann) (Morales et al., 2007; Pascual-Villalobos et al., 2006). *Episyrphus balteatus* is the most abundant hoverfly species in Europe and is even commercially available as a biological control agent.

2.2.2. Orius spp.

Anthocorids of the genus *Orius* Wolff, commonly called "pirate bugs" or "flower bugs" are also generalist predators present in vegetable crops (Riudavets, 1995b), including lettuce crops. They have mainly been associated with thrips (Brown et al., 1999; Riudavets and Castañé, 1998), and with *F. occidentalis* in particular (Baez et al., 2004; Riudavets, 1995a). Some *Orius* species, like *O. majusculus* and *O. laevigatus* have even been succesfully used to control *F. occidentalis* in pepper and cucumber greenhouses in Europe (Bosco et al., 2008; Bosco and Tavella, 2008; Trottin-Caudal et al., 1991). Aphids could also be an important component of *Orius* diets. Some *Orius* predation studies on aphids have been conducted under laboratory conditions (Ahmadi et al., 2009; Paik et al., 2010) and in field conditions (Kabicek and Hejzlar, 1996). No evidence has been found that show *Orius* predation on *N. ribisnigri*. Some *Orius* species can also eat pollen, which enables them to be present in pollen bearing crops without any prey (Atakan, 2010; Lundgren, 2009b; Oveja et al., 2012; Pumariño and Alomar, 2012; Shakya et al., 2010; Wackers, 2005).

Orius females lay 1-3 eggs per day embedded in the plant tissue. After eclosion, they have five nymphal stages with conspicuous red eyes (Fig. 3). In the later stages, nymphs gradually become a darker color. Larva development time is around three weeks (depending on the temperature), and adults can live for three to four weeks (Bahsi and Tunc, 2012). Both nymphs and adults are fluid feeders and practice extraoral digestion (Gurr et al., 2007). Some *Orius* species are quite difficult to differentiate morphologically, particularly the immature stages. The genital clasper in males and the copulatory tube in females are commonly used to discriminate some of these species, but the differences are not always evident. The females of some species can also be identified by inspecting the opercula structure of the oviposited eggs (Schuldiner-Harpaz and Coll, 2012).

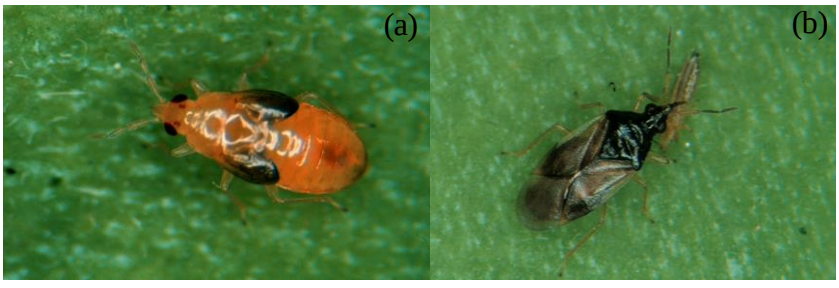

Fig. 3.- Nymph (a) and adult (b) of *Orius majusculus*.

Some *Orius* species have been reported to colonize Mediterranean vegetable crops naturally, like *O. majusculus* (Reuter), *O. laevigatus* (Fieber), *O. niger* (Wolff), *O. albidipennis* (Reuter), *O. minutus* (L.), *O. horvathi* (Reuter) and *O. laticollis* (Reuter) (Ferragut and González-Zamora, 1994; Goula et al., 1993; Morales et al., 2007; Riudavets and Castañé, 1998; Tommasini et al., 2004). The Nearctic species *Orius insidiosus* (Say) was released in Europe in the nineteen nineties to control *F. occidentalis* on cucumber and pepper (Fejt and Jarosik, 2000; Meiracker et al., 1991; Veire and Degheele, 1993), as well as on ornamental plants (Beekman et al., 1991; Fransen et al., 1993; Sorensson and Nedstam, 1993). Results were not as positive as expected because the endemic species were better adapted to the European environmental conditions (Tommasini, 2003).

2.2.3. Coccinellids

Coccinellids are small beetles, commonly called "ladybugs" or "ladybirds", with four developmental stages (egg, larva (four instars), pupa and adult) (Fig. 4). Larvae are present for 2-4 weeks, whereas pupae last for 5-7 days. The larva attaches itself to a leaf and pupates, and within 3 to 12 days the adult emerges, which has a life span of a few months. Most of them have dome-shape bodies, flat underside and depending on the species, the elytra may display spots or stripes (Nedved and Honek, 2012). In the Mediterranean basin, they enter in diapause during winter (Hodek and Evans, 2012), often being among the first insects to appear in the crops in spring. Most of the species in this family are predacious in both adult and larva stage and their mouthparts are modified for chewing (Hodek and Evans, 2012). However, some species also consume nectar, honeydew, pollen, fruit, vegetation and fungus. These non-prey foods are used to increase survival when prey is scarce, reduce migration and enhance reproductive capacity (Lundgren, 2009a).

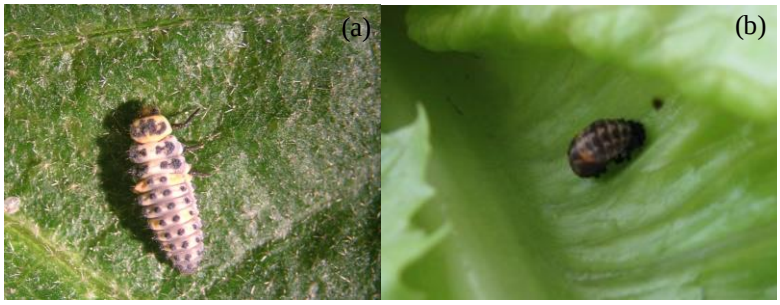

Fig. 4.- Larva (a) and pupa (b) of *Coccinella septempunctata*

This family includes many beneficial species, which are known to be voracious predators of pestiferous aphids (Giorgi et al., 2009). Some studies have shown that their conservation and augmentation within cropland help to suppress aphid outbreaks (Berthiaume et al., 2007; Nyukuri et al., 2012; Obrycki et al., 2009). Only one study has been found about coccinellid predation on *N. ribisnigri*, which was conducted in red and black currant, as well as in lettuce (Nunnenmacher and Goldbach, 1996). Some coccinellids have been reported to prey on thrips (Deligeorgidis et al., 2005; Sabelis and Van Rijn, 1997), including *F. occidentalis* (Sabelis and Van Rijn, 1997 ; Deligeorgidis *et al.,* 2005). The most abundant aphidophagous coccinellids in the Mediterranean basin belong to four genera: *Adalia* Mulsant, 1850; *Coccinella* Linnaeus, 1758; *Scymnus* Kugelann, 1794; and *Propylea* Mulsant, 1846 (Kavallieratos et al., 2004; Urbaneja et al., 2005).

2.2.4. Spiders

Spiders spend all their larval development inside the egg and hatch as spiderlings, very small and sexually immature but similar in shape to adults (Foelix, 1982). They have fangs and most of them use venom to immobilize their prey (King, 2004). They are very common generalist predators in agroecosystems (Nyffeler and Sunderland, 2003), which can be divided into two groups: "hunting and ambush spiders" and "web-building spiders", which means that some spiders are active hunters that patrol the plants and the ground for prey, and others sit camouflaged waiting to catch prey (Enders, 1975; Morse, 1984). In European agroecosystems, “web-building spiders” like Linyphiidae, Theridiidae and Araneidae; and “hunting and ambush spiders” like Salticidae, Thomisidae and Tetragnathidae are commonly present (Clough et al., 2005; Seyfulina, 2005).

Some species of these families are reported to feed on aphids and it has even been suggested that some of them decrease aphid abundance in crops (Chapman et al., 2013; Greenstone and Shufran, 2003; Harwood and Obrycki, 2007; Harwood et al., 2004; Nyffeler et al., 1994). No studies about spider predation on *N. ribisnigri* have been found in the literature. On the other hand, Sabelis and Van Rijn (1997) predicted that thrips would most likely be important components of their diet. The species *Xysticus kochi* Thorell (Araneae: Thomisidae) has been suggested as a potential predator of *F. occidentalis* (Ban et al., 2007; Miliczky and Horton, 2011; Nagy et al., 2010; Zrubecz et al., 2008), as well as the Linyphidae family (Miliczky and Horton, 2011). However, their ecological significance for natural pest control is still largely unexplored. Linyphid spiders have also been described as

feeding on non-pest prey, such as springtails (Agustí et al., 2003), which helped to maintain spider populations in cereal crops when no aphid populations were present.

2.2.5. Other predators

Other less common, but potentially present, predators in lettuce crops are mites, earwigs and green lacewings. Some mites have been described as feeding on aphids, like *Typhlodrompis swirskii* (Athias-Henriot) (Acari: Phytoseiidae) on the aphid *Aphis duranta* Theo (Ali and Zaher, 2007), but no studies of mites predation on *N. ribisnigri* have been found in the literature. Some other phytoseiid species have been reported in several studies as potential natural enemies of *F. occidentalis* in greenhouses, but their role in lettuce crops has never been studied (Chow et al., 2008; Chow et al., 2010; Shipp and Wang, 2003; Wittmann and Leather, 1997; Xu and Enkegaard, 2010). Other ectoparasitic mites from the family Erythraeidae have been also described as feeding on *F. occidentalis* (Goldarazena et al., 2000; Munoz-Cardenas et al., 2014), as well as other species of soil-dwelling predatory mites, like *Hypoaspis acuileifer* (Canestrini) and *Hypoaspis miles* (Berlese) (Mesostigmata: Laelapidae) (Berndt et al., 2004; Borgemeister et al., 2002; Thoeming and Poehling, 2006).

Earwigs have chewing mouth parts (McPartland et al., 2000) and can feed on both plants and animals (Albouy and Caussanel, 1990). They have been described as active aphid predators, feeding on *Eriosoma lanigerum* (Hausmann) (Mueller et al., 1988; Nicholas et al., 2005; Stap et al., 1987); and *Aphis gossypii* Glover and *Aphis spiraecola* Patch (Romeu-Dalmau et al., 2012), but no studies have been found about earwig predation on *N. ribisnigri*.

Green lacewing females lay their tiny, oblong eggs on silken stalks attached to plant tissues (Lucas, 1998). Their larvae are voracious predators, attacking most insects of suitable size, particularly soft-bodied ones, whereas adults feed on nectar and pollen (Bahar et al., 2013; Satpathy et al., 2012). They have been described as feeding on *N. ribisnigri* and *F. occidentalis* under laboratory conditions, but very little is known because only one study has been published about them. This study showed *Chrysoperla carnea* (Stephens) predation on both pests, but particularly on *N. ribisnigri* (Shrestha and Enkegaard, 2013).

Some thrips, like the genus Haplothrips, Scolothrips, Franklinothrips and Aeolothrips have also been indicated as potential predators of *F. occidentalis* (Al-Duhawi et al., 2006; Riudavets, 1995a; Riudavets, 1995b), but very few studies have been conducted, some on greenhouses (Fukuda et al., 2008) and some others under laboratory conditions (Kakimoto et al., 2006; Zegula et al., 2003).

3. Prey-predator interaction studies

Trophic relationships, defined by (Paine, 1996) as ‘‘the ecologically flexible scaffolding around which communities are assembled and structured’’, are defined by interactions among parasitods, predators and their prey. Prey-predator interactions are often embedded in rich communities of multiple interacting prey and natural enemies and the interactions among these species affect the efficacy of biological pest control (Janssen et al., 1998; Prasad and Snyder, 2006). Therefore, predator diversity, as well as the diversity of herbivorous prey and alternative non-pest prey, may affect the suppression of a particular pest species. While specialist predators are mainly dependent on a specific prey species (monophagous), or on only a limited variety of species (oligophagous), generalist predators are able to switch among alternative prey according to their current abundance and/or profitability (polyphagous). Generalist predators maintain relatively constant vital rates because they can shift to alternative prey and therefore should display a more stable population than specialist predators (Redpath and Thirgood, 1999). Hence, designing effective biological control programs for more than one pest species requires an understanding of all interactions occurring among species within biocontrol communities, not just those among pests and their natural enemies or among different species of natural enemies.

Intraguild predation (IGP) is a widespread interaction in ecological communities affecting different taxa and trophic levels. It can occur between biological control agents affecting the abundance, distribution and evolution of many species (Arim and Marquet, 2004; Polis et al., 1989; Rosenheim et al., 1995). According to Rosenheim et al. (1995), IGP involves two different species of organisms that share a prey or host, and have some interaction between them (parasitism or predation). This interaction represents a combination of predation and competition, because both species feed on the same prey resources and also benefit from preying upon one another. The predator that kills and eats the other natural enemy is called the intraguild predator and the other natural enemy is the intraguild prey (Holt and Polis, 1997; Polis et al., 1989).

3.1. The role of alternative non-pest prey

Sometimes generalist predator feeding habits can result in the rejection of the target pest in favor of preferred and often more nutritious non-pest prey, which would be negative for the

BC by reducing the levels of pest consumption (Harper et al., 2005; Harwood and Obrycki, 2005; Harwood et al., 2001). However, at other times, the effect of consuming non-pest species by a generalist predator can be positive for BC. For example, it could enhance predator fecundity by colonizing habitats prior to the arrival of the pest (Lundgren, 2009a) or can maintain predator populations in the crop when pest preys are not present (Agustí et al., 2003). It is therefore important to maximize the diversity of NE to control crop pests in the field; to determine the feeding capacity of the different predatory groups for pest control; and to evaluate the potential interference of alternative non-pest preys when they are available in the field.

3.1.1. Collembola

Springtails (Hexapoda: Collembola) have the widest distribution of any hexapod group, occurring throughout the world, including Antarctica. With at least 6500 species (Hopkin, 1997), they colonize all soil habitats that provide enough humidity and food. They are very abundant in agroecosystems, where most springtail species are soil or litter dwellers, whilst only a few species can live on the surface or in the vegetation, like the family Entomobryidae (Hopkin, 1997).

They are soft-bodied, oval or roundish shaped, primitive insects (Fig. 5). Their bodies consist of six or fewer segments and they lack wings. Although many species have small eyes, some are nearly or totally blind. They occur in a wide range of colors including whitish, yellowish, brown, gray, bluish or black, and they may be mottled. They have biting mouthparts mostly retracted into the head. Some springtails have mandibles with well-developed molars. Others are fluid feeders, having stylet-like mouthparts. On the ventral side of the first abdominal segment, there is a tube-like structure called collophore, which is the site of water uptake. A forked structure or furcula is located on the ventral side of the fourth abdominal segment. This structure is used to propel themselves through the air and to jump away (Hopkin, 1997).

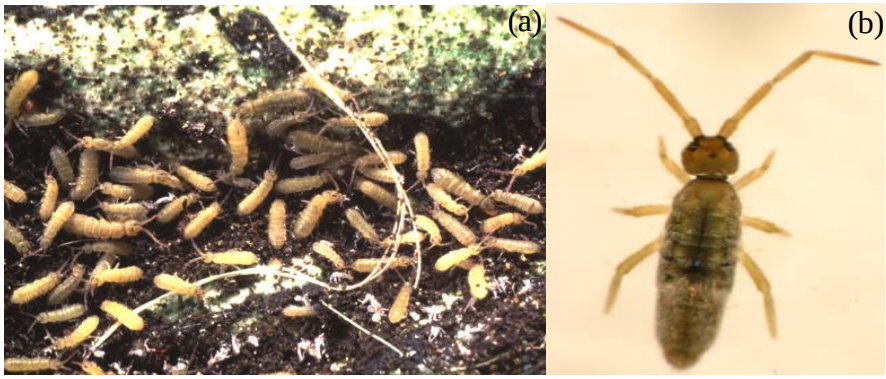

Fig.5- Springtails (a) and adult of the family Entomobrydae (b).

Some predators have been reported to feed on springtails. For example, species of hemipters like *Anthocoris nemorum* (Linnaeus) or *Anthocoris nemoralis* (Fabricius) (Hemiptera: Anthocoridae) (Sigsgaard, 2010), species of coleopters like *Cantharis* spp. (Coleoptera: Cantharidae), *Nebria brevicollis* (Fabricius) (Coleptera: Carabidae) and *Bembidion* spp.(Coleoptera: Carabidae) and scydmaenine beetles like *Scydmaenus tartasus* Muller & Kunze and *Scydmaenus hellwigii* (Herbst) (Coleoptera: Scymaenidae) (Eitzinger and Traugott, 2011; Holopainen and Helenius, 1992; Jaloszynski, 2012). Some spider families, like Theridiidae, Lyniphidae, Lycosidae and Oxyopidae (Agustí et al., 2003; Bardwell and Averill, 1997; Chapman et al., 2013; Harwood et al., 2004; Kuusk and Ekbom, 2012; Opatovsky et al., 2012) have also been reported as feeding on springtails.

3.2. Prey-predator interactions in Mediterranean lettuce crops

As explained in the previous sections, several generalist predators can be found in Mediterranean lettuce crops, among the most abundant being the anthocorids of the genus *Orius*, syrphids, coccinellids and spiders. The trophic relationships explained in the previous sections between predators, pests and non-pest prey in Mediterranean lettuce crops are summarized in Fig. 6. In summary, syrphids are well-known predators of aphids and of *N. ribisnigri* in particular. They have also been described as preying on thrips, but predation on *F. occidentalis* has never been investigated (see section 2.2.1). It is well known that *Orius* feed on *F. occidentalis*, but no evidence of prey on *N. ribisnigri* has been found yet (see section 2.2.2). Coccinellids feed on *N. ribisnigri* and some species can even prey on thrips, including *F. occidentalis* (see section 2.2.3). Spiders feed on *F. occidentalis*, springtails and aphids, but no studies have shown spiders' predation on *N. ribisnigri* (see section 2.2.4).

Some other potential interactions have never been studied, like the predation of *Orius* spp., coccinelid and syrphids on springtails.

As previously mentioned, IGP can play an important role in the dynamics of predation by NEs and their role in BC programs, but the IGP between the predator species present in Mediterranean lettuce crops has hardly been studied. For instance, it is not well known *Orius* can feed on coccinellids. *Orius laevigatus* was not able to feed on eggs of the coccinellid *Adalia bipunctata* (Linnaeus) or *Harmonia axyridis* (Pallas) in laboratory conditions (Santi and Maini, 2006). However, other species, like *O. insidiosus* have been observed to consume *H. axyridis* in field conditions (Harwood et al., 2009). Also, no studies have been published about *Orius* predation on syrphids, even if it has been demonstrated that other heteropterans, like mirid bugs preyed on them (Frechette et al., 2007; Lucas and Alomar, 2000). No studies have been published either about *Orius* spp. predation on spiders. It has been described that syrphids fed on coccinellids in laboratory conditions (Hindayana et al., 2001), but no assay has been published about syrphids preying on *Orius* spp. or spiders. Also, no studies have been published about coccinellids preying on *Orius* spp., syrphids or spiders. Spiders are well-known ladybird predators (Yasuda and Kimura, 2001), but no studies about predation on *Orius* spp. or syrphids have been found in the literature. On the other hand, it is also known that IGP could occur between species of the same genus. For instance, IGP has been demonstrated between *O. laevigatus* and *O. insidiosus* (Tommasini et al., 2002), different species of coccinellids (Rondoni et al., 2012) and different species of spiders (Denno et al., 2004). In the case of syrphids, there are no studies about predation on other syrphid species.

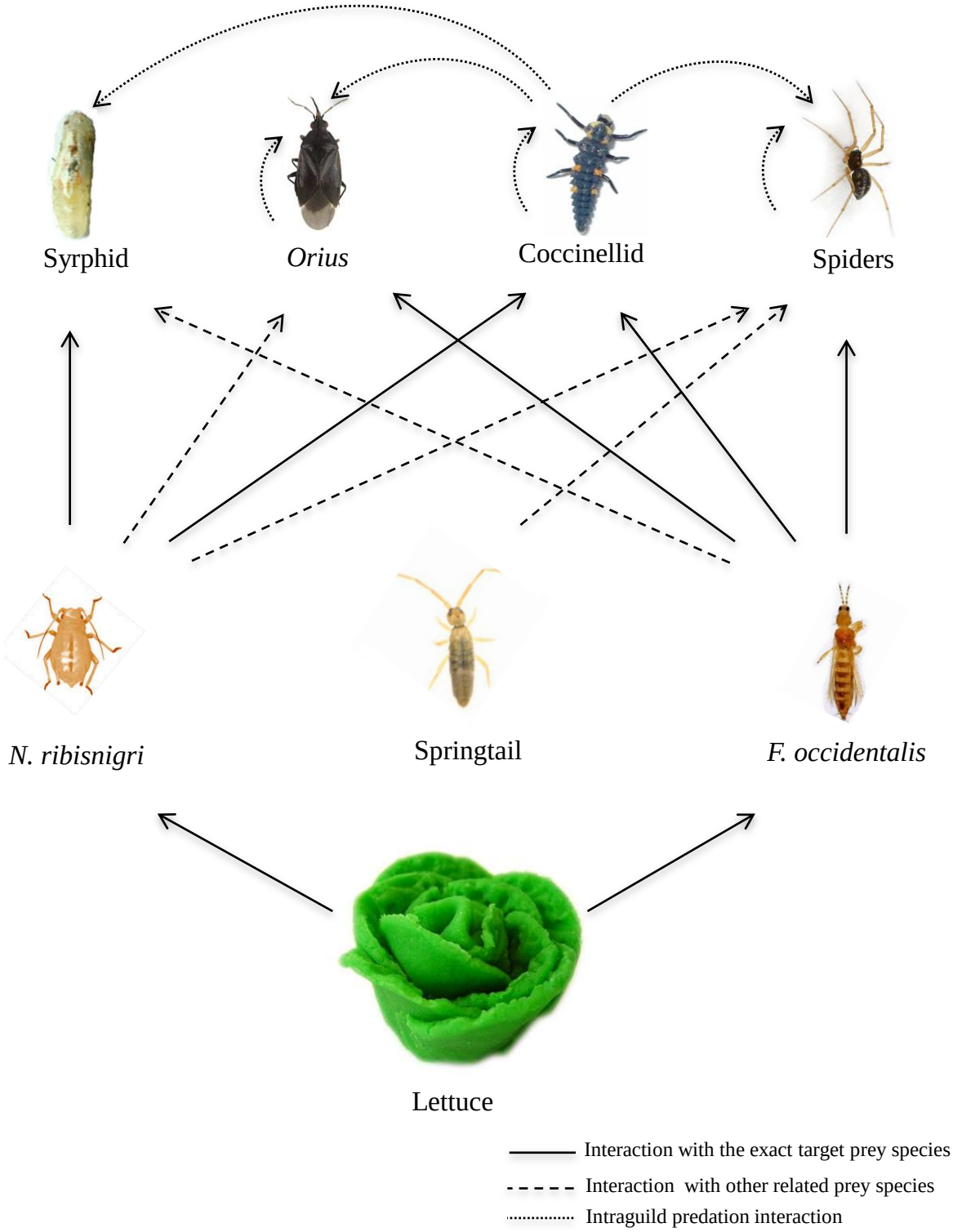

Fig. 6.- Known trophic interactions between the main predators (*Orius* spp., syrphids, coccinellids and spiders), pests (*N. ribisnigri* and *F. occidentalis*) and non-pest prey (springtails) present in Mediterranean lettuce crops. Solid lines indicate an interaction with the exact target prey species. Discontinuous lines indicate an interaction with other related prey species. Dotted lines indicate an intraguild predation interaction.

3.3. The use of molecular methods to study prey-predator interactions

Laboratory experiments are unable to recreate real field conditions, and even in field conditions there is a possible alteration of predation due to observation (Symondson, 2002). Direct observation, which means observing predation directly in the field, can be complicated for small and cryptic invertebrate predators and particularly tedious and time-consuming. The fact that some predators may have nocturnal habits can make this observation even more difficult. Performing a post-mortem analysis of predators collected in the field and studying the ingested prey can avoid these problems. These post-mortem methods can be classified as direct and indirect methods. Direct methods are based on gut dissection and morphological identification of prey remains. However, in those cases where predators are fluid feeders or perform an extraoral digestion followed by suction of the liquified content, this is not possible. This is the case of many polyphagous predators used in BC programs. In these cases, an alternative indirect method to identify prey remains based on the use of molecular techniques should be used.

3.3.1. Conventional and real-time PCR

Different molecular methods have been used to identify food remains in animal guts. They can be classified in two main groups depending on the type of molecules detected, which can be either proteins (including electrophoretic and serological methods) or DNA (Deoxyribonucleic acid) (Agustí, 1998). Even if serological methods, and particularly the use of monoclonal antibodies, were the most used in the past century (80's and 90's), the most common method for diet analysis nowadays is the use of Polymerase Chain Reaction (PCR). Several PCR-based approaches have been used to amplify food remain DNA (reviewed in Symondson (2002); King *et al.* (2008)), but conventional PCR combined with the use of prey-specific primers has been the most frequently used method until now, allowing the visualization of prey DNA products as size-specific bands on an agarose gel (McPherson and Møller, 2000).

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 (Saunders, 2009). It is important to point out that conventional PCR gives a qualitative assessment. On the contrary, real-time PCR can add information to the interpretation of PCR-based gut

analysis based on the accumulation of the amplified product as the reaction progresses (Lundgren and Fergen, 2011; Weber and Lundgren, 2009); which could represent a measurement of prey consumption. The main controversy concerning real-time PCR is the fact that even if allows quantifying, it is not possible to distinguish between the amount of prey DNA truly eaten or the post-ingestion elapsed time. This, together with the fact that it is a more expensive technique than conventional PCR, has meant that real-time PCR has not been the most common technique in predation studies (Lundgren, 2009a; Lundgren et al., 2009; Lundgren and Fergen, 2011; Weber and Lundgren, 2009). Besides of being able to quantify consumption, it is well known that real-time PCR is a more sensitive technique than conventional PCR. Therefore, it is still interesting to use this method in the evaluation of predation because predation rates can be increased with respect to those obtained by conventional PCR.

3.3.2. Next Generation Sequencing (NGS)

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 (conventional and real-time PCR) can establish trophic linkages, but only focused on specific consumer-food interactions based on primer sequences that amplify the prey-specific DNA. To overcome these barriers, an entirely new technology comes on the scene: *Next Generation Sequencing* (NGS).

The most important advantage of NGS is that it can be used to examine dietary breadth without the need to design species-specific primers for each prey. Instead, a targeted DNA fragment from all food items present in a gut is amplified using universal primers. Those amplicons are then sequenced, and the identities of the ingested organisms can be established by comparing with sequence databases, like GenBank, (www.ncbi.nlm.nih.gov) or BOLD (www.barcodinglife.com). The potential of NGS for simultaneously characterizing many species from an environmental sample through sequencing of DNA barcodes is enormous (Pompanon et al., 2012).

Sanger sequencing, which is often considered the "first generation sequencing" technology, relies on a technique known as capillary electrophoresis, which separates fragments of DNA by size and then sequences them by detecting the final fluorescent base on each fragment (Sanger et al., 1977). After a series of technical innovations, the Sanger method has reached the capacity to read through 1000–1200 basepairs (bp). This technology,

which has become widely adopted in laboratories across the world and is still extremely important today, has always been hampered by inherent limitations in throughput, scalability, speed and resolution. The fact of sequencing individual specimens made Sanger technology inadequate for processing complex environmental samples, especially for large-scale studies. Although Sanger sequencing has provided the most efficient method for the development of large DNA barcode reference libraries, the number of individuals in an environmental sample is beyond the scope of its ability (Hajibabaei et al., 2011). The high demand for low-cost sequencing has driven the development of NGS technologies that produce thousands or millions of sequences concurrently.

NGS technologies can be classified into two main groups: (1) PCR-based technologies and (2) non-PCR-based technologies. The first group includes several commercially available platforms, like Roche 454 Genome Sequencer (Roche Diagnostics Corp.), HiSeq 2000 (Illumina Inc.), AB SOLiD System (Life Technologies Corp.) and Ion Torrent Personal Personal Genome Machine (PGM) (Life Technologies). The non-PCR-based group, called 'single-molecule' sequencing (SMS), does not include an amplification step prior to sequencing and is very recent. Two single-molecule sequencing systems have been announced recently: HeliScope (Helicos BioSciences Corp.) and PacBio RS SMRT system (Pacific Biosciences) (Liu et al., 2012; Mardis, 2008; Metzker, 2010; Shendure and Ji, 2008; Shokralla et al., 2012; Zhang et al., 2011).

Regarding the "PCR-based group", there are also three groups depending on the technology used: (1) sequencing by synthesis technology (based on light measurement) and known as "pyrosequencing" (like in Roche 454 Genome Sequencer or HiSeq 2000 platform), in which the light emitted from phosphate molecules during nucleotide incorporation is recorded as the polymerase synthesizes the DNA strand (Fuller et al., 2009; Liu et al., 2012; Shokralla et al., 2012); (2) sequencing by ligation technology (based on fluorescence measurement), in which an emulsion PCR approach with small magnetic beads is used to amplify DNA fragments and fluorescently labelled oligonucleotides hybridize to their complementary sequence adjacent to the primed template (like in AB SOLiD System platform) (Liu et al., 2012; Metzker, 2010; Shokralla et al., 2012); and (3) sequencing by post-light sequencing technology (based on pH measurement). In this case, instead of using light as an intermediary or fluorescent as label, it uses a semiconductor chip. Each time a nucleotide is incorporated into the DNA strand, one hydrogen ion is released. The charge from that ion changes the pH of the solution, which can be detected by the ion sensor and then directly translates chemical signals into digital format (see below the Ion Torrent PGM

section) (Hajibabaei et al., 2011; Liu et al., 2012; Merriman et al., 2012; Rothberg et al., 2011).

In recent years, NGS technologies have increased the speed and throughput capacities of DNA sequencing and as a result, dramatically reduced overall sequencing costs (Mardis, 2008; Metzker, 2010; Schuster, 2008; Shendure and Ji, 2008; Zhang et al., 2011). At present, NGS only provides 50–500 continuous basepair reads, which is why sequencing results are defined as short reads. These short reads are a major limitation in current technology; however, identification of prey species is based on the amplification of short DNA fragments (100-350 bp), characteristic of those obtained from stomach contents or feces, making the NGS approach very appropriate for the gut analysis of arthropods (Pompanon et al., 2012; Valentini et al., 2009). On the other hand, as NGS technology spreads and reduces costs, ecologists are turning it into a powerful tool for ecological studies including dietary analyses. Some NGS technologies, like 454 Roche platform, have been used up to now to study the diet of vertebrates and invertebrates (Bohmann et al., 2011; Brown et al., 2012; Deagle et al., 2009; Raye et al., 2011; Shehzad et al., 2012; Valentini et al., 2009).

3.3.3. Ion Torrent PGM

Ion Torrent Personal Genome Machine (PGM) technology (Rothberg et al., 2011) represents an entirely new approach to sequencing based on the post-light sequencing technology previously mentioned. The Ion Torrent PGM platform can use one of the three available chips (314, 316 or 318), which can generate up to 10 Mb, 100 Mb or 1 Gb, respectively (Shokralla et al., 2012). The major advantages of the Ion Torrent PGM platform are its relatively short run time, its relatively low cost per run and its low-cost equipment compared with the other platforms previously cited (Loman et al., 2012; Quail et al., 2012).

Very few studies of animal diets have used the Ion Torrent PGM platform until now (Deagle et al., 2013; Piñol et al., 2014; Welker et al., 2014). The steps to follow are shown in Fig. 7. After sample collection (e.g. arthropod specimens), DNA was extracted (prey and predator DNA in this case) and amplified using general primers (e.g. invertebrate primers, like those described in Zeale *et al.* (2011)). After sequencing with the Ion Torrent PGM, thousands of prey sequences were obtained together with numerous non-informative predator reads. The last step was processing and analyzing the data obtained by comparing the sequence reads obtained with the sequences from databases, which ended with the final taxonomic assignation of each Ion Torrent PGM sequence read.

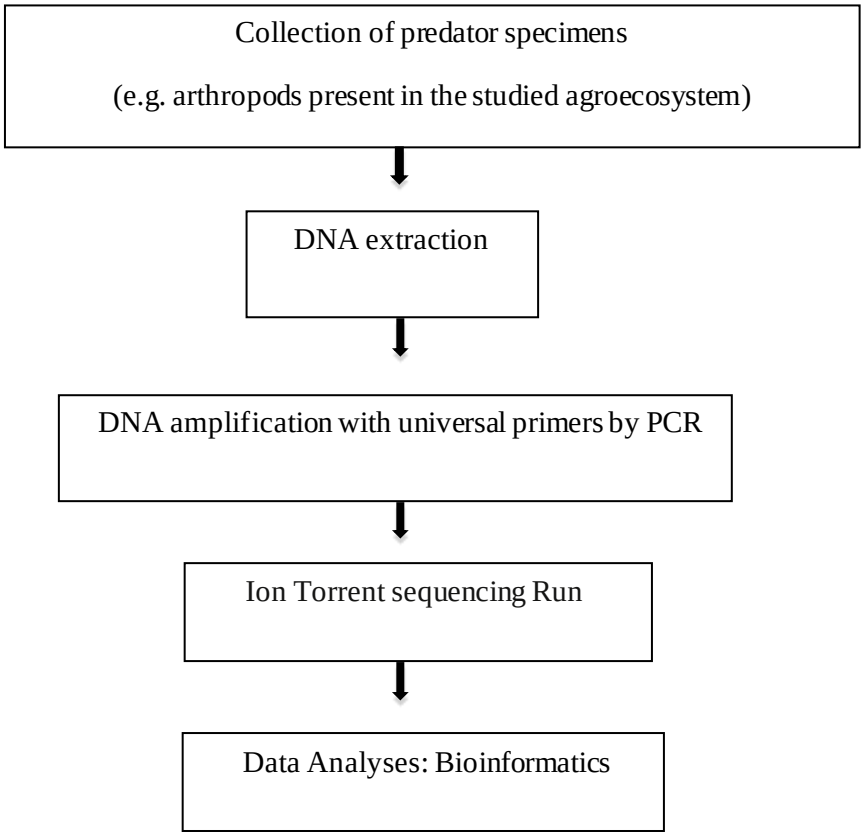

Fig.7. - Flowchart diagram showing the main steps of the use of Ion Torrent PGM approach for assessing predator diet composition.

Objectives

This Thesis is focused on the study of the trophic interactions present in Mediterranean lettuce crops, which is necessary to develop, apply and improve CBC programs. The principal objectives are to:

1. develop molecular diagnostic methods for the identification of the most abundant predator species present in Mediterranean lettuce crops in all developmental stages: hoverflies (**Chapter 1**) and *Orius* (**Chapter 2**).

2. describe the trophic links present in Mediterranean lettuce crops under field conditions among the main pests species, non-pest prey and generalist arthropod predators; which are hoverflies (**Chapters 3 and 5**), *Orius* (**Chapters 4 and 5**), spiders and coccinellids (**Chapter 5**).

This Doctoral Thesis has been organized in 5 chapters, which corresponded to 5 papers (2 published, 1 in revision and 2 submitted for publication). More information about each paper is given below together with the impact factor of each journal (2012 Journal Citation Reports, ISI Web of Knowledge):

Chapter 1:

Gomez-Polo P, Traugott M, Alomar O, Castañé C, Rojo S and Agustí N. (2014). Identification of the most common predatory hoverflies of Mediterranean vegetable crops and their parasitism using multiplex PCR. Journal of Pest Science (87): 371-378.
(2012 Journal Impact factor: 2.174).

Chapter 2:

Gomez-Polo P, Alomar O, Castañé C, Riudavets J and Agustí N. (2013). Identification of *Orius* spp. (Hemiptera: Anthocoridae) in vegetable crops using molecular techniques. Biological Control (67): 440-445.
(2012 Journal Impact factor: 1.917).

Chapter 3:

Gomez-Polo P, Alomar O, Castañé C, Lundgren J G, Piñol J and Agustí N. (2014). Molecular assessment of predation by hoverflies (Diptera: Syrphidae) in Mediterranean lettuce crops. Submitted In revision into Pest Management Science.
(2012 Journal Impact factor: 2.594).

Chapter 4:

Gomez-Polo P, Alomar O, Castañé C, Aznar-Fernández T, Lundgren J G, Piñol J and Agustí N. (2014). Understanding predation by *Orius* spp. in lettuce crops by molecular methods. Submitted to Biocontrol.
(2012 Journal Impact factor: 2.215).

Chapter 5:

Gomez-Polo P, Alomar O, Castañé C and Agustí N. (2014). Molecular tracking of arthropod predator-prey interactions in Mediterranean lettuce crops. Submitted to Biological Control.
(2012 Journal Impact factor: 1.917).

Chapter 1: Identification of the most common predatory hoverflies of Mediterranean vegetable crops and their parasitism using multiplex PCR.

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ORIGINAL PAPER

Identification of the most common predatory hoverflies of Mediterranean vegetable crops and their parasitism using multiplex PCR

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Abstract The larvae of many hoverflies (Diptera: Syrphidae) are important polyphagous predators used in integrated pest management programs. Because the accurate identification of preimaginal stages by morphological characters is difficult, we have developed a multiplex PCR to identify the immature and/or adult stages of the most common syrphid species in Mediterranean vegetable crops: *Episyrphus balteatus*, *Scaeva pyrastri*, *Eupeodes corollae*, *Meliscaeva auricollis*, *Sphaerophoria scripta,* and *Sphaerophoria rueppellii*. The latter two species were amplified by the same primer pair due to the high similarity of their cytochrome oxidase subunit I sequences. Additionally, the assay included a primer pair targeting *Diplazon laetatorius*, a common koinobiont ichneumonid endoparasitoid of predatory syrphid larvae. The multiplex PCR assay proved to be highly specific and sensitive, and it was used to study the assemblage of hoverfly species in larval stage in two Mediterranean lettuce crops in two consecutive years. The molecular analysis revealed that *Eu. corollae*, *Ep. balteatus*, and *Sph. scripta/Sph. rueppellii* were the species present in the investigated fields. Species composition differed depending on sampling date and whether the larvae were collected on the plants or on the ground. The parasitoid *D. laetatorius* was not detected in any of the analyzed hoverfly larvae, suggesting low-parasitism pressure in the studied syrphid populations. The wide distribution of most of these syrphid species makes this multiplex PCR assay an ideal tool to deepen our knowledge on the ecology of these polyphagous hoverfly species in preimaginal stages and to improve the use of hoverflies to control insect pests.

Keywords Syrphidae · COI · *Diplazon laetatorius* · Molecular species identification · Lettuce crops · Diagnostic PCR

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Introduction

Hoverflies (Diptera: Syrphidae) are an abundant group of insects present in natural and agriculture related ecosystems. With about 750 species recorded in Europe (Speight 2011), at least 355 species are recorded from Spain (Marcos-García et al. 2002). Their adults provide crucial ecosystem services as important pollinators, obtaining their energy requirements by feeding on nectar and pollen (Haslett 1983; Branquart and Hemptinne 2000; Jauker et al. 2012). The larvae of about 35 % of the species of the family of syrphids are polyphagous predators of a broad range of soft-bodied arthropods, including coleopteran and lepidopteran larvae, mites, thrips, and hemipterans (e.g.,

coccids, psyllids, whiteflies and particularly aphids) being the preferred prey (Rojo et al. 2003; Rotheray and Gilbert 2011). Predatory larvae of many syrphid species hide under bark or underneath soil particles during the day and are mostly active at dawn and dusk. This behavior makes them less conspicuous than other natural enemies (Hagen et al. 1999). In Spain, 124 syrphid species with predaceous larvae have been reported by Marcos-García et al. (1998), most of them commonly found in the Mediterranean basin (Speight 2011). Some predatory hoverflies are abundant in different agroecosystems such as fruit orchards, woodlands, grasslands, scrublands, as well as in arable and vegetable crops (Ghahari et al. 2008; Haenke et al. 2009; Hopper et al. 2011). Less than 30 syrphid predaceous species had been related to herbaceous plants at the Iberian Peninsula (Rojo and Marcos-García 1998; Rojo et al. 2003). Six of these hoverfly species are commonly found in Mediterranean vegetable crops such as lettuce (Rojo 1995; Pascual-Villalobos et al. 2006; Morales et al. 2007): *Episyrphus balteatus* (De Geer), *Scaeva pyrastri* (Linnaeus), *Eupeodes corollae* (Fabricius), *Meliscaeva auricollis* (Meigen), *Sphaerophoria scripta* (Linnaeus), and *Sphaerophoria rueppellii* (Wiedemann).

Syrphid larvae may be attacked by a wide range of hymenopteran parasitoids belonging to the families Ichneumonidae, Encyrtidae, Pteromalidae, Megaspilidae, and Figitidae (Scott 1939; Rotheray and Gilbert 2011). However, the most common endoparasitoids of predatory species belong to the family Ichneumonidae and the subfamily Diplazontinae (Bordera et al. 2000, 2001). Particularly *Diplazon laetatorius* (Fabricius) has been reported as the most important natural enemy of hoverflies in terms of abundance of the taxon around the world (Greco 1997; Jankowska 2004). This species is a koinobiont endoparasitoid that oviposits into the syrphid eggs or first instars larvae, with the imago emerging from the syrphid puparium (Mayadunnage et al. 2009).

Although the larvae of many hoverfly species are important biocontrol agents (Rojo et al. 2003; Hopper et al. 2011), it is quite difficult to obtain accurate identification of preimaginal stages, particularly in the first larval stages, using exclusively morphological characters (Bastian 1986; Laska et al. 2006). Moreover, larvae of many species are unknown and the color pattern of common species it is not retained after preserving them in ethanol (Rotheray 1993). Rearing field-collected larvae to the adult stage is recommended for a correct morphological identification (Gilbert 1993), however, this is a time-consuming process which can be accompanied by a high mortality (Jankowska 2004). At the same time, it is also difficult to discriminate between parasitized and nonparasitized hoverfly larvae to assess how parasitoids may impact hoverfly larval populations and their biocontrol success (Hazell et al. 2005). Hence, an alternative technique is needed which allows identifying hoverflies in their larval stage and to detect parasitism by *D. laetatorius*.

To date, two DNA-based approaches are most widely used for species identification: (i) DNA barcoding (Hebert et al. 2003), where species-specific sequences are generated and identified via a reference database (e.g., Mengual et al. 2008; Stahls et al. 2009; Benefer et al. 2013), or (ii) diagnostic PCR where species-specific primers may be used either individually in one PCR amplifying just one target species (singleplex PCR) or simultaneously in a multiplex PCR which enables the parallel identification of several species (King et al. 2011; Staudacher et al. 2011). While the former approach can be limited by the sequence barcode information available in databases such as GenBank or Bold to identify the sequence, the latter technique, is particularly useful once species-specific primers have been developed and when large sample numbers have to be screened because it is cost-effective and quick. Results obtained by multiplex PCR are usually not corrupted by the presence of endoparasitoid DNA, which can be a problem when using the barcoding approach because the mixture of different sequences may foil species identification (Traugott et al. 2013). On the other hand, multiplex PCR can only identify those taxa for which primers have been developed, which means that this approach needs to be carefully checked for cross-reactivity to ensure accurate results.

The aims of this study were: (1) to design species-specific primers for the six most common hoverfly species found in Mediterranean vegetable crops, as well as the parasitoid *D. laetatorius*; (2) to embed these primers in a multiplex PCR assay to easily and rapidly identify these syrphid species including the detection of parasitoid DNA; and (3) to use this molecular tool to identify which of these hoverflies species are present in larval stage in two Mediterranean lettuce crops in two consecutive years as well as to assess the levels of parasitism by *D. laetatorius*.

Materials and methods

Insects

Twenty hoverfly species commonly present in European agricultural environments (Table 1) were used for designing species-specific primers targeting the most common hoverfly species found in Mediterranean vegetable crops: *Ep. balteatus*, *Sc. pyrastri*, *Eu. corollae, M. auricollis, Sph. scripta*, and *Sph. rueppellii*. These specimens were collected in several locations of Spain and Germany (Table 1).

Table 1 Syrphid, potential prey, and parasitoid species tested in the specificity test with the hoverfly- and parasitoid-specific primers described in Table 2

Order	Family	Species	Location (country)
Diptera	Syrphidae	*Dasysyrphus albostriatus* (Fallén)	Butenbock (G)
		Epistrophe nitidicollis (Meigen)	Butenbock(G)
		Episyrphus balteatus (De Geer)	Ruthe (G), Cabrils, lab rearing (S)
		Eupeodes corollae (Fabricius)	Ruthe (G), Alicante (S)
		Eupeodes lucasi (Marcos-García&Laska)	Alicante (S)
		Eupeodes luniger (Meigen)	Niedernwöhren (G)
		Melanostoma mellinum (Linnaeus)	Ruthe (G)
		Melanostoma scalare (Fabricius)	Ruthe (G)
		Meliscaeva auricollis (Meigen)	Alicante (S)
		Meliscaeva cinctella (Zetterstedt)	Ruthe (G)
		Paragus tibialis (Fallén)	Alicante (S)
		Platycheirus albimanus (Fabricius)	Ruthe (G)
		Platycheirus clypeatus (Meigen)	Ruthe (G)
		Scaeva albomaculata (Macquart)	Niedernwöhren (G)
		Scaeva pyrastri (Linnaeus)	Niedernwöhren (G), Alicante (S)
		Scaeva selenitica (Meigen)	Niedernwöhren (G)
		Sphaerophoria rueppellii (Wiedemann)	Valencia (S)
		Sphaerophoria scripta (Linnaeus)	Ruthe (G),Valencia (S)
		Syrphus ribesii (Linnaeus)	Valencia (S)
		Xanthandrus comtus (Harris)	Valencia (S)
Hemiptera	Aphididae	*Nasonovia ribisnigri* (Mosley)	Madrid, lab rearing (S)
		Aphis gossypii (Glover)	Madrid, lab rearing (S)
		Aulacorthum solani (Kaltenbach)	Madrid, lab rearing (S)
		Hyperomyzus lactucae (Linnaeus)	Madrid, lab rearing (S)
		Macrosiphum euphorbiae (Thomas)	Madrid, lab rearing (S)
		Myzus persicae (Sulz.)	Madrid, lab rearing (S)
Thysanoptera	Thripidae	*Frankliniella occidentalis* (Pergande)	Cabrils, lab rearing (S)
		Thrips tabaci Lindeman	Cabrils, lab rearing (S)
Collembola	Entomobryidae	*Entomobrya* sp.	Cabrils (S)
Hymenoptera	Ichneumonidae	***Diplazon laetatorius*** (Fabricius)	Alicante (S)

Target species are highlighted in bold

G Germany, *S* Spain

Sequencing and primer design

A nondestructive DNA extraction method was used to avoid morphological damage to the adult syrphid samples (Staudacher et al. 2011), and a minimum of one adult specimen per species was sequenced. The adult hoverflies were incubated overnight at 58 °C with 180 µl of buffer ATL and 20 µl of Proteinase K (10 mg ml^{-1}, AppliChem, Darmstadt, Germany). DNA was extracted from this solution using the DNeasy Tissue Kit (Qiagen, Hilden, Germany; protocol for animal tissues) following the manufacturer's protocol and stored at −20 °C. One negative extraction control was included in each batch of 30 samples. All syrphids were amplified using the universal primers LC01490/HC02198 described in Folmer et al. (1994), obtaining fragments of the cytochrome *c* oxidase subunit I (COI) gene of approximately 700 bp in length. Each 10 µl PCR contained 1.5 µl of DNA extract, 5 µl of 2× Multiplex PCR Master Mix (Qiagen), 1 µM of each primer, and 1.5 µl of PCR-grade RNase-free water (Qiagen). Thermocycling was done using Mastercycler Gradient PCR machines (Eppendorf, Hamburg, Germany); the thermocycling program consisted of an initial denaturation step of 15 min at 95 °C, followed by 35 cycles of 20 s at 94 °C, 30 s at 52 °C, 45 s at 72 °C, and a 3 min final extension at 72 °C. PCR products were electrophoresed on 1.5 % agarose gels stained with GelRed™ (Biotium, Hayward, USA) and visualized under UV light. PCR products were purified with ExoSAP®-IT (GE Helthcare, Little Chalfont, UK) following the manufacturer's

recommendation and sequenced according to the dideoxychain-termination method. Sequences were aligned and edited manually using Bioedit Sequence Alignment Editor v. 7.0.9.0 (Hall 1999). The obtained sequences were submitted to GenBank database (see Table s1 for accession numbers). These sequences were also aligned with other sequences from the GenBank database (Table s1) using CLUSTALW2 (www.ebi.ac.uk/Tools/msa/clustalw2) and checked for species-specific primer-binding sites. All primer pairs (five for the six hoverfly target species and one for the parasitoid *D. laetatorius*) were designed using Primer Premier 5 (Premier Biosoft International, CA, USA).

Multiplex PCR and specificity assay

All field-collected larval syrphid specimens tested by multiplex PCR were also DNA extracted using the DNeasy Tissue Kit (QIAGEN; protocol for animal tissues). Total DNA was eluted in 100 µl of AE buffer provided by the manufacturer and stored at −20 °C. Two negative extraction controls were added to each set of 28 samples. Multiplex PCR was optimized testing different concentrations of primers and thermocycling conditions. The final reaction volumes (10 µl) contained 1.5 µl of DNA extract, 5 µl of 2× Multiplex PCR Master Mix (Qiagen), 1 µl of 10× primer mix, 1 µl of 5× Q-solution, and 1.5 µl of PCR-grade RNase-free water (Qiagen). Primer concentrations in the primer mix were different depending on the species (see Table 2). In a 2720 thermocycler (Applied Biosystems, CA, USA), the DNA extracts were subjected to 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 64 °C for 90 s, and 72 °C for 60 s and a final extension of 72 °C for 10 min. Target DNA and water were always included as positive and negative controls, respectively. PCR products were separated by electrophoresis in 3.6 % agarose gels stained with ethidium bromide and visualized under UV light.

Primer specificity was evaluated not only by testing the six target hoverfly species, but also the other 14 nontarget hoverfly species (1–4 individuals/species) used for primer design. Additionally, nine potential hoverfly prey species which are commonly found in Mediterranean lettuce crops, including aphids, thrips, and collembolans, as well as the hoverfly parasitoid *D. laetatorius*, were tested (3 individuals/species) (Table 1).

Different concentrations of *D. laetatorius* DNA were analyzed to characterize the sensitivity of the primer pair targeting the parasitoid. The initial DNA concentration tested with the multiplex PCR protocol described above was 0.4 ng/µl which was twofold diluted down to 2.5 pg/µl. DNA concentrations were measured in a Qubit Fluorometer (Invitrogen, CA, USA) using the Quant-iT^{TM} dsDNA HS assay kit (Invitrogen).

Analysis of field-collected hoverfly larvae

Two lettuce fields (var. Maravilla) located in El Maresme area (Barcelona, Spain) were sampled. One was an experimental field at IRTA (41°31′4.33″N, 2°22′37.87″E) and the other one was a commercial field in 50-km distance (41°28′26.07″N, 1°57′34.52″E).

In the experimental field, two consecutive lettuce crops were planted: one from beginning of April until end of May and another from beginning of June until beginning of August, both in 2009 and 2010. Twenty to thirty lettuces were collected on May18th and 19th 2009; July 7th 2009; and May 11th, 18th, and 25th 2010. All lettuces were brought individually in plastic bags to the laboratory, where all syrphid larvae were collected. On May 12th 2009, the experimental field was also manually sampled

Table 2 Syrphid- and parasitoid-specific primer pairs. Columns show target species, primer names (F and R denotes forward and reverse primers, respectively), sequences, product sizes, and the primer concentrations used in the multiplex PCR

Target species	Primer name	Sequence (5′–3′)	Size (bp)	Con. (µM)
Meliscaeva auricollis	Mel-aur-F1	TGAACAGTTTATCCTCCTCTTTCTT	96	0.4
	Mel-aur-R2	TGATGATATACCTGCTAAATGTAAAGAG		
Sphaerophoria scripta/*Sphaerophoria rueppellii*	Sph-rue-scr-F2	GATTATTACCTCCTTCTYTAACATTACTT	165	0.4
	Sph-rue-scr-R1	TTGATGATATTCCTGCTAAATGTAAT		
Scaeva pyrastri	Sca-pyr-F3	TATTTTTTCTCTACATTTAGCTGGTATG	314	0.3
	Sca-pyr-R1	TGGATCTCCTCCTCCTGCA		
Eupeodes corollae	Eup-cor-F2	TGATTATTACCTCCATCTTTAACTCTT	395	0.2
	Eup-cor-R2	GATGATATTCCAGCTAAATGAAGG		
Episyrphus balteatus	Epi-bal-F1	GCAGAACTTGGTCATCCTGGT	754	0.2
	Epi-bal-R1	GGTATTCGATCATAAGTAATTCCATG		
Diplazon laetatorius	Dip-F2	CTGTATATCCCCCTTTATCTTCTAATT	220	0.8
	Dip-R3	GGGAACTGCTAATAATAATAAAATTGT		

once for syrphid larvae found on the ground. In the commercial field, also twenty to thirty lettuces were sampled once on April 22th 2009. All collected larvae were stored at −20 °C until molecular analyses.

All syrphid larvae were individually analyzed by multiplex PCR to study parasitism by *D. laetatorius* and the syrphid larval species composition depending on the sampled season (spring and summer), year (2009 and 2010) and substrate (lettuce or ground). Species percentages were calculated and compared in order to determine whether they were influenced by the season, year, substrate and sample location.

Results

Multiplex PCR and specificity assay

COI sequences of 21 hoverfly species were generated and submitted to GenBank (accession numbers are shown in Table s1). Six specific primer pairs were designed for the hoverflies *Ep. balteatus*, *Eu. corollae*, *M. auricollis*, *Sc. pyrastri, Sph. scripta/Sph. rueppellii* and the parasitoid *D. laetatorius* (Table 2). *Sphaerophoria scripta* and *Sph. rueppellii* were covered by one primer pair as their sequences were very similar (97.6 % sequence identity for a 570 bp long stretch of COI sequence). The hoverfly primers generated DNA fragments ranging from 96 to 754 bp depending on the species (Fig. 1; Table 2). The parasitoid *D. laetatorius* was also detected with the parasitoid primers, amplifying a specific 220 bp fragment. Detection of the parasitoid was possible down to a DNA concentration of 0.4 pg/µl PCR.

When these primers were tested in the multiplex PCR for cross-amplification against the other hoverfly species and potential prey of hoverfly larvae (Table 1), all non-target samples were negative, demonstrating the specificity of the assay.

Analysis of field-collected hoverfly larvae

Diagnostic PCR allowed identifying 169 field-collected syrphid larvae from both fields and years. Only three taxa (*Eu. corollae, Ep. balteatus* and *Sph. scripta/Sph. rueppellii*) were found. Overall, *Eu. corollae* dominated the catches (74 % of all collected larvae), followed by *Sph. scripta/Sph. rueppellii* (14 %) and *Ep. balteatus* (12 %). On the lettuce plants in spring 2009, the species assemblage in the experimental field was very similar to that in the commercial field (Fig. 2). On the ground however, *Eu. corollae* was found almost exclusively when searching for hoverfly larvae on the soil surface. In spring 2010, only *Eu. corollae* and *Ep. balteatus* were captured while in summer 2009, *Sph. scripta/Sph. rueppellii* were the taxa with the highest representation, followed by *Eu. corollae*, whereas, *Ep. balteatus* was only occasionally found (Fig. 2). None of the syrphid larvae tested positive for DNA of the parasitoid *D. laetatorius*.

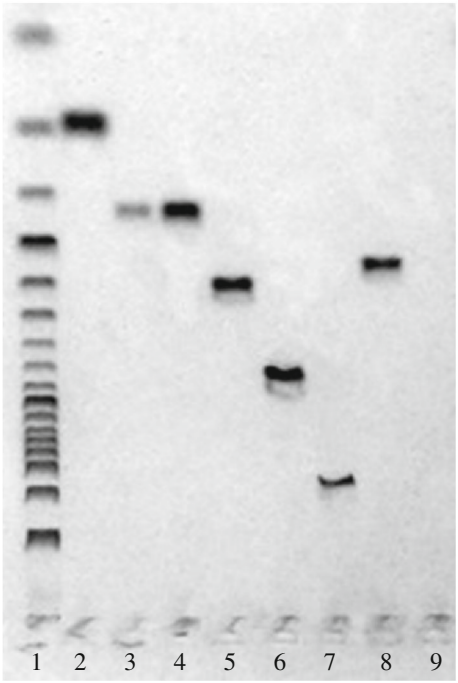

Fig. 1 DNA fragments obtained by multiplex PCR amplification using the specific syrphid- and parasitoid-specific primers. *Lane 1* DNA size marker (50 bp ladder), L2: *Meliscaeva auricollis* (96 bp), *L3 Sphaerophoria scripta* (165 bp), *L4 Sphaerophoria rueppellii* (165 bp), *L5 Scaeva pyrastri* (314 bp), *L6 Eupeodes corollae* (395 bp), *L7 Episyrphus balteatus* (754 bp), *L8 Diplazon laetatorius* (220 bp), and *L9* negative control

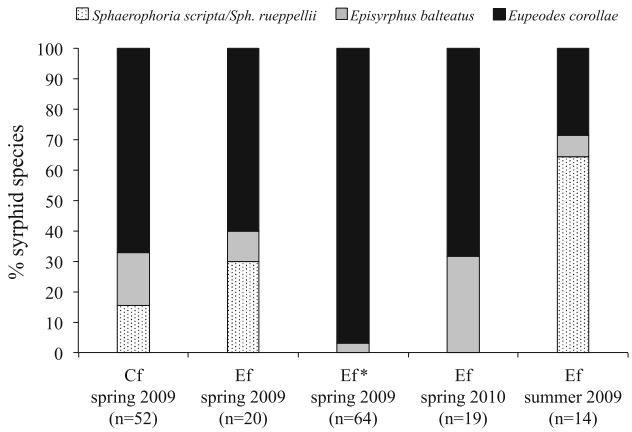

Fig. 2 Syrphid species composition found in two fields (*Cf* commercial field, *Ef* experimental field), two seasons (spring and summer), 2 years (2009 and 2010) and collected either on lettuce plants or on the ground (*asterisk*)

Discussion

The multiplex PCR assay developed in this study allows unambiguous identification of the five most common predatory hoverfly taxa present in Mediterranean vegetable crops. Moreover, the assay includes a primer pair for the parasitoid *D. laetatorius*, a common ichneumon-parasitoid of hoverfly larvae. A primer pair for the parasitoid has been included in the assay because parasitoid eggs and larvae are easily missed when inspecting the hoverfly larvae under a dissecting microscope which can lead to an underestimation of the real parasitism rate (Moreno-Ripoll et al. 2012). Compared to an identification of the larvae via a DNA barcode (Jinbo et al. 2011), the current approach has the advantage that whole body DNA extracts which might also contain DNA of prey and/or parasitoids can be tested. This nonsyrphid DNA would cause problems for DNA barcoding if general invertebrate/metazoan primers are used to generate the COI fragment used as the barcode DNA region. Using Sanger sequencing, sequence-based identification of one type of DNA in the sample is preferable. A mixture of syrphid, prey and/or parasitoid DNAs can lead to unreadable sequences or preferential amplification of parasitoid DNA (Lee and Lee 2012) and prohibit species identification. This could be avoided by using Next Generation Sequencing technologies, which have been also recently used to identify a wide range of insect prey items present in the gut of predaceous syrphid larvae (author's unpublished results).

When the designed primers were tested for specificity, none of the other syrphid species potentially present in Mediterranean vegetable crops nor any other potential prey species yielded false positives. The latter were tested because of the possibility of amplifying prey remains from the gut content of the hoverflies. The lack of amplification demonstrated that the PCR products were exclusive from the syrphid taxa. The assay developed here can be used to identify all developmental stages, and even parts or remains of the targeted species, which makes its possibility of application manifold. In the case of the primers that produce a band smaller than 400 bp [i. e., *M. auricollis* (96 bp), *Sphaerophoria* spp. (165 bp), *Sc. pyrastri* (314 bp), and *Eu. corollae* (395 bp)], they could also be used to test other predators for consumption of these hoverfly species.

When the multiplex PCR assay designed here was used to study the composition of hoverfly larvae communities in Mediterranean lettuce fields, only three syrphid taxa were found: *Eu. corollae*, *Ep. balteatus*, and *Sph. scripta/Sph. rueppellii*. Previous studies conducted also in lettuce crops in Spain confirm these results (Pascual-Villalobos et al. 2006; Morales et al. 2007), being also the main syrphid species found. Other species, such as *M. auricollis* have also been observed in lettuce crops in Spain, but in much less proportion (Rojo and Marcos-García 1998).

The multiplex PCR assay also detected temporal differences in the hoverfly species assemblages. *Eupeodes corollae* and *Ep. balteatus* were more abundant in spring whereas *Sph. scripta/Sph. rueppellii* densities peaked in summer. The same temporal pattern (*Eu. corollae/Ep. balteatus/Sph. rueppellii*) was found in a previous study on aphidophagous syrphid population dynamics in pepper greenhouses in the southeast Spain (Pineda and Marcos-García 2008). *Eupeodes corollae*, *Ep. balteatus*, and *Sph. scripta* are highly migratory species (Speight 2011) that move to Central Europe during summer and the mated females returning to South Europe in autumn (Rotheray and Gilbert 2011). On the other hand, *Sph. rueppellii* is a resident Mediterranean species which is well adapted to high-ambient temperatures (Pineda and Marcos-García 2008; Amorós-Jiménez et al. 2012). In relation with these biological traits, larvae of both species of the genus *Sphaerophoria* were found in Spanish lettuce crops during spring, but only *Sph. scripta* was found in autumn (Morales et al. 2007). For this reason, those syrphid larvae which were collected in summer 2009 in this study and which were assigned by the multiplex PCR approach to the two molecularly indistinguishable species *Sph. scripta/Sph. rueppellii* probably belong to *Sph. rueppellii.*

When analyzing the syrphid larvae collected on the ground, we did not find a complex of syrphid species like on the lettuce plants. Instead, *Eu. corollae* was the most abundant species. *Episyrphus balteatus* and *Sph. scripta/Sph. rueppellii* were hardly and not found on the ground, respectively. This behavior is also related with the preference of these hoverfly species (like most Syrphinae) to pupate on the plant on which their prey occur. However, according to Dusek and Laska (1961), *Eu. corollae* overwinters as pupa, which is unusual for aphidophagous hoverflies (Stubbs and Falk 1983).

From all syrphid larvae analyzed here, none was found to be parasitized by *D. laetatorius,* suggesting that the syrphid populations in the investigated fields did not experience top-down pressure by this endoparasitoid. Note, however, that the current result could also be explained by the comparably low number of syrphid larvae analyzed, as parasitism rates are usually not very high in hoverfly larvae. For example, in lettuce crops, Smith and Chaney (2007) found less than 5 % of parasitism by *D. laetatorius* after analyzing 1,087 syrphid larvae collected in Californian crops. Krawczyk et al. (2011) reported that 3 % of the syrphid pupae inspected ($n = 538$) were parasitized in maize fields in Poland, where the dominant syrphid parasitoid was *Pachyneuron grande* (Hymemoptera: Pteromalidae). In cabbage fields, also in Poland, parasitism by *D. laetatorius* was found as high as 22 % when 410 syrphid

larvae and pupae were analyzed (Jankowska 2004). Lacking parasitoid DNA detection in diagnostic PCRs could also be ascribed to a low sensitivity of the assay (Traugott and Symondson 2008).The sensitivity of the current multiplex PCR for detecting parasitoid DNA, however, is highly comparable to previous assays which allowed detection of eggs and early instar larvae of parasitoids (e.g., Traugott et al. 2006). Therefore, we think that the current results are not due to a methodological artifact but represent a nonexisting/very low level of parasitism of these hoverfly larvae by *D. laetatorius.*

The multiplex PCR approach described here is an efficient tool for the rapid identification of the main hoverfly species present in Mediterranean vegetable crops. Because the larvae of these hoverfly species are known to be important predators of several insect pests, and the species studied in the present study have been identified in other agroecosystems (Jansen 2000; Marshall and West 2007; Sajjad et al. 2008) or forest ecosystems (Kehlmaier and Martínez de Murguía 2004), this molecular method will be particularly useful for further studies on population dynamics, distribution, and abundances of these syrphid species. A molecular tool for detecting *D. laetatorius* parasitism within syrphid larvae has also been described here, allowing to further examine which effect this parasitoid has on syrphid populations and their ability to control pest populations. A better understanding of the identity of the predators and their feeding activities would allow to better conserve key predators in conservation biological programs in vegetable crops.

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Identification of *Orius* spp. (Hemiptera: Anthocoridae) in vegetable crops using molecular techniques.

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Identification of *Orius* spp. (Hemiptera: Anthocoridae) in vegetable crops using molecular techniques

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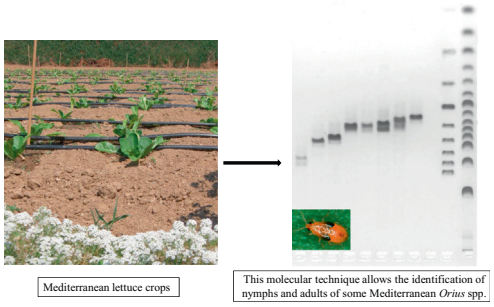
HIGHLIGHTS

- Immature stages of *Orius* spp. cannot be identified by morphological traits.
- Adults of *Orius* spp. can be morphologically identified only by their genitalia.
- This molecular method allows the discrimination of some Mediterranean *Orius* spp.
- *Orius laevigatus*, *O. majusculus* and *O. niger* were the most abundant in Spanish lettuce crops.

GRAPHICAL ABSTRACT



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Lobularia maritima

ABSTRACT

The species of the genus *Orius* Wolff are well known as generalist predators able to control pest outbreaks in several agroecosystems. Correct species identification can be problematic given their similarities, particularly in the immature stage. A pair of primers previously designed from the internal transcribed spacer-1 (ITS-1) region was able to discriminate between seven *Orius* species commonly present in Mediterranean vegetable crops (*Orius majusculus*, *Orius laevigatus*, *Orius minutus*, *Orius laticollis*, *Orius horvathi*, *Orius albidipennis* and *Orius niger*), as well as to correctly identify *O. majusculus* and *O. laevigatus* from commercial colonies. This molecular tool was used for the discrimination of *Orius* spp. present in two lettuce crops, as well as in a *Lobularia maritima* flower margin in northeast Spain in 2009 and 2010. Molecular analyses revealed that *O. laevigatus*, *O. majusculus* and *O. niger* were the most common species present in both lettuce plots and the *L. maritima* border, although there was some variation depending on the plant and year. This molecular tool permits unambiguous identification of these species and allows proper implementation of biological control programs based on conservation.

1. Introduction

The genus *Orius* Wolff 1811 has 70 described species present in the Oriental, Ethiopian, Palaearctic, Neotropical and Nearctic regions, and the importance of this genus in efficient pest control is widely recognized (Horton, 2008). Several species are considered generalist predators that feed on a wide range of prey and are capable of very effective control of thrips (Riudavets and Castañé, 1998), as well as aphids, psyllids, scale insects, psocids, mites and the eggs of Lepidoptera, Coleoptera, and Diptera (Horton, 2008). They occur naturally in various agroecosystems including cotton, soybean, fava bean, potato, wheat, alfalfa, maize, and orchards (Veres et al., 2012). Seven *Orius* spp. have been reported to be present in vegetable crops of the Mediterranean basin: *Orius majusculus* (Reuter), *Orius laevigatus* (Fieber), *Orius albidipennis* (Reuter), *Orius niger* (Wolff), *Orius minutus* (L.), *Orius horvathi* (Reuter) and *Orius laticollis* (Reuter) (Ferragut and González-Zamora, 1994; Riudavets and Castañé, 1994; Tommasini, 2004). Like other heteropterans, some *Orius* spp. often show a lack of clear

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morphological differences, particularly in the immature stages (Gaskin et al., 2011). The genital clasper in males and the copulatory tube in females are commonly used to discriminate some of these species, but the differences in these features are not always evident. The females of some species can also be identified by inspecting the opercula structure of the oviposited eggs (Schuldiner-Harpaz and Coll, 2012), but eggs are not always available and rearings have to be carried out to identify the adults. These taxonomic characters for species discrimination only concern the morphology of eggs, males or females, making the identification of the nymphs impossible.

An alternative strategy to correctly identify nymphs and adults is based on molecular techniques, which overcomes the problems inherent to conventional morphological identification. Some molecular approaches, like RAPD-PCR, RFLP-PCR, microsatellites, as well as the use of other nuclear and mitochondrial regions have been used to study *Orius* strains and species (Gozlan et al., 1997; Hinomoto et al., 2009; Honda et al., 1999; Jung and Lee, 2011; Muraji et al., 2000a,b, 2004). The internal transcribed spacer-1 (ITS-1) region of nuclear ribosomal DNA has been also used for molecular characterization of *Orius* species (Hinomoto et al., 2004; Honda et al., 1998; Muraji et al., 2004; Sayed and Montaser, 2012; Sayed et al., 2013). Particularly, in Hinomoto et al. (2004) the variation of the length of the entire ITS1 region, which was amplified using the universal primers F2 and R2 allowed the discrimination of two *Orius* species from Japan: *O. minutus* and *Orius tantillus* (Motschulsky). Of these species, only *O. minutus* is also present in the Mediterranean. Therefore we herein tested how the same primers designed by Hinomoto et al. (2004) from the ITS-1 region might discriminate among Spanish *Orius* spp. On the other hand, *Orius insidiosus* (Say), a Nearctic species present in various crops such as soybean, corn, sorghum, alfalfa and cotton (Iglinsky and Rainwater, 1950) was also tested for the potential use of this molecular marker in other regions. As it has been experimentally released also in Europe, it could have become established in vegetable crops as part of the complex of the European *Orius* species.

Therefore, the main aim of this study was to set up a quick and cheap diagnostic technique to identify a large number of field-collected *Orius* specimens to be used for discriminate seven *Orius* species: *O. majusculus*, *O. laevigatus*, *O. minutus*, *O. laticollis*, *O. horvathi*, *O. albidipennis* and *O. niger*, and to determine their presence in two lettuce plots in northeast Spain as an example of the use of the developed molecular method. The use of molecular tools for analyzing the ingested prey is very much used nowadays in order to identify key predators used in conservation biological control. Conducting a previous fast molecular identification of the species involved facilitate these studies, particularly because the same DNA extraction can be used for both (identification and gut analysis), and because the morphological identification of each specimen (based on the observation of the genital structures) would delay the gut content analysis and then lose information about the ingested prey.

2. Materials and methods

2.1. DNA extraction and amplification

DNA was individually extracted from all insects using the DNeasy Tissue Kit (Qiagen, Hilden, Germany; protocol for animal tissues). Total DNA was eluted in 100 μl of AE buffer provided by the manufacturer and stored at −20 °C. Negative controls without insect DNA were added to each DNA extraction set. PCR reaction volumes (25 μl) contained 4 μl of resuspended DNA, 0.6 U of *Taq* DNA polymerase (Invitrogen), 200 μM dNTPs (Promega), 0.6 μM of each primer (F2: 5′-GTCGCTACTACCGATTGAATGG-3′ and R2: 5′-GTGTCCTGCAGTTCACATGG-3′) (Hinomoto et al., 2004) and 5 μM $MgCl_2$ in the manufacturers' reaction buffer. Samples were amplified for 35 cycles of 94 °C for 30 s; 50 °C for 30 s; and 72 °C for 45 s with a first cycle of denaturation at 94 °C for 2 min and a final extension at 72 °C for 5 min. Amplifications were conducted in a 2720 thermal cycler (Applied Biosystems, CA, USA). Target DNA and water were always included as positive and negative controls, respectively. PCR products were separated by electrophoresis using 2.4% agarose gels stained with ethidium bromide that were visualized under UV light. The sizes of the PCR products were confirmed experimentally by comparison to DNA markers using ImageJ (Image Processing and Analysis in Java, http://rsb.info.nih.gov/ij/) software.

2.2. Specificity of the primers

Five adult specimens each of *O. majusculus*, *O. laevigatus*, *O. minutus*, *O. laticollis*, *O. horvathi*, *O. albidipennis*, and *O. niger* were morphologically identified following the descriptions of Péricart (1972). These were collected in two different localities of northeast Spain separated 120 km from each other: La Selva del Camp (Tarragona) (*O. minutus*, *O. laticollis*, *O. horvathi*) and El Maresme (Barcelona) (*O. majusculus*, *O. laevigatus*, *O. albidipennis*, *O. niger*). Also five specimens of *O. insidiosus* (Say) collected in Brookings, South Dakota, USA were tested, as well as some specimens from commercial colonies ($n = 5$/colony): *O. laevigatus* from Agrobío (Spain), Syngenta (UK) and Biobest (Belgium) and *O. majusculus* from Syngenta and Biobest.

To ensure that the amplified bands were exclusively from *Orius* spp. and not from a prey present in their gut, potential prey species found in lettuce crops were also tested ($n = 5$/species). These included *Frankliniella occidentalis* (Pergande), *Thrips tabaci* (Lindeman) (Thysanoptera: Thripidae) and *Entomobrya* sp. (Collembola) from our facilities (IRTA); *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) from CRAG (Center for Research in Agricultural Genomics; Bellaterra (Barcelona), Spain); and *Macrosiphum euphorbiae* (Thomas), *Myzus persicae* (Sulz.), *Hyperomyzus lactucae* (L.), *Aphis gossypii* (Glover) and *Aulacorthum solani* (Kaltenbach) from ICA-CSIC (Institute of Agricultural Sciences-Spanish National Research Council; Madrid, Spain). Some faint bands were obtained for *N. ribisnigri* and *F. occidentalis* and therefore, *O. majusculus* was also tested after feeding on either species. Females were placed into 1.5 ml tubes for 24 h of starvation at 25 °C, then into small transparent plastic boxes (2.5 cm diameter) with 4 individuals of either *N. ribisnigri* (wingless) or *F. occidentalis* (second-instar larvae). Predators were allowed to feed on the prey for a maximum of 2 h at 25 °C. Those that had consumed 2–4 items were frozen immediately after the prey ingestion. Ten *O. majusculus* fed on *N. ribisnigri* and other ten fed on *F. occidentalis* were analyzed by PCR using the F2 and R2 primers described in Section 2.1.

2.3. Field-collected Orius analysis

A lettuce plot (var. Maravilla) situated in the El Maresme area (northeast Spain) was sampled in 2009 and 2010. The plot was an experimental field at our research institute (IRTA) that was planted from the beginning of June to the beginning of August of both years. Twenty to thirty lettuces were collected twice in 2009 (on July 7th and 14th). This was repeated in 2010 (on July 20th and August 3rd). *Lobularia maritima* plants were transplanted in early spring at one border of the experimental plot as a refuge for predators, and they were sampled once on July 27th 2010 by beating them 3 times on a white tray (25 × 15 cm). A commercial field located 5 km from the experimental plot was also sampled by collecting 20–30 lettuces once on July 12th 2010.

Lettuces were placed in individual plastic bags and brought to the laboratory where all *Orius* spp. were collected, and nymphs

and adults were separated under the microscope. All *Orius* specimens from lettuce and *L. maritima* were frozen at −20 °C until molecular analysis, which were conducted as described in Section 2.1. To confirm the presence of DNA in those *Orius* specimens that were negative using primers F2/R2, the universal primers C1-J-1718 and C1-N-2191 (Simon et al., 1994) were used in order to amplify a fragment of the cytochrome c oxidase subunit I (COI) gene. The method described by Agustí et al. (2003) was used for those amplifications with an annealing temperature of 55 °C instead of 58 °C. Target DNA and water were always included as positive and negative controls, respectively. PCR products were separated by electrophoresis using 2.4% agarose gels stained with ethidium bromide that were visualized under UV light.

3. Results

3.1. Specificity of the primers

Results showed successful amplifications for the eight *Orius* species tested, being clearly distinguished by the size of the resulting PCR products (*O. minutus* (two bands of 943 and 874 bp), *O. laevigatus* (721 bp), *O. majusculus* (686 bp), *O. horvathi* (two bands of 636 and 596 bp), *O. albidipennis* (two bands of 612 and 578 bp) and *O. niger* (563 bp)), except for *O. insidiosus* (618 bp) and *O. laticollis* (614 bp) (Fig. 1), which amplified a very similar band in size. All *O. laevigatus* and *O. majusculus* specimens from the commercial colonies (Agrobío, Syngenta and Biobest) were also amplified and identified as *O. laevigatus* and *O. majusculus*. Of the potential prey species, only *N. ribisnigri* and *F. occidentalis* were amplified (~800 and 1050 bp, respectively). However, no bands could be amplified when analyzing *O. majusculus* that had consumed 2–4 of either prey (Fig. 2).

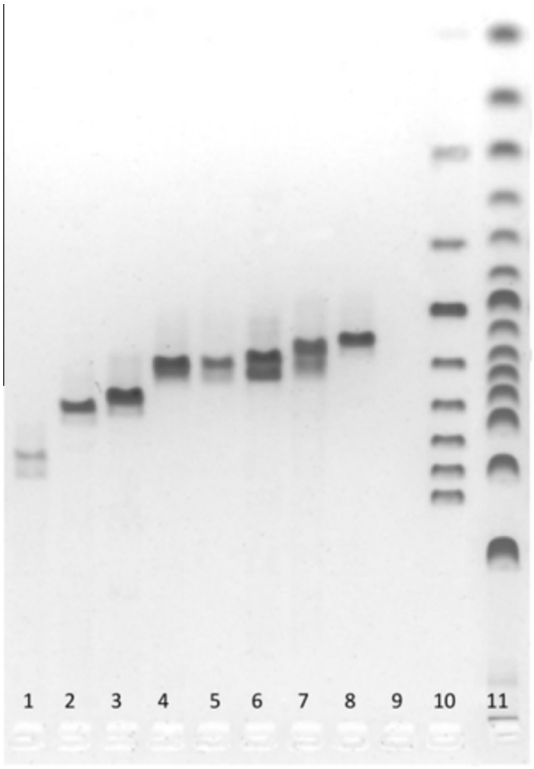

Fig. 1. PCR amplified fragments of eight *Orius* species using F2/R2 primers. Lane 1 (L1): *O. minutus*, L2: *O. laevigatus*, L3: *O. majusculus*, L4: *O. insidiosus*, L5: *O. laticollis*, L6: *O. horvathi*, L7: *O. albidipennis*, L8: *O. niger*, L9: negative control, L10: DNA size marker (100 bp ladder), L11: DNA size marker (50 bp ladder).

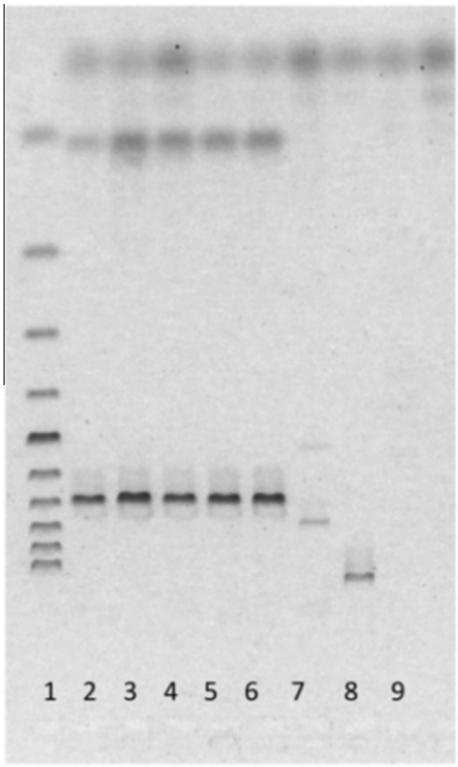

Fig. 2. PCR amplified fragments of starved *O. majusculus* and after feeding on *F. occidentalis* and *N. ribisnigri* using F2/R2 primers. Lane 1 (L1): DNA size marker (100 bp ladder), L2: *O. majusculus* starved 24 h, L3 and L4: *O. majusculus* after eating 2–4 items of *N. ribisnigri*, L5 and L6: *O. majusculus* after eating 2–4 items of *F. occidentalis*, L7: *N. ribisnigri*, L8: *F. occidentalis*, L9: negative control.

3.2. Field-collected Orius analysis

A total of 137 *Orius* specimens were collected in the field, and of these 131 (96%) showed a specific band pattern that allowed assignment to one of the seven species. A complex of 5–6 *Orius* spp. was found in the IRTA experimental plot in both years tested. Such complex was also found in 2010 in the *L. maritima* margin, as well as in the commercial field. The predominant species varied according to the date and location sampled. Regarding the adult specimens (males and females) detection percentages obtained in the experimental plot in 2009 showed a higher presence of *O. laevigatus*, followed by *O. niger* and *O. majusculus*, with *O. minutus* and *O. albidipennis* in minor proportions (Fig. 3). In the same experimental plot in 2010, the species with the highest representation were again *O. niger* and *O. laevigatus*, while others were present in minor proportions. The species presence in the *L. maritima* margin in 2010 was similar to that of the nearby experimental plot except that *O. laticollis* was found instead of *O. minutus*. Finally, in the commercial plot in 2010, *O. laevigatus* was again the most abundant species, while others were found in minor proportions (Fig. 3). Regarding the nymphs, the *Orius* species present were similar to the adults on lettuce, which were *O. majusculus, O. niger*, *O. laevigatus* and *O. minutus*. In the case of the *L. maritima* border, also *O. albidipennis* was present, as happened with the adults (Fig. 3).

From all *Orius* collected, females were more abundant than males and nymphs in lettuce. Surprisingly, a lower proportion of all *Orius* specimens found on *L. maritima* were females, where nymphs were the most abundant (Fig. 4). Finally, it was found that 4% of the analyzed *Orius* ($n = 6$) from the experimental and commercial plots in 2010 did not show PCR amplification with F2/R2 primers. As they screened positive for DNA with the general

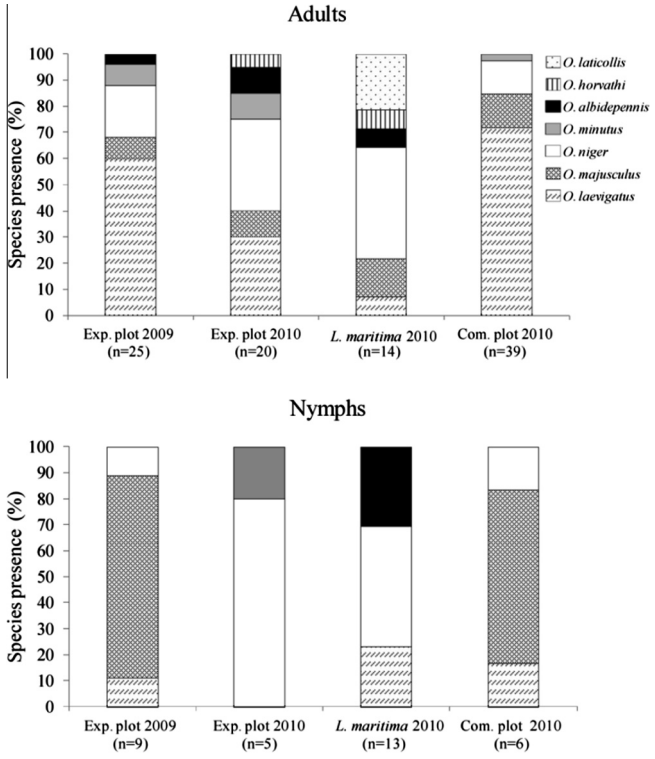

Fig. 3. Composition of *Orius* spp. (adults and nymphs) found in the experimental plot in 2009 and 2010, in the *L. maritima* border 2010, and in the commercial plot in 2010. Exp. plot: experimental plot, Com. plot: commercial plot.

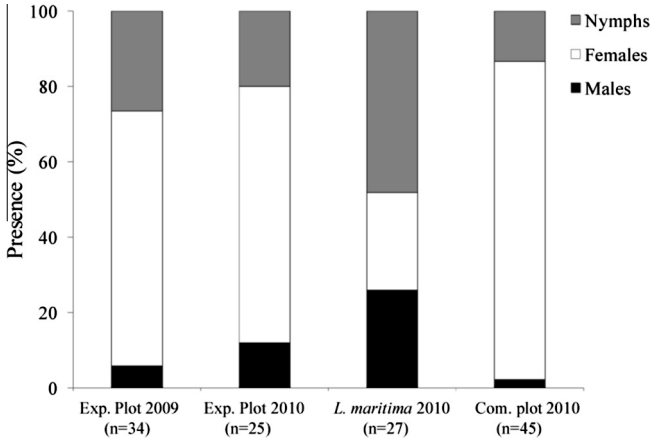

Fig. 4. Percentages of nymphs, females and males of *Orius* spp. in the experimental plot in 2009 and 2010, in the *L. maritima* border in 2010, and in the commercial plot in 2010. Exp. plot: experimental plot, Com. plot: commercial plot.

primers C1-J-1718 and C1-N-2191 (Simon et al., 1994), it is probable that they were in fact other *Orius* species or even other anthocorids.

4. Discussion

Molecular analyses conducted in this study allowed discrimination among the seven most common *Orius* spp. present in Mediterranean vegetable crops, based on the different sizes of the amplified DNA fragments that characterizes each species. In only one case, involving *O. laticollis* (614 bp) and *O. insidiosus* (618 bp), the bands were too close in size to permit inter-species discrimination. The latter is a well known Nearctic species (Barber, 1936; Fisher and Bellows, 1999) that was released in Europe for the control of *F. occidentalis*. Notwithstanding, no evidence of establishment has been recorded up to now (Bosco and Tavella, 2008; Lynch et al., 2001) probably because of being gradually replaced by native related species (Fejt and Jarošík, 2000; van de Veire and Degheele, 1992). For these reasons, we assume in this case that those individuals amplifying a band of this size were *O. laticollis*, but this should be taken into account in future studies where both species could be present.

In the study of Hinomoto et al. (2004), the PCR-amplified fragment for *O. minutus* was ~910 bp. We believe that the reason we detected two bands (943 and 874 bp) for *O. minutus* was the result of longer electrophoresis or even a potential intraspecific variability between Spanish and Japanese *O. minutus* populations. Our results also showed that some faint bands obtained for *N. ribisnigri* and *F. occidentalis* were not amplified when analyzing *O. majusculus* after being fed. Both prey were likely not detected in this case due to the digestion process, as demonstrated in predation studies where prey detection was analyzed with time (Romeu-Dalmau et al., 2012). Therefore, the presence of either prey in the gut of *Orius* specimens does not interfere with *Orius* identification using F2/R2 primers.

This methodology represents a quick and cheap tool for the discrimination of the *Orius* species present in two Mediterranean lettuce crops and in a *L. maritima* border in northeast of Spain, as well as in the commercial colonies tested. It was observed that these predators are present on lettuce in a complex of 4–6 species, where 2–3 of them are the dominant (*O. laevigatus*, *O. niger* and *O. majusculus*). This agrees with previous findings on Mediterranean vegetable and ornamental crops (Ferragut and González-Zamora, 1994; Goula et al., 1993; Riudavets and Castañé, 1994; Tommasini, 2004). The species found in the present study at lower proportions were *O. albidipennis*, *O. minutus* and *O. horvathi*. Other studies conducted in the same area also found *O. albidipennis* in low quantities (Goula et al., 1993; Riudavets and Castañé, 1994; Sánchez and Lacasa, 2006). This was expected, as this species prefers meridional (warmer) regions like southern Spain, the Canary Islands and Israel (Carnero et al., 1993; Chyzik et al., 1995; Riudavets and Castañé, 1994). Although both *O. minutus* and *O. horvathi* are common in the Palearctic region, they are more abundant in crops other than lettuce. As shown in previous studies, *O. minutus* was frequently found on beans in Italy (Bosco and Tavella, 2008) and on potatoes in Iran (Fathi and Nouri-Ganbalani, 2010). *O. horvathi* was mainly found on trees, such as citrus orchards in northeast Spain (Ribes et al., 2004), as well as almond and peach orchards in Syria (Almatni and Khalil, 2008). It is also fundamental to correctly identify the *Orius* spp. present in the field margins, to ensure the presence of species that are important for the success of conservation biological control programs. In this study we found almost the same spectrum of species in *L. maritima* and in the adjacent lettuce crop. Only one species present on *L. maritima* was not found on lettuce (*O. laticollis*). This species probably stays on *L. maritima* because of finding other food sources such as pollen or nectar. Further studies should be conducted in order to increase the sample size of the assay when the goal will be to study *Orius* species abundance, as well as to sample other locations to verify potential intraspecific variability. On the other hand, when other locations will be studied new positive controls of other potentially present *Orius* species and previously morphologically identified should be always included.

The presence of different stages of *Orius* spp. on lettuce and *L. maritima* was also studied, and it was shown that females were more abundant on lettuce, whereas young stages were more abundant in the *L. maritima* border. The fact that the *L. maritima* border was established before the lettuce crops would have allowed

females of *Orius* spp. to lay eggs and settle on *L. maritima* before than on lettuce. It is well known that *L. maritima* is more attractive than lettuce for *Orius*, offering pollen and nectar to predators (Alomar et al., 2008; Hogg et al., 2011). It is also known for being a good reproductive plant from where nymphs could colonize the crop (Pumariño and Alomar, 2012). Lundgren et al. (2009) recently showed that *O. insidiosus* females clearly distinguish among plants for oviposition in the field, laying nearly twice as many eggs on non-crop plants as on soybean. On the other hand, the different sampling method (beating *L. maritima* plants on a tray versus enclosing lettuces in plastic bags) could also explain the differences found between *L. maritima* border and lettuce crops. The fact that *Orius* adults can fly when the *L. maritima* plants are beaten could have altered the results. Nymph analysis revealed that the major species on lettuce were also *O. majusculus* and *O. niger*. Notwithstanding, even the fact that nymphs sample size was low ($n \leqslant 13$), it was possible to observe a high diversity of *Orius* species indicating the importance of these 2–3 main *Orius* species in future conservation biological control programs.

Only 4% of the *Orius* analyzed could not be identified because the PCR analysis using F2/R2 primers did not yield any product. Amplification using the COI primers of Simon et al. (1994) confirmed the presence of DNA, indicating that these individuals may have been other species. Other *Orius* species that could also be present in this area (in less proportions) include: *Orius vicinus* (Ribaut), *Orius lindbergi* (Wagner) and *Orius pallidicornis* (Reuter) (Goula et al., 2010). In previous studies, *O. vicinus* was mainly found in tree canopy (Fauvel, 1999; Kondorosy et al., 2010). *O. lindbergi* was present in very low abundance on carnation flowers (Riudavets, 1995) and watermelon plants (Ferragut and González-Zamora, 1994), and *O. pallidicornis* were exclusively found on the wild plant *Ecballium elaterium* (Tommasini, 2004). Although the likelihood that these three *Orius* species were present on vegetable crops is low, it is possible that they were present on lettuce. On the other hand, it will be also possible that this 4% belongs to other anthocorid genus.

This study shows that the use of F2/R2 primers represents a quick and cheap tool to correctly discriminate among *Orius* spp collected in northeast Spain lettuce crops, as well as among some commercial *Orius* populations. This tool has been useful not only for adults, but particularly for the immature stages, which cannot be identified by morphological traits. Given that both adults and nymphs of *Orius* spp. are important predators of several insect pests, and that the species reported in the present study have been identified also in other vegetable agroecosystems and ornamental crops (Riudavets and Castañé, 1994), this molecular approach may be particularly useful for population dynamics studies in future pest control programs, as well as for further *Orius* spp. predation studies that contribute as well to the selection of key predators for conservation biological control programs of vegetable crops, as been done in Gomez-Polo et al. (2013). Because it has been able to discriminate between commercially available *Orius* mass reared colonies, this technique could also be used for the quality control of *Orius* spp. colonies when contamination with other species could be suspected.

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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 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. *Frankliniella 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 (IGP) on other hoverflies species, as well as on anthocorids, spiders and even aphid parasitoids.

CONCLUSIONS: Conventional 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 (NGS).

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1 INTRODUCTION

Understanding trophic linkages in a community can facilitate the development of conservation biological control (CBC) programs 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 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 micro-organisms.[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 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 visualizes 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 do not require equipment associated with gel analysis used in conventional PCR. Instead, positive samples are distinguished based on 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 this kind of studies.

Next Generation Sequencing (NGS) technologies offer the opportunity for describing the dietary breadth of an arthropod, not possible using conventional PCR or qPCR. Using generalized 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 of 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, like 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]

Summarizing, the aims of this study were: 1) to analyze 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; and 2) to compare predation percentages obtained by conventional and qPCR; and 3) to use Ion Torrent PGM to more completely describe 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 (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 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), Light: Dark 16:8 and 25 ± 2º C, except *N. ribisnigri* which was reared at 19 ± 2º 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 Agusti *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).

Table 1. Prey-specific primers used: target species, sequence (5'-3'), amplified fragment length, region targeted and study were 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	Kuusk & Agusti (2008)
	Col5R	TCTTGGCAAATGCTTTCGCAGTA			

The following sequences from the GenBank database (www.ncbi.nlm.nih.gov) were used for primers 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, CA, USA). Reaction volumes (25 μl) contained resuspended DNA (4 μl), *Taq* DNA polymerase (0.6 U) (Invitrogen, CA, USA), dNTPs (0.2 mM) (Promega, WI, USA), 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 94º C for 30 s; 58º C (Fo1F/Fo1R) or 62º C (Col4F/Col5R, Nr1F/Nr2R and Nr3F/Nr3R) for 30 s; and 72º C for 45 s. For all reactions, the first denaturation cycle was at 94º C for 2 min, and the final extension cycle was at 72º C for 5 min. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualized 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, like 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 (2^{nd}-3^{rd} instar) were placed into 1.5 ml tubes with a moistened piece of cotton and starved for 48 h at 25º C. Next, they were placed in small transparent plastic boxes (2.5 cm diameter) with eight individuals of *N. ribisnigri* (wingless), *F. occidentalis* (2^{nd} instars) or Collembola (*Entomobrya* sp. 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 25º C for 2, 4, or 8 h and frozen at -20º C until PCR analysis. Ten individuals of *E. balteatus* were analyzed for each time period and food. Each predator was tested up to three times and considered 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.

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.

Order	Family	Species	Primers		
			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*	-	-	-

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 to 30 lettuce plants were collected in spring 2009 (May 18th and 20th), summer 2009 (July 7th and 14th), spring 2010 (May 11th, 18th and 25th, June 1st) and

summer 2010 (July 13rd, 20th, 27th and August 3rd). 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 (May 14th, n = 3 cages), summer 2009 (June 24th, n = 5; July 2nd, n = 1; 23th, n = 3), spring 2010 (May 13th, n = 1; 20th, n = 1; 28th, n = 1) and summer 2010 (July 3rd, n = 2). Each cage enclosed four lettuce plants (which were not cleaned of endemic arthropod community), on which were introduced: 2-3 *E. balteatus* larvae, 25-70 *N. ribisnigri* and 30-75 *F. occidentalis* per plant. After 48 h, lettuces were cut, individualized 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 based on 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 analyzed by conventional PCR for the presence of *N. ribisnigri*, *F. occidentalis* and Collembola in their guts were also analyzed using qPCR at the North Central Agricultural Research Laboratory (USDA-ARS, Brookings, SD, USA) 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 (June 24th 2009 [n = 14], July 3rd 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 nmol/L), template DNA (1 µl), and PCR water (9.5 µl). Reactions were run on a MX3000P qPCR thermocycler (Stratagene, CA, USA) using the following qPCR optimal conditions: 95º C for 15 min, followed by 50 cycles of 94º C for 15 s, 53º C for 30 s and 72º 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-templates 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.[35,36] When preliminary PCR analyses were conducted using these general invertebrate primers with 21 arthropod species tested, 5 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 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 were performed on *E. balteatus* DNA using primers ZBJ-ArtF1c and ZBJ-ArtR2c and adding different concentrations of the blocking primer BloEb2. Total volume of reactions (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 from 1 to 6 times the concentration of PCR primers during amplification. Samples were amplified for 40 cycles at 94º C for 30 s; 45º C for 45 s; and 68º C for 45 s. A single initial denaturation cycle of 94º C for 5 min, and a final extension at 68º C for 10 min was carried out. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualized under UV light.

2.5.2 Analysis of field samples

Fusion primers were also designed following the Ion Torrent recommendations[37] (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 one having a different 10-bp barcode (tag) before the ZBJ-ArtF1c primer to allow multiplexing 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 June 25th 2009 and July 3th 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 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 QIA-quick PCR Purification Kit (Qiagen). Fragments of the expected size (157 bp) were selected (E-Gel® Size Select 2% Agarose Gel, Invitrogen), quantified (DNA High Sensitivity kit, Bioanalyzer 2100, Agilent Technologies, CA, USA) 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). A single 314 chip was used with the sequencing chemistry for a 200 bp read length, as well as the version 2.2 of the Torrent Suite software for base calling (Ion Torrent, Life Technologies).

Table 3. Fusion primers used for sequencing *E. balteatus* specimens in the Ion Torrent PGM. 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 (Zeale et al., 2011). F = Forward, R = Reverse.

Primer Name	Sequence
AkT5 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*CAGAAGGAACAGATATTGGAACWTTATA
AkT6 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*CTGCAAGTTCAGATATTGGAACWTTATA
trP1-ZBJ (R)	CCTCTCTATGGGCAGTCGGTGATWACTAATCAATTWCCAAATCCTCC

2.5.3 Processing and analysis of data

The output of the massive sequencing process was treated bioinfomatically 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[38]. Sequences shorter than 150 bp were discarded, then trimmed to 150 bp, and finally discarded those with a mean quality score lower than 25 (all using PRINSEQ[39]). 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 [40]) to explore the taxonomical content of the data set.

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).

When these primers were used to analyze *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).

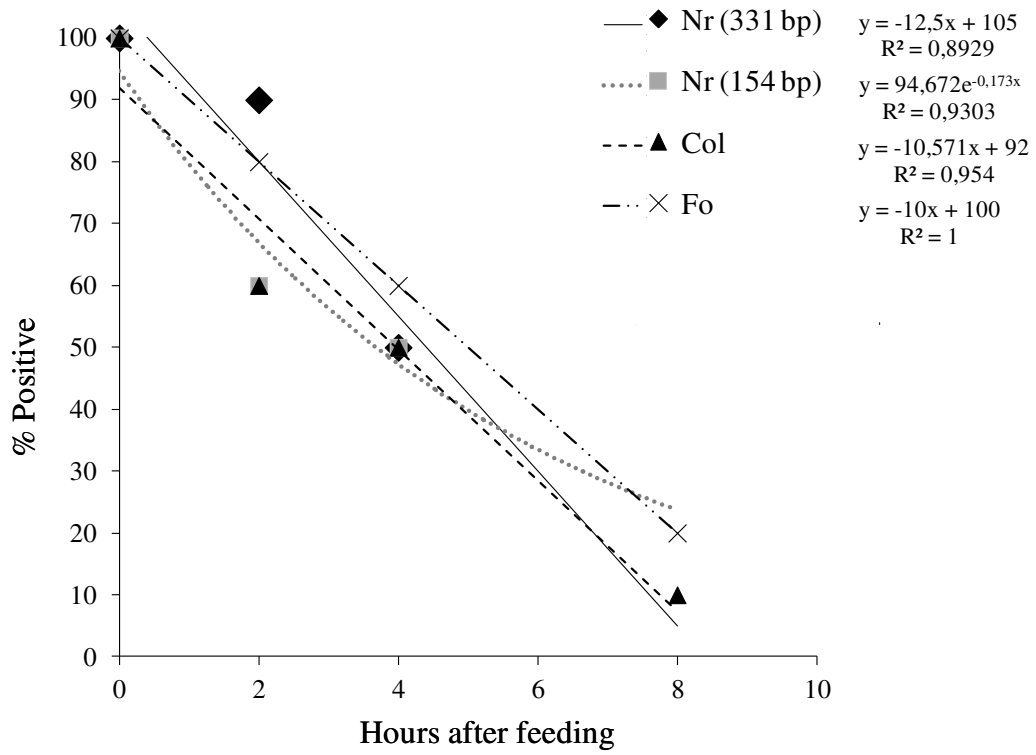

Fig. 1 Detection curves of ingested *Nasonovia ribisnigri* (with primers Nr1F/Nr2R [331bp] and Nr3F/Nr3R [154 bp]), *Frankliniella occidentalis* (with primers Fo1F/Fo1R [292 bp]) and *Entomobrya* sp. (with primers Col4F/Col5R [177 bp]) at different times after ingestion within *Episyrphus balteatus.*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

3.2 Field experiment

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

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 of being 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 like *E. corollae* (n = 32 and n = 2 in

spring and summer, respectively), *Sphaerophoria* sp. (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* sp. 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* sp. consumed only one species, whereas in summer the rate of detection of multiple prey increased (Fig. 2).

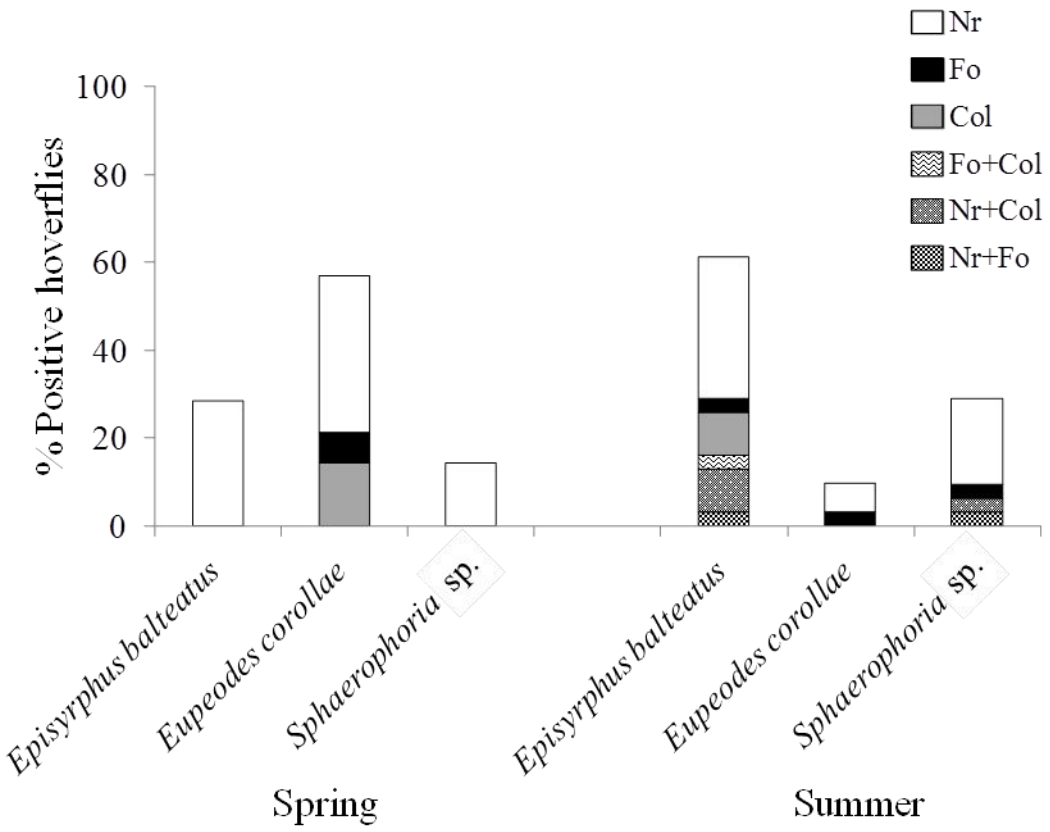

Fig 2. Percentages of PCR detection of *Nasonovia ribisnigri* (Nr), *Frankliniella occidentalis* (Fo) and Collembola (Col) within positive *Episyrphus balteatus*, *Eupeodes corollae* and *Sphaerophoria* spp. larvae from the cages placed in the experimental lettuce plot in spring and summer 2009-2010.

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 analyzed by qPCR, *N. ribisnigri* detection percentages were higher than those obtained by conventional PCR using the same *N. ribisnigri*-specific primers (154 bp). qPCR revealed a higher percentage of *E. balteatus* larvae positive for *N. ribisnigri* also in field cages than using conventional PCR. The percentage of spring-collected *E. balteatus* that tested positive for *F. occidentalis* was again higher when the samples were analyzed 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.

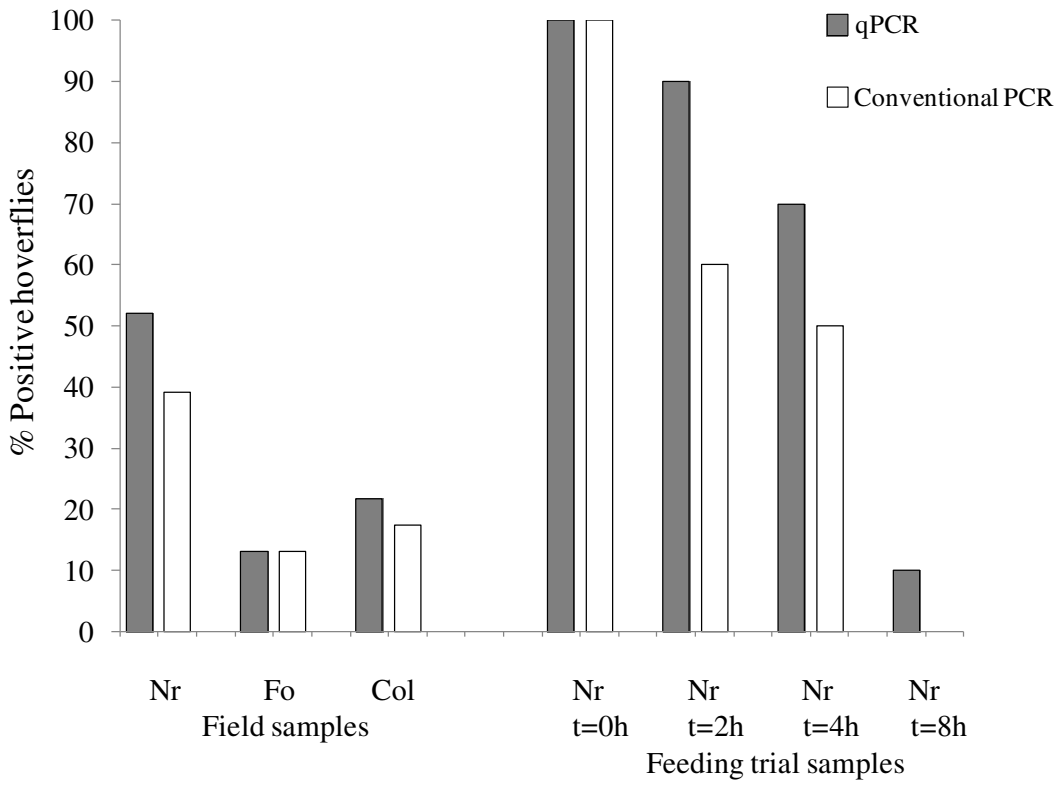

Fig 3. Percentages of qPCR and conventional PCR detection of *Nasonovia ribisnigri* (Nr), *Frankliniella occidentalis* (Fo) and Collembola (Col) within *Episyrphus balteatus* larvae from the field cages placed in the experimental lettuce plot, as well as from the feeding trials conducted in the laboratory.

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 despite 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 of 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, like 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* sp.) were detected, showing a certain 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 = 12 specimens positives for *N. ribisnigri*, *F. occidentalis* and/or *Entomobrya* sp. by conventional and qPCR with blocking primer; bulk 2 = the same as bulk 1 without adding blocking primer. BP = blocking primer BloEb2.

	Bulk 1	**Bulk 2 (no BP)**
Num. reads (raw)	108470	128072
Num. reads (good)	38976	35912
Num reads (non- *E. balteatus*)	471	424
Detected prey	**Percentages (%)**	
Sphaerophoria sp. (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 sp. (Hemiptera: Anthocoridae)	0.2	2.1
Cimicoidea	0.4	0.5
Aphidius colemani (Hymenoptera: Braconidae)	1.5	1.3

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 and [41]). They did not amplify other syrphids species present in the system either, making these a useful tool for studying predation by these, as well as other predators, like *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 relative short median detection times obtained. In the sampled plot (outside the cages), *N. ribisnigri* was more abundant in spring (31.07 individuals/lettuce) than in summer (0.16 individuals/lettuce), whereas *F. occidentalis* had lower abundances in spring (0.05 individuals/lettuce) becoming more abundant in summer (2.13 individuals/lettuce). *Nasonovia ribisnigri* was the most detected prey in spring as well as in summer when *N. ribisnigri* was much less present outside of 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* sp. The diminishing natural infestation of *N. ribisnigri* during summer might cause this diet diversification of hoverfly larvae. Albeit 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[41] 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, both reflecting the relative prey abundance during summer but also this species' affinity for thrips as prey. Predation on Collembola was notably higher than on *F. occidentalis*, probably due to their higher abundance in both seasons.

When syrphid larvae found inside the cages were molecularly analyzed for species identification[5], several hoverfly species were found (*E. balteatus*, *E. corollae*, *M. auricollis* and *Sphaerophoria* sp.). These are all very common species in Mediterraean vegetables,[42] particularly in Spanish lettuce and pepper crops.[3-5,43] Even if the most abundant species inside the cages in spring and summer was *E. balteatus* because of being the species introduced, *E.*

corollae and *Sphaerophoria* sp. were also found. *Eupeodes corollae* was more abundant in spring and *Sphaerophoria* sp. in summer. *Meliscaeva auricollis* abundance was trivial. The present study has demonstrated that *E. balteatus, E. corollae* and *Sphaerophoria* sp. fed on *N. ribisnigri*, *F. occidentalis* and Collembola; being 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 larvae 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,[41] as well as in other disciplines.[44-49] 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.[49] However, depending on the aim of the study, conventional PCR is still a powerful tool that can effectively answer a number of ecological questions, like the qualitative evaluation of predation, which can help narrow down which predators may be important targets for BC programs 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.[26,35,50] 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, like 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, being in fact the most abundant one [5]. Other BC agents, like mirid bugs, earwigs, lacewings, coccinellids, hymenopteran parasitoids and even a entomopathogenic fungal-infected aphids have been cited to be consumed by hoverfly larvae.[7,51-56] 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 NE, particularly some parasitoids, like Cecidomyiids and the braconid *A. colemani* were detected through the 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 of *E. balteatus* predation showing other interactions, like intraguild trophic links in this agroecosystem which should be considered in order to develop, apply or improve new CBC programs.

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Understanding predation by *Orius* spp. in lettuce crops by molecular methods.

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Abstract

The aphid *Nasonovia ribisnigri* and the thrips *Frankliniella occidentalis* are common pests in Mediterranean lettuce crops, where *Orius* spp. are common generalist predators. Predation by *Orius* was studied in a lettuce plot by conventional PCR and qPCR analyses, which 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 Next Generation Sequencing (NGS) revealed predation on other natural enemies (Intraguild Predation (IGP)), showing a broader diet of *Orius* within the ecosystem studied. The detected predation by *Orius* on alternative prey, as well as on other natural enemies present in Mediterranean lettuce crops could compromise the biological control of *N. ribisnigri* and *F. occidentalis*.

Keywords: Conventional PCR; Gut-content analysis; NGS; *Orius;* qPCR; trophic relationships.

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 IPM (Integrated Pest Management) systems is based on the use of polyphagous predators, like the genus *Orius* Wolff 1811 (Hemiptera: Anthocoridae). Seven *Orius* species have been reported to naturally colonize 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) (Goula et al. 1993; Ferragut and González-Zamora 1994; Riudavets and Castañé 1998; Tommasini et al. 2004; Gomez-Polo et al. 2013). Even though *Orius* spp. have mainly been associated with thrips (Riudavets and Castañé 1998), aphids may also be consumed and could be an important component of their diets (Alomar et al. 2008). 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 (Agustí et al. 2003; Kuusk and Agustí 2008). There are many laboratory predation studies of *Orius* spp. on thrips (Fritsche and Tamo 2000; Montserrat et al. 2000; Blaeser et al. 2004; Arnó et al. 2008; Bonte and De Clercq 2010; Messelink et al. 2013), as well as some under field conditions in the Mediterranean basin and the Middle East (Riudavets and Castañé 1998; Atakan 2010; Bosco and Tavella 2010; Fathi and Nouri-Ganbalani 2010). 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 characterization of gut contents, but this is only feasible when solid remains are present (Moreno-Ripoll et al. 2012). This is not possible for many arthropod predators, like *Orius* spp., which are fluid feeders that practice extraoral digestion (Gurr et al. 2007). PCR-based techniques provide alternative approaches for establishing trophic links between arthropod predators and their prey. 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 feces, gut contents or regurgitates (King et al. 2008). This approach has been applied to a wide range of vertebrate and invertebrate 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 (Lundgren et al. 2009; Weber and Lundgren 2009; Lundgren and Fergen 2011).

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. 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, these amplicons are sequenced and the identities of the organisms eaten can be established by using sequence databases. The potential of NGS to characterize 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 (Valentini et al. 2009; Pompanon et al. 2012; Gomez-Polo et al. 2014b). 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 (Vestheim and Jarman 2008).

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

Materials and Methods

Arthropods

A colony of *N. ribisnigri* was established with specimens from CRAG (Center for Research in Agricultural Genomics; Bellaterra, Barcelona, Spain) on lettuce plants, and colonies of *Thrips tabaci* (Linderman), *F. occidentalis* and *O. majusculus* were established from specimens captured in vegetable crops from El Maresme area (Barcelona, Spain) on green bean pods. All these species were maintained under controlled conditions of 70 ± 10% relative humidity (RH), 16 h photoperiod and 25 ± 2º C, except *N. ribisnigri,* which was maintained at 19 ± 2º C. *Orius majusculus* were fed with *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs (Biotop, Valbonne, France). Collembola were collected in an

experimental lettuce plot at IRTA facilities (described below), where *Entomobrya* was the most abundant genus. In the same plot, the hoverflies *Episyrphus balteatus* (De Geer), *Eupeodes corollae* (Fabricius), *Sphaerophoria scripta* (Linnaeus) and *Sphaerophoria rueppellii* (Wiedemann) (Diptera: Syrphidae) were also collected. The aphids *Aphis gossypii* (Glover), *Aulacorthum solani* (Kaltenbach), *Hyperomyzus lactucae* (Linnaeus), *Macrosiphum euphorbiae* (Thomas) and *Myzus persicae* (Sulzer) were provided by ICA-CSIC (Institute of Agricultural Sciences-Spanish National Research Council; Madrid, Spain). *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). The predator *Aphidoletes aphidimyza* (Rondani) and the parasitoids *Aphelinus abdominalis* (Dalman) and *Aphidius colemani* (Dalman) were provided by Biobest (Westerlo, Belgium).

Field experiments

An experimental lettuce plot (var. Maravilla) was established at IRTA facilities (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 analyzed, 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 (May 14th and 21st, n = 3 *O. majusculus* nymph cages per date), summer 2009 (July 2nd, 6th, 9th and 16^{th,} with n = 4 nymph cages, n = 3 nymph cages, n = 3 female cages and n = 2 nymph cages, respectively), and summer 2010 (July 23rd and August 8th, n = 2 female cages per date). After 48 h, lettuce plants were cut and individually placed in 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 lettuces sampled and some of them are difficult to identify by morphological methods (particularly the nymphs), molecular identification analyses were conducted individually by conventional PCR using the 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 (Gomez-Polo et al. 2013).

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

Conventional PCR analyses

DNA was extracted from individual arthropods using the DNeasy Tissue Kit (QIAGEN; Hilden, Germany; protocol for animal tissues). 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, CA, USA). PCR reaction volumes (25 µl) contained 4 µl of template DNA, 0.6 U of *Taq* DNA polymerase (Invitrogen, CA, USA), 0.2 mM of dNTPs (Promega Corporation, 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 to target the mitochondrial cytochrome oxidase I (COI) region (Gomez-Polo et al., 2014b). These primers produced amplicons of 331 bp 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 (Kuusk and Agustí, 2008). Samples were amplified for 35 cycles at 94º C for 30 s; 58º C (Fo1F/Fo1R) or 62º C (Col4F/Col5R, Nr1F/Nr2R and Nr3F/Nr3R) for 30 s; and 72º C for 45 s. A denaturation cycle of 94º C for 2 min initiated the PCR, and the reaction was terminated with a final extension at 72º 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 visualized under UV light.

Primer specificity and prey DNA decay rates

Nasonovia ribisnigri, *F. occidentalis* and Collembola primers were screened by conventional PCR against 2-5 individuals of common non-target species potentially present in vegetable crops in the area of study (Albajes et al. 1999; Nebreda et al. 2005; Alomar et al. 2008; Jacas and Urbaneja 2008), including other potential prey and natural enemies (predators and parasitoids) (Table 1).

Table 1 Species tested for cross-reactivity using specific primers for *Nasonovia ribisnigri* (Nr1F/Nr2R [331bp] and Nr3F/Nr3R [154 bp]), *Frankliniella occidentalis* (Fo1F/Fo1R [292 bp]) and Collembola (Col4F/Col5R [177 bp]) used in the present study. Also other species tested in another previous study are included. In bold, the target species detected with their respective specific-primers. These positive amplifications are indicated showing the length of the amplified fragments (bp = base pairs).

Order	Family	Species	Nr1F/Nr2R Nr3F/Nr3R	Fo1F/Fo1R	Col4F/Col5R	Reference
Hemiptera	Anthocoridae	*Orius majusculus*	-	-	-	present study
		Orius laevigatus	-	-	-	present study
		Orius albidipennis	-	-	-	present study
		Orius horvathi	-	-	-	present study
		Orius laticollis	-	-	-	present study
		Orius minutus	-	-	-	present study
		Orius niger	-	-	-	present study
Diptera	Syrphidae	*Dasysyrphus albotriatus*	-	-	-	Gomez-Polo et al., 2014b
		Epistrophe nitidicollis	-	-	-	Gomez-Polo et al., 2014b
		Episyrphus balteatus	-	-	-	Gomez-Polo et al., 2014b
		Eupeodes corollae	-	-	-	Gomez-Polo et al., 2014b
		Eupeodes lucasi	-	-	-	Gomez-Polo et al., 2014b
		Eupeodes luniger	-	-	-	Gomez-Polo et al., 2014b
		Melanostoma mellium	-	-	-	Gomez-Polo et al., 2014b
		Melangyna cincta	-	-	-	Gomez-Polo et al., 2014b
		Meliscaeva auricollis	-	-	-	Gomez-Polo et al., 2014b
		Meliscaeva cinctella	-	-	-	Gomez-Polo et al., 2014b
		Paragus tibialis	-	-	-	Gomez-Polo et al., 2014b
		Platycheirus albimatus	-	-	-	Gomez-Polo et al., 2014b
		Platycheirus clypeatus	-	-	-	Gomez-Polo et al., 2014b
		Scaeva albomaculata	-	-	-	Gomez-Polo et al., 2014b
		Scaeva pyrastri	-	-	-	Gomez-Polo et al., 2014b
		Scaeva selenitica	-	-	-	Gomez-Polo et al., 2014b
		Sphaerophoria rueppellii	-	-	-	Gomez-Polo et al., 2014b
		Sphaerophoria scripta	-	-	-	Gomez-Polo et al., 2014b
		Syrphus ribesii	-	-	-	Gomez-Polo et al., 2014b
		Xanthandrus comptus	-	-	-	Gomez-Polo et al., 2014b
	Cecidomyiidae	*Aphidoletes aphidimyza*	-	-	-	Gomez-Polo et al., 2014b
Hemiptera	Aphididae	*Aphis gossypii*	-	-	-	Gomez-Polo et al., 2014b
		Aulacorthum solani	-	-	-	Gomez-Polo et al., 2014b
		Hyperomyzus lactucae	-	-	-	Gomez-Polo et al., 2014b
		Macrosiphum euphorbiae	-	-	-	Gomez-Polo et al., 2014b
		Myzus persicae	-	-	-	Gomez-Polo et al., 2014b
		Nasonovia ribisnigri	**+**	-	-	Gomez-Polo et al., 2014b
Thysanoptera	Thripidae	***Frankliniella occidentalis***	-	**+**	-	Gomez-Polo et al., 2014b
		Thrips tabaci	-	-	-	Gomez-Polo et al., 2014b
Collembola	Entomobrydae	***Entomobrya* sp.**	-	-	**+**	Gomez-Polo et al., 2014b
Hymenoptera	Aphelinidae	*Aphelinus abdominalis*	-	-	**-**	Gomez-Polo et al., 2014b
	Braconidae	*Aphidius colemani*	-	-	**-**	Gomez-Polo et al., 2014b

Orius majusculus feeding trials were performed to establish prey decay rates within the predator guts. Ten females of *O. majusculus* were analyzed for each time period and each prey species (*N. ribisnigri*, *F. occidentalis* and Collembola). Individual females were placed in 1.5 ml tubes and starved for 24 h with a moistened piece of cotton at 25º C. After that, they were placed in transparent plastic boxes (2.5 cm diameter) with four individuals of *N. ribisnigri* (wingless), *F. occidentalis* (second-instars) or Collembola (*Entomobrya* spp. adults). Predators were allowed to consume them for up to 2.5 h at room temperature. Individuals that had consumed 2-4 items were immediately frozen (t = 0 h) or maintained individually without prey at 25º C for 2, 4, or 8 h and frozen at -20º C until PCR analysis.

Each predator was tested up to three 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.

qPCR 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 May 14th, 28 nymphs from May 21st, 29 nymphs from July 2nd and 30 females from July 9th). qPCR reactions (25 µl) contained 12.5 µl 2× Brilliant SYBR Green qPCR master mix (Qiagen), 0.3 µM of each primer, 1 µl template DNA, and 9.5 µl of PCR-grade water. Reactions were run on a MX3000P qPCR thermocycler (Stratagene, La Jolla, CA, USA) using the following conditions: 95º C for 15 min, followed by 50 cycles of 94º C for 15 s, 53º C for 30 s and 72º C for 30 s.

NGS analyses

Few *O. majusculus* specimens collected in spring inside the field cages were tested by NGS. They were analyzed using the Ion Torrent Personal Genome Machine (PGM) platform with the general arthropod primers ZBJ-ArtF1c and ZBJ-ArtR2c (Bohmann et al., 2011; Zeale et al., 2011), 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* (Bohmann et al., 2011; Zeale et al., 2011; Gomez-Polo et al. 2014b). 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: Theridiidade); *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); *A. gossypii* and *Aphis spiraecola* Patch (Hemiptera: Aphididae); *Entomobrya* sp. Rondani (Arthropoda: Collembola); and *A. colemani*.

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 (2008) and Deagle et al. (2009). 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 Platinum® PCR SuperMix High Fidelity (Invitrogen) and 0.6 µl template DNA. The blocking primer was included at 1 to 6 times the concentration of PCR primers during amplification. Samples were amplified for 40 cycles at 94º C for 30 s; 45º C for 45 s; and 68º C for 45 s. A first denaturation cycle of 94º C for 5 min, and a final extension at 68º C for 10 min was carried out. PCR products were separated by electrophoresis in 2.4% agarose gels stained with ethidium bromide and visualized under UV light.

Fusion primers needed for the NGS analyses were prepared following the Ion Torrent recommendations (Life Technologies Corporation, 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).

Table 2. Fusion primers used for sequencing *Orius majusculus* field-collected specimens in the Ion Torrent PGM. 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 (Zeale et al., 2011). F = Forward, R = Reverse.

Primer Name	Sequence
AkT1 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*CTAAGGTAACAGATATTGGAACWTTATATTTTATTTTTGG
AkT2 (F)	**CCATCTCATCCCTGCGTGTCTCCGAC***TCAG*TAAGGAGAACAGATATTGGAACWTTATATTTTATTTTTGG
trP1-ZBJ (R)	CCTCTCTATGGGCAGTCGGTGATWACTAATCAATTWCCAAATCCTCC

Samples were analyzed in two bulks as follows: 22 *O. majusculus* from spring 2009 field cages (May 14th n = 14 nymphs and 21st 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); and 18 *O. majusculus* from spring 2009 field cages (May 14th n = 8 nymphs and 21st 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), quantified (DNA High Sensitivity kit, Bioanalyzer 2100, Agilent Technologies, 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.

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 same methodology explained in Gómez-Polo et al. (2014b),

based on the use of BLAST (NCBI website) and MEGAN (MEtaGenomics ANalyzer; Huson et al. 2007) to compute and explore the taxonomical content of the data set.

Results

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 target prey (Table 1). When the four pairs of primers were tested for cross-amplification against the seven most common *Orius* species in the area and crop studied, as well as against other potential prey (Gomez-Polo et al. 2014b), only the target species were detected, showing high specificity (Table 1).

When these primers were used to analyze *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 25º 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. ribisnibri*-specific primers (Nr3F/Nr3R; 331 bp) and *F. occidentalis*-specific primers, and a linear decay for *N. ribisnibri*-specific primers (Nr1F/Nr2R; 154 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. ribinigri* (Nr1F/Nr2R; 331 bp) and 8.6 h for *F. occidentalis.*

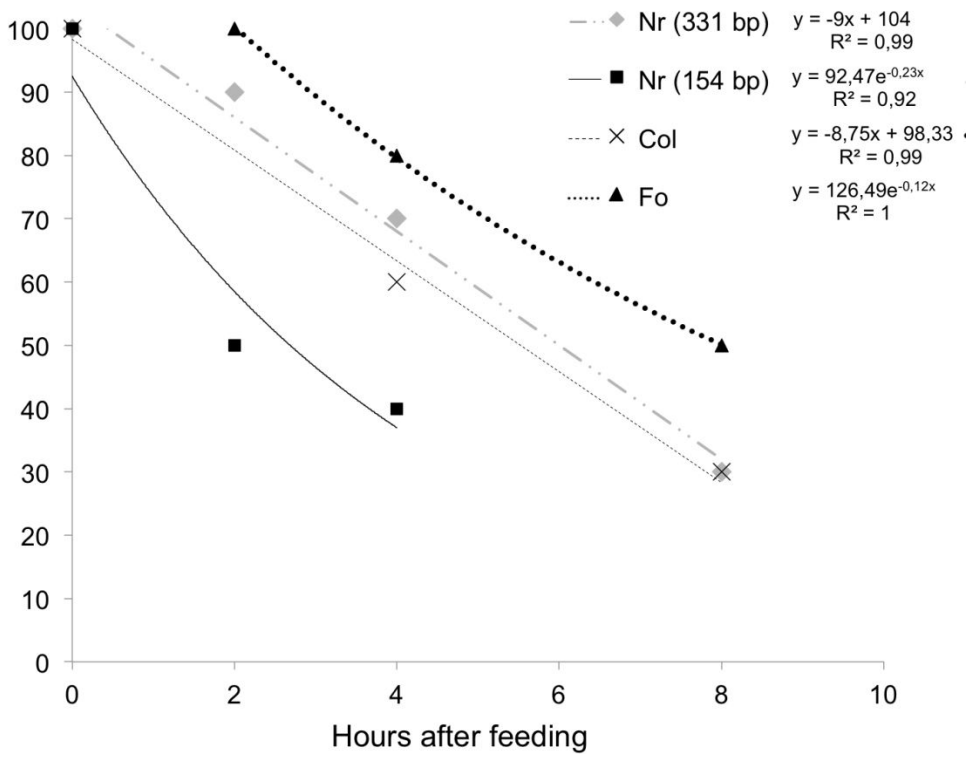

Fig 1. Detection curves of ingested *Nasonovia ribisnigri* (primers Nr1F/Nr2R [331bp] and Nr3F/Nr3R [154 bp]), *Frankliniella occidentalis* (primers Fo1F/Fo1R [292 bp]) and *Entomobrya* spp. (primers Col4F/Col5R [177 bp]) by *Orius majusculus* at different times after ingestion. Nr: *Nasonovia ribisnigri;*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

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. (2013). 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, respectively). However, due to the natural colonization 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 *Orius* sampled (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. Percentages are presented according to the prey detected, *Orius* species and period (spring 2009 (n = 53 nymphs), summer 2009 (n =105 nymphs), and summers 2009 + 2010 (n=62 females)). A higher predation was recorded on *N. ribisnigri* than *F. occidentalis* or Collembola in spring, whereas in summer the detection rate for *F. occidentalis* was higher than *N. ribisnigri* or Collembola. When *Orius* nymphs and females were analyzed 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% were 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 *Orius* females were positive for all three preys.

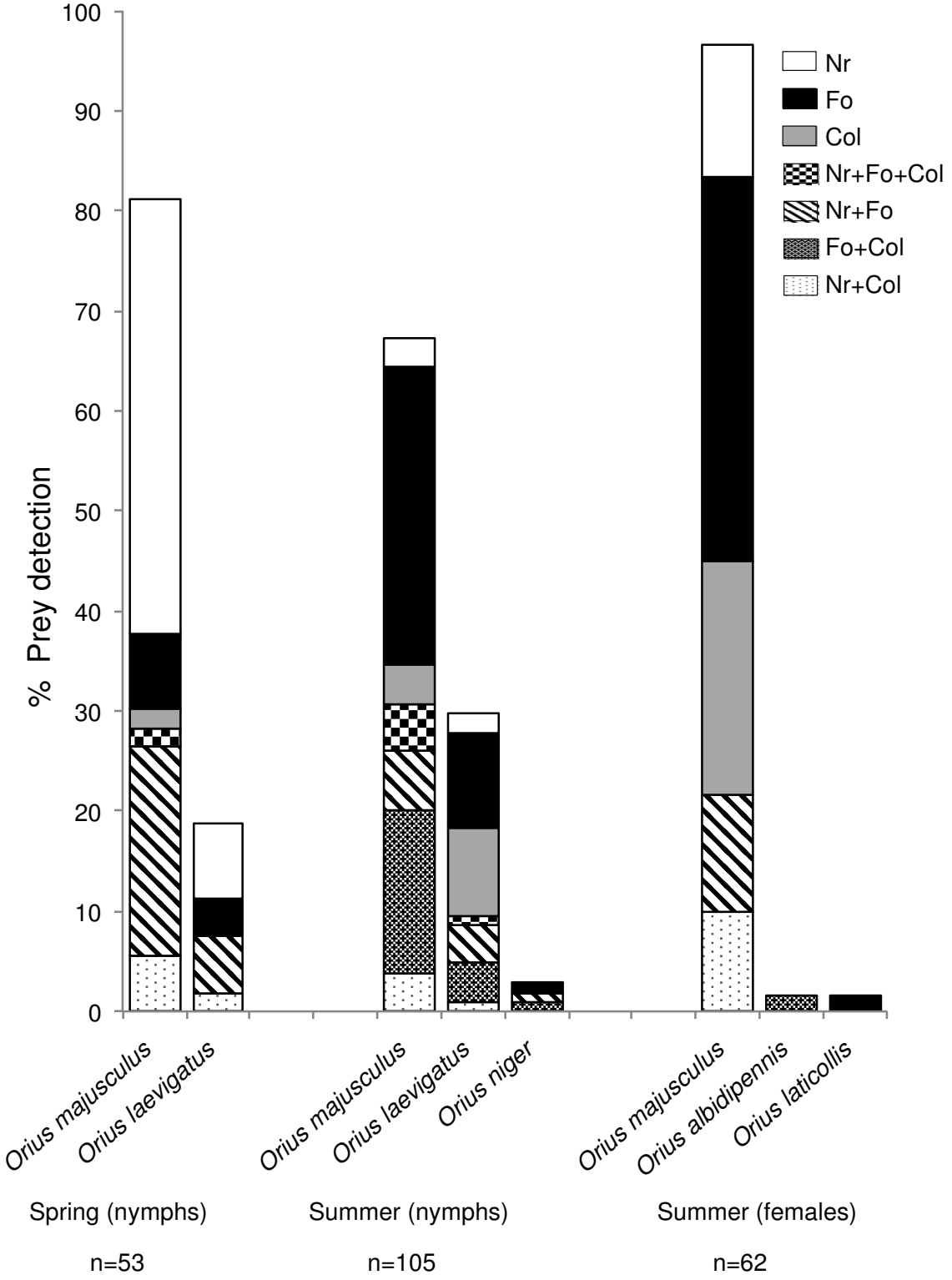

Fig 2. Molecular prey percentages obtained within the positive *Orius* nymphs and females collected in the field cages of the experimental lettuce plot in three different periods: spring 2009 (nymphs), summer 2009 (nymphs) and summer 2009 + 2010 (females). Nr: *Nasonovia ribisnigri;* Fo: *Frankliniella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

qPCR analyses of laboratory and field-collected *Orius* spp.

When *N. ribisnigri* feeding trials by *O. majusculus* were analyzed with 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 (see 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 analyzed with 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 analyzed using conventional PCR (15% in spring and 44% in summer), again showing a higher sensitivity using qPCR compared with conventional PCR.

NGS analyses of field-collected *Orius 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).

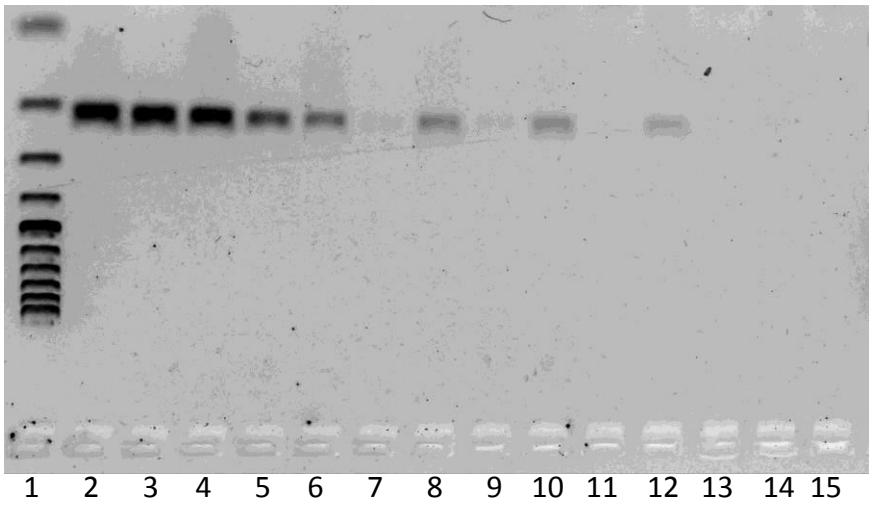

Fig 3. Conventional PCR amplifications using different concentrations of the *Orius majusculus* blocking primer (BloOm2). Lane 1: DNA size marker (100 bp ladder); even-numbered lanes correspond to an *O. majusculus* specimen fed on *Entomobrya* spp. (Collembola) and frozen immediately after consuming three individuals; odd lanes correspond to a starved (24h, 25ºC) *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×.

To be conservative, 2.5× the amount of blocking primer was added to the Ion Torrent reactions. The Ion Torrent PGM platform run produced two FASTQ files and the quality control process reduced the number of reads, but despite 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 prey sequences obtained were clearly dominated in both bulks by hoverflies of the tribe Syrphini and the species *Episyrphus balteatus* in particular (Table 3). The other prey sequences obtained were all from species known to be present in the area studied, 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.

Table 3. Number of sequences and prey percentages 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
Num. reads (raw)	75401	92915
Num. reads (good)	36648	47319
Num 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

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 to describing trophic interactions. As discussed in Pompanon et al. (2012), NGS provides an excellent tool for initial screening of predators supplying an invaluable guide to the composition and range of species consumed. It can then be followed by complementary PCR analyses based upon species- and group-specific primers aimed 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 (from 5.5 - 8.6 h). These half-lives were all within the same order of magnitude, as they were in a companion study with the same prey fed by the hoverfly predator *E. balteatus* (Gomez-Polo et al. 2014b). Therefore, it is not expected that the interpretation of the predation percentages obtained was strongly biased. On the other hand, half-life values obtained in both studies were quite short, as were those obtained with other predators, like *M. pygmaeus* (Pumariño et al. 2011), probably due to their small size, physiology of their digestive tract and feeding habits. However, other predator/prey-independent factors could also be related to the length of the detection period, like the amplicon size or the primer biochemical properties. On the contrary, other predators, like the earwig *F. auricularia* fed on aphids have shown a half-life of 24 h (Romeu-Dalmau et al. 2012), and the spider *Pardosa cribata* Simon (Araneae: Lycosidae) fed on *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) showed a half-life of 78 h (Monzó et al. 2010).

When *Orius* spp. collected from the field cages were analyzed, 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 a companion study in the same study system (Gomez-Polo et al. 2014b), showing a higher level of detection for *Orius* fed on 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 (Gomez-Polo et al., 2014b). Inside the cages *N. ribisnigri* and *F. occidentalis* abundances were modified to ensure prey availability (similar numbers were introduced in both seasons), and under this situation predators consumed all three preys (*N. ribisnigri*, *F. occidentalis* and Collembola) either alone or in different combinations (Fig. 2), showing that Collembola were also present in their diet. Consumption of non-pest prey, like Collembola, may contribute to the maintenance of predator populations (Agustí et al. 2003), 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, shown in 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 to the different temperatures present in the plot in both seasons.

Generalist predators need to diversify food intake to balance nutritional needs (Portillo et al. 2012; Pumariño and Alomar 2012). 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. (2009), 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 (Venzon et al. 2002).

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* (Gomez-Polo et al. 2014b). Other studies have obtained similar results using both techniques (Cullen et al. 2002; Lees et al. 2002; Apfalter et al. 2003; Hernandez et al. 2003; Minerdi et al. 2008; Frosth et al. 2012; de Morais et al. 2013). 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 equipments of these two approaches.

Ion Torrent NGS was used in this study to further investigate dietary breadth of *O. majusculus* collected in spring field cages placed at the experimental lettuce plot. As mentioned in the results section, 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 to elaborate on the diet of *O. majusculus* beyond them. Prey sequences belonging to the tribe Syrphini were found in their 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 (Gomez-Polo et al. 2014a; Gomez-Polo et al. 2014b). *Episyrphus balteatus* was the most detected species (with more than 90% of detection in each bulk), which had been described as one of the most abundant hoverfly species at that experimental plot in spring (Gomez-Polo et al. 2014a; Gomez-Polo et al. 2014b). The present study is the first to show hoverfly predation by *O. majusculus*, a form of IGP that merits further attention for its implications on the biological control of the target pests. Other prey sequences were also obtained, corresponding to species potentially present in the study system, like lyniphid spiders, lepidopterans, coccinellids, other dipterans and even another *Orius* species (*O. laevigatus*), highlighting the high suitability of NGS in identifying unknown trophic links between species. Previous studies conducted under laboratory conditions revealed that *Orius* spp. was able to prey on other natural enemies, like phytoseids, coccinellids, spiders, other hemipterans and even parasitoids (Jakobsen et al. 2004; Madadi et al. 2009; Sohrabi et al. 2013). Some were even conducted under field and greenhouse conditions (Harwood et al. 2009; Venzon et al. 2001; Hosseini et al. 2010; Wong and Frank 2012; Messelink et al. 2013). The species detected to be fed by *Orius* spp. in the present field study using NGS were common predators present in lettuce crops of the area studied, like hoverflies, coccinellids and spiders (Gomez-Polo et al. 2014b; Gomez-Polo et al. 2014c). The information obtained in the present study about *O. majusculus* predation by NGS reveals unknown trophic interactions not only with pest species present in the crop, but also with other biological control agents. This IGP should be further investigated in order to determine potential positive or negative effects on the biological control of these target pests in this agroecosystem.

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Molecular tracking of arthropod predator-prey interactions in Mediterranean lettuce crops.

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Abstract

The feeding habits of the generalist arthropod predators in agroecosystems are often difficult to reveal, as they are small, mobile and live among the vegetation or in the soil. DNA-based gut-content analysis is a powerful tool that enables to study arthropod predator–prey interactions. Predation on two of the main pests of Mediterranean lettuce crops, the aphid *Nasonovia ribisnigri* and the thrips *Frankliniella occidentalis*, as well as on Collembolla, the most abundant non-pest prey has been studied. Generalist arthropods were collected in four lettuce plots in two different seasons (spring and summer). All hoverflies, *Orius* spp., coccidellids and spiders found were collected and analysed by conventional PCR using *N. ribisnigri*, *F. occidentalis* and Collembola-specific primers. Results showed that coccinellids fed mainly on *N. ribisnigri*; *Orius* spp. mainly on *F. occidentalis;* spiders mainly on Collembola, and hoverflies on both pests besides the non-pest prey. Molecular analyses also revealed that the studied predator communities occupied different niches depending on the season. This study shows a deeper knowledge of the trophic relationships present in Mediterranean lettuce crops, providing the groundwork for the design of a proper implementation of biological control programs based on conservation.

Keywords: Predator–prey interactions, Nasonovia ribisnigri, Frankliniella occidentalis, Collembola, gut-content analysis, PCR.

Introduction

Generalist predators can play a major role in controlling pest populations and preventing pest outbreaks in many agroecosystems (Symondson, 2002; Welch et al., 2012). Detailed knowledge about generalist predator diets is fundamental in the development of conservation biological control (CBC) programs. In Mediterranean lettuce crops, the aphid *Nasonovia ribisnigri* (Mosley) (Hemiptera: Aphididae) and the thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) are two of the main pests (Alomar et al., 2008; Gomez-Polo et al., 2014a; Gomez-Polo et al., 2014b; Mou, 2008). Their CBC is based on the use of generalist predators.

Hoverflies (Diptera: Syrphidae) are usually present in Mediterranean lettuce crops, being *Episyrphus balteatus* (De Geer), *Scaeva pyrastri* (L.), *Eupeodes corollae* (F.), *Meliscaeva auricollis* (Meigen), *Sphaerophoria scripta* (L.) and *Sphaerophoria rueppellii* (Wiedemann) the most common species (Gomez-Polo et al., 2014c; Morales et al., 2007; Pascual-Villalobos et al., 2006). While adults mainly consume pollen and nectar, larvae of many species are polyphagous predators of a broad range of soft body insects, such as coleopteran and lepidopteran larvae (Ingels and De Clercq, 2011), as well as aphids, which are a preferred prey of most hoverflies (Rojo et al., 2003). *Orius* spp. (Hemiptera: Anthocoridae) are common polyphagous predators in agroecosystems of the Mediterranean area where they have usually been associated with thrips (Riudavets and Castañé, 1998). Seven *Orius* species have been reported to naturally colonize 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) (Ferragut and González-Zamora, 1994; Gomez-Polo et al., 2013; Goula et al., 1993; Riudavets and Castañé, 1998; Tommasini et al., 2004). Spiders are ubiquitous in terrestrial ecosystems, both in natural and agricultural habitats (Nyffeler and Benz, 1987; Turnbull, 1973), and they have been suggested to decrease herbivore abundance (Bell et al., 2008; Chapman et al., 2013; Greenstone and Shufran, 2003; Harwood and Obrycki, 2007; Harwood et al., 2004; Nyffeler et al., 1994). Even if the Linyphiidae family is a major component of the generalist predator community within arable crops (Agusti et al., 2003a; Ludy and Lang, 2004; Romero and Harwood, 2010; von Berg et al., 2012; Welch et al., 2011), other spider families like Theridiidae, Philodromidae, Araneidae, Salticidae, Clubionidae and Thomisidae have been also described to be present in agroecosystems (Mestre et al., 2013; Nyffeler, 1999; Piñol et al., 2010; Young and Edwards,

1990). Other generalist predators, like lady beetles (Coleoptera: Coccinelidae) are also abundant in vegetable crops. In the Mediterranean area, the most important species belong to four genera: *Scymnus* Kugelann, 1794; *Adalia* Mulsant, 1846; *Coccinella* Linnaeus, 1758, and *Propylaea* Mulsant, 1846 (Kavallieratos et al., 2004; Urbaneja et al., 2005). Some species like *Adalia bipunctata* (Linnaeus), *Coccinella septempunctata* (Linnaeus), *Propylaea quatordecimpuctata* (Linnaeus) and *Hippodamia variegata* (Goeze) are the most abundant, particularly in orchards (Cotes et al., 2010; Dib et al., 2010; Kourdoumbalos et al., 2006; Molinari et al., 1999; Pasqualini and Civolani, 2003; Prodanovic et al., 2010), as well as in vegetable crops, like lettuce (Sengonca et al., 2002). They have been described to feed on hemipterans, such aphids and scale insects (Urbaneja et al., 2005). All these generalist predators may feed not only on pests, but also on non-pest food, which may be particularly important when focal pest populations are scarce. One of the main non-pest food in arable ecosystems are springtails (Collembola), which may serve as alternative prey for the biological control agents of pests (Agusti et al., 2003a; Kuusk and Agusti, 2008).

Agricultural habitats are artificially created and are often characterized by high levels of disturbance as a consequence of frequent harvesting and planting regimes. In particular, Mediterranean lettuce crops are short-term crops, which make them a simple model for studying trophic links. An important step for the development of predator conservation programs is establishing their trophic linkages with focal pests. Traditional methods of visual observation have been used for determining trophic linkages througt gut dissection and microscopic characterization of the gut contents, but they can be only applied when solid remains are present (Moreno-Ripoll et al., 2012; Symondson, 2002). Conversely, when the studied generalist predators are fluid feeders (e.g. hoverfly larvae, *Orius*, spiders, etc.), PCR-based techniques are more suitable approaches for establishing trophic links, as they detect prey DNA within the gut of their predators (Agusti et al. 2003b; King et al., 2008; Lundgren et al., 2009; Sint et al., 2011).

The overall purpose of this work is to describe the trophic interactions present in Mediterranean lettuce crops. A PCR-based gut content analisis has been conducted to study predation by the most common generalist predators (hoverflies, *Orius* spp., spiders and coccinellids) on the two main pests of lettuces (*N. ribisnigri* and *F. occidentalis*), and the most abundant alternative prey (Collembola), under real field conditions and in two seasons of the year. This study provides an important knowledge of the main predatory natural

enemies present in Mediterranean lettuce crops and for improving CBC programs in those crops.

Materials and Methods

DNA amplification and primers specificity

DNA was extracted from individual predators using the DNeasy Tissue Kit (QIAGEN; Hilden, Germany; protocol for animal tissues). Total DNA was eluted into 100 ml of AE buffer and stored at -20° C. Negative controls were added to each DNA extraction set. Predation on *N. ribisnigri*, *F. occidentalis* and Collembola was analysed by conventional PCR using specific primers previously developed for the detection of *N. ribisnigri* (Nr1F/Nr2R) and *F. occidentalis* (Fo1F/Fo1R) (Gomez-Polo et al., 2014a). They were designed from the cytochrome oxidase I (COI) mitochondrial region and produced an amplicon of 331 bp and 292 bp for *N. ribisnigri* and *F. occidentalis*, respectively. Collembola-specific primers (Col4F/Col5R) were designed from the 18S region and produced an amplicon of 177 bp (Kuusk and Agusti, 2008). PCR reactions (volume of25 µl) contained 4 µl of template DNA, 0.6 U of *Taq* DNA polymerase (Life Technologies, CA, USA), 0.2 mM of dNTPs (Promega Corporation, WI, USA), 0.6 µM of each primer and 5 mM of $MgCl_2$ (50 mM) in 10× manufacturer's buffer. Samples were amplified in a 2720 thermal cycler (Applied Biosystems, CA, USA) for 35 cycles at 94º C for 30 s; 58º C (Fo1F/Fo1R) or 62º C (Nr1F/Nr2R and Col4F/Col5R) for 30 s; and 72º C for 45 s. A denaturation cycle of 94º C for 2 min initiated the PCR, and a final cycle extension was conducted at 72º 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 visualized under UV light.

Nasonovia ribisnigri, *F. occidentalis* and Collembola primers were screened by PCR for specificity against 2-5 individuals of several non-target species (Table 1); like other prey, predators and parasitoids potentially present in agroecosystems of the studied area.

Table 1. Predator (n=36), prey (n=6), and parasitoid (n=2) species tested for cross-reactivity using the *Nasonovia ribisnigri* (Nr1F/Nr2R), *Frankliniella occidentalis* (Fo1F/Fo1R) and Collembola (Col4F/Col5R) specific primers used in the present study. Other species tested in two previous studies with the same primers are also included. In bold, the three target species amplified with the respective specific-primers. The length of the amplified fragments is also included.

Order	Family	Species	Nr1F/Nr2R	Fo1F/Fo1R	Col4F/Col 5R	Reference
Hemiptera	Anthocoridae	*Orius majusculus*	-	-	-	Gomez-Polo *et al*., 2014b
		Orius laevigatus	-	-	-	Gomez-Polo *et al*., 2014b
		Orius albidipennis	-	-	-	Gomez-Polo *et al*., 2014b
		Orius horvathi	-	-	-	Gomez-Polo *et al*., 2014b
		Orius laticollis	-	-	-	Gomez-Polo *et al*., 2014b
		Orius minutus	-	-	-	Gomez-Polo *et al*., 2014b
		Orius niger	-	-	-	Gomez-Polo *et al*., 2014a
Diptera	Syrphidae	*Dasysyrphus albotriatus*	-	-	-	Gomez-Polo *et al*., 2014a
		Epistrophe nitidicollis	-	-	-	Gomez-Polo *et al*., 2014a
		Episyrphus balteatus	-	-	-	Gomez-Polo *et al*., 2014a
		Eupeodes corollae	-	-	-	Gomez-Polo *et al*., 2014a
		Eupeodes lucasi	-	-	-	Gomez-Polo *et al*., 2014a
		Eupeodes luniger	-	-	-	Gomez-Polo *et al*., 2014a
		Melanostoma mellium	-	-	-	Gomez-Polo *et al*., 2014a
		Melangyna cincta	-	-	-	Gomez-Polo *et al*., 2014a
		Meliscaeva auricollis	-	-	-	Gomez-Polo *et al*., 2014a
		Meliscaeva cinctella	-	-	-	Gomez-Polo *et al*., 2014a
		Paragus tibialis	-	-	-	Gomez-Polo *et al*., 2014a
		Platycheirus albimatus	-	-	-	Gomez-Polo *et al*., 2014a
		Platycheirus clypeatus	-	-	-	Gomez-Polo *et al*., 2014a
		Scaeva albomaculata	-	-	-	Gomez-Polo *et al*., 2014a
		Scaeva pyrastri	-	-	-	Gomez-Polo *et al*., 2014a
		Scaeva selenitica	-	-	-	Gomez-Polo *et al*., 2014a
		Sphaerophoria rueppellii	-	-	-	Gomez-Polo *et al*., 2014a
		Sphaerophoria scripta	-	-	-	Gomez-Polo *et al*., 2014a
		Syrphus ribesii	-	-	-	Gomez-Polo *et al*., 2014a
		Xanthandrus comptus	-	-	-	Gomez-Polo *et al*., 2014a
Coleoptera	Coccinellidae	*Adalia bipunctata*	-	-	-	current study
		Coccinella septempuctata	-	-	-	current study
		Propylea quartuodecimpunctata	-	-	-	current study
Araneae	Araneidae		-	-	-	current study
	Linyphidae		-	-	-	current study
	Lycosidae		-	-	-	current study
	Salticidae		-	-	-	current study
	Theridiidae		-	-	-	current study
	Thomisidae		-	-	-	current study
Hemiptera	Aphididae	*Aphis gossypii*	-	-	-	Gomez-Polo *et al*., 2014a
		Aulacorthum solani	-	-	-	Gomez-Polo *et al*., 2014a
		Hyperomyzus lactucae	-	-	-	Gomez-Polo *et al*., 2014a
		Macrosiphum euphorbiae	-	-	-	Gomez-Polo *et al*., 2014a
		Myzus persicae	-	-	-	Gomez-Polo *et al*., 2014a
		Nasonovia ribisnigri	**+/(331 bp)**	-	-	Gomez-Polo *et al*., 2014a
Thysanoptera	Thripidae	***Frankliniella occidentalis***	-	**+/(292 bp)**	-	Gomez-Polo *et al*., 2014a
		Thrips tabaci	-	-	-	Gomez-Polo *et al*., 2014a
Collembola	Entomobrydae	***Entomobrya* sp.**	-	-	**+/(177 bp)**	Gomez-Polo *et al*., 2014a
Hymenoptera	Aphelinidae	*Aphelinus abdominalis*	-	-	**-**	Gomez-Polo *et al*., 2014a
	Braconidae	*Aphidius colemani*	-	-	**-**	Gomez-Polo *et al*., 2014a

PCR analyses of field-collected predators

Four lettuce plots (var. Maravilla) located in El Maresme (plots 1 and 2) and Baix Llobregat (plots 3 and 4) areas were sampled. Both areas are close to Barcelona (Spain) and separated around 35 Km from each other. Plot 1 was an experimental field at IRTA (41.518 N, 2.377 E).

The rest were commercial plots located in Vilassar de Mar (41.497 N, 2.374 E; plot 2), Castellbisbal (41.474 N, 1.959 E; plot 3) and Molins de Rei (41.398 N, 2.024 E; plot 4).

Sixteen to 30 lettuces were collected in plot 1 in spring (May 18th and May 19th 2009; and May 11th, May 18th, May 25th and June 1st 2010) and in summer (July 7th and July 14th 2009; and July 13th, July 20th, July 27th and August 3th 2010). On May 12th 2009, the same plot was also sampled once for ground-dwelling predators. Plot 2 was only sampled once on July 12th 2010, when 20 lettuces were collected. Plot 3 was sampled twice, on April 22th and May 5th 2009, when 25 and 14 lettuces were sampled, respectively; as well as also some ground predators . Plot 4 was sampled once on May 5th 2009 by collecting 20 lettuces, as well as some predators on the ground. All lettuces were brought individually in plastic bags to the laboratory, where they were examined. Densities of *N. ribisnigri*, *F. occidentalis* and Collembola in the four plots were recorded. Collected predators were stored at -20ºC until molecular analyses. Prior to DNA extraction, all predators were checked for attached prey remains under a microscope. After PCR analyses, predation percentages obtained with *N. ribisnigri*, *F. occidentalis* and Collembola specific primers were calculated. The molecular identification of all *Orius* and hoverfly specimens collected on those lettuces was conducted as explained in Gomez-Polo *et al.* (2013) and Gomez-Polo *et al.* (2014c), respectively. Spiders and coccinellids were morphologically identified using the identification keys of Barrientos and Ferrández, (1985) and Plaza (1986), respectively.

Results

Specificity of the primers

All primers tested Nr1F/Nr2R (*N. ribisnigri*), Fo1F/Fo1R (*F. occidentalis*) and Col4F7/Col5R (Collembola) showed successful amplifications of the target prey. When they were tested for cross-amplification against 37 predator species, 2 parasitods and 6 other potential prey (some of them already tested in other previous studies), only the target species were detected, showing a high specificity (Table 1).

PCR analyses of field-collected predators

When lettuces from plots 1 to 4 were examined for *N. ribisnigri* and *F. occidentalis* abundances in spring and summer 2009 and 2010, it was found that *N. ribisnigri* populations were the highest in spring and *F. occidentalis* in summer in all plots (Table 2). Collembola

were present both in spring and summer, but in different abundances depending on the sampled plot (Table 2).

Table 2. Mean ± SE of the prey (*Nasonovia ribisnigri*, *Frankliniella occidentalis* and Collembola (specimens/lettuce)) present in the four sampled plots in spring and summer 2009-2010.

Prey	Year	Plot	Spring	Summer
N. ribisnigri	2009	1	34.5 ± 9.47	0.3 ± 0.30
		3	11.4 ± 14.09	-
		4	2.8± 0	-
	2010	1	29.4 ± 46.44	0.08 ± 0.07
		2	-	0.05 ± 0
F. occidentalis	2009	1	0.1± 0.12	2.4± 0.47
		3	0.1 ± 0.14	-
		4	0.2 ± 0	-
	2010	1	0.04 ± 0.05	2.0 ± 1.15
		2	-	4.1 ±0
Collembola	2009	1	19.5 ± 6.36	21.1± 0.45
		3	1.7± 0.99	-
		4	4.5± 0	-
	2010	1	4.5± 3.53	7.2± 4.61
		2	-	7.2± 0

The 421 collected predators were hoverfly larvae (n=117), *Orius* spp. (n=104), spiders (n=167) and coccinellid larvae (n=33). Hoverflies were mostly found in spring (n=103), than in summer (n=14); *Orius* spp. were only found in summer (n=104); spiders mainly in spring (n= 131 in spring and n=36 in summer) and coccinellids only in spring (n=33) (Table 3). Some of the hoverflies were found on lettuce (n=53), and some on the ground (n=64). These hoverfly larvae belonged to three taxa: *E. corollae* (78%), *S. scripta/S. ruepelli* (13%) and *E. balteatus* (9%). However, *E. corollae* was the only species found on the ground. When these predators were analysed by PCR, prey DNA was detected in 34% of all hoverfly larvae. From all prey-positive hoverflies, 78% were positive for one prey species, 17% for two and 5% for all three prey.

Table 3. Mean ± SE of predators present (hoverflies, *Orius* spp., spiders and coccinellids (specimens/lettuce) in the four sampled plots in spring and summer 2009-2010.

Predatory group	Year	Plot	Spring	Summer
Hoverflies	2009	1[a]	28 ± 31.43	14 ± 0
		3[b]	-	-
		4[c]	-	-
	2010	1[d]	9.5 ± 6.39	-
		2[e]	-	-
Orius spp.	2009	1[f]	-	17±11.31
		3	-	-
		4	-	-
	2010	1	-	12.5 ± 2.12
		2	-	45 ± 0
Spiders	2009	1	-	-
		3	14± 13.36	-
		4	44±0	-
	2010	1[g]	7.75 ± 6.40	9 ± 4.76
		2	-	-
Coccinellids	2009	1[h]	33 ± 0	-
		3	-	-
		4	-	-
	2010	1	-	-
		2	-	-

a) Three sampling dates in spring (n= May 12^{yh*}, 18th and 19th) and one in summer (n=July 7th)
b) Four sampling dates in spring (x= April 22th and 22^{th*}, May 5th and 5^{th*})
c) One sampling date in spring (n=May 5^{th8})
d) Two sampling dates in spring (n=May 11th and 18th) and two in summer (n=July 20th and August 3th)
e) One sampling date in summer (n=July 12th)
f) Two sampling dates in summer (n=July 7th and 14th)
g) Four sampling dates in spring (x= May 11th, 18 thand 25^{th*}and July 1st) and four sampling dates in summer (x= July 13th, 20th and 27^{th*}and August 3st)
h) One sampling date in spring (n=May 5th)

Table 4 shows the prey detection rates obtained for each prey combination in each season, and in Fig. 1 (A) this information is detalied per each hoverfly taxa. Although *E. corollae* and *S. rueppellii*/*S. scripta* were positive mainly for *F. occidentalis* in spring, *N. ribisnigri* and Collembola were also detected. In the case of *E. balteaus* only *N. ribisnigri* was detected. Detection of two preys (*N. ribisnigri* + *F. occidentalis* and *F. occidentalis* + Collembola) was found within *E. corollae* and *S. rueppellii*/*S. scripta*. The three prey were detected simultaneously only within *E. corollae* in spring. In summer all hoverflies were positive for only one prey species, which was mainly *F. occidentalis,* and *N. ribisnigri* in minor proportion.

Table 4. PCR detection percentages of *Nasonovia ribisnigri* (Nr), *Frankliniella occidentalis* (Fo), Collembola (Col), and their combinations within detected prey-positive hoverflies, *Orius* spp., spiders and coccinelids in spring and summer 2009-2010.

Group of predators	% of prey-positive predators	Nº prey detected	% of prey detection	Prey Detected	% prey detection in spring	% prey detection in summer
Hoverflies	34	1	78	Nr	22	20
				Fo	23	0
				Col	10	0
		2	17	Nr+Fo	10	0
				Fo+Col	7	0
		3		Nr+Fo+Col	5	3
Orius spp.	66	1	97	Nr	0	94
				Fo	0	0
				Col	0	3
		2	3	Nr+Fo	0	0
				Fo+Col	0	0
		3		Nr+Fo+Col	0	0
Spiders	72	1	95	Nr	0	3
				Fo	3	14
				Col	75	0
		2	5	Nr+Fo	0	3
				Fo+Col	2	0
		3		Nr+Fo+Col	0	0
Coccinellids	77	1	100	Nr	86	0
				Fo	14	0
				Col	0	0
		2	0	Nr+Fo	0	0
				Fo+Col	0	0
		3		Nr+Fo+Col	0	20

These percentages seem to be according with prey relative abundances present in the studied plots in summer (high *F. occidentalis* and low *N. ribisnigri*) (see Table 2).

Regarding to *Orius* specimens, they were all found in summer. They belonged to the following species with the following percentages: *O. laevigatus* (49%), *O. niger* (22%), *O. majusculus* (19%), *O. albidepennis* (3%), *O. minutus* (6%) and *O. horvathi* (1%). When they were analysed by PCR, prey DNA was detected in 66% of all *Orius* sampled (n=104). When examining these positive *Orius*, 97% of them were positive for only one prey species and 3% for two (Table 4). Prey detection rates calculated per each *Orius* species are presented in Fig. 1 (B), showing a higher predation on *F. occidentalis* in all of them.

Most of the spiders were collected in spring (n=131) and some in summer (n=36). They were found in similar numbers on lettuce plants (n=75), as well as on the ground (n=92). They belonged to Linyphidae (91%) and Thomisidae (9%) families.

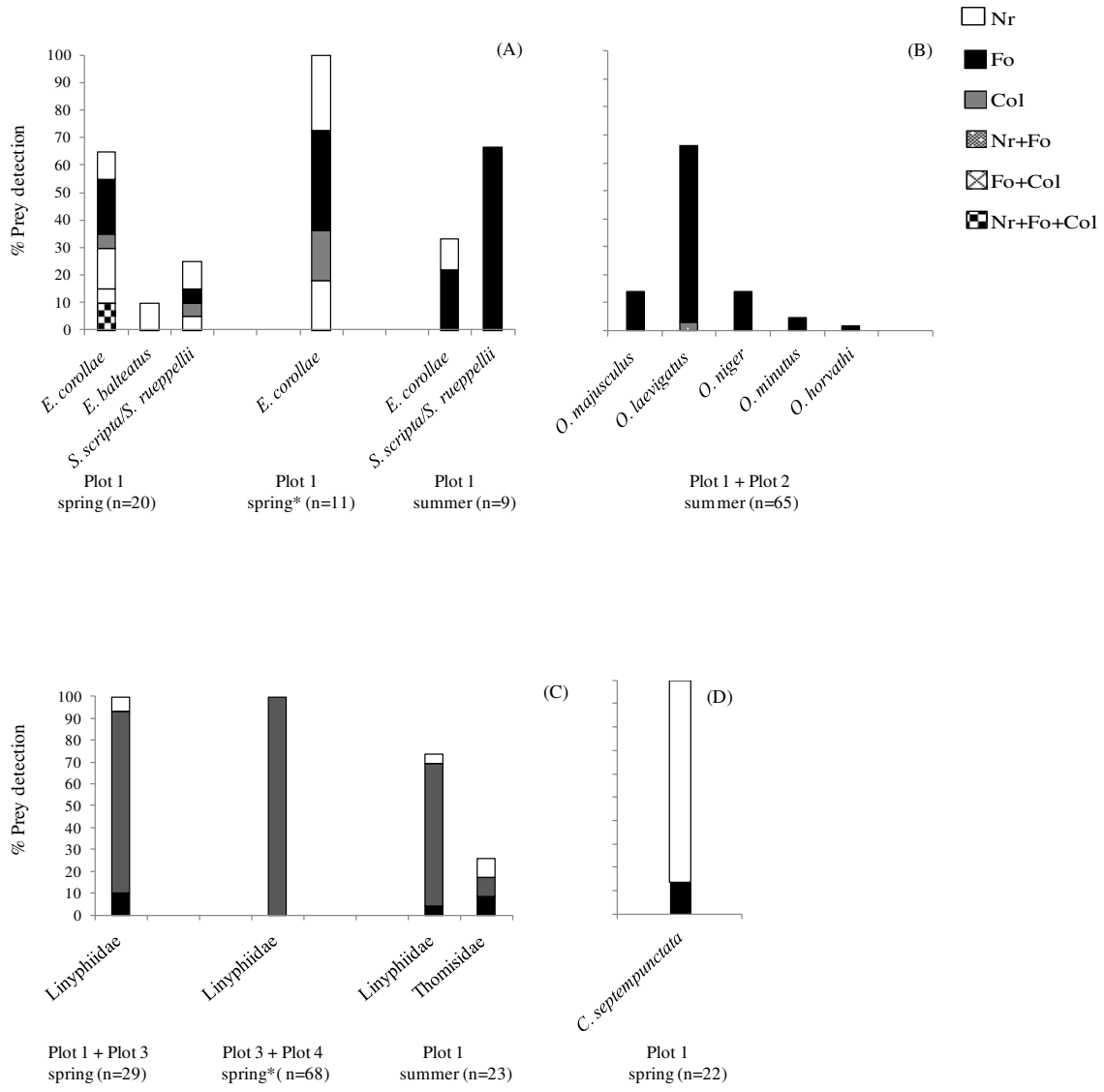

Fig 1. PCR detection percentages of *Nasonovia ribisnigri* (Nr), *Frankliniella occidentalis* (Fo) and Collembola (Col) and their combinations within those prey-positive hoverfly species (*E. corollae*, *E. balteatus* and *S. scripta/S. rueppellii*) (A), *Orius* species (*O. majusculus, O. laevigatus, O. niger, O. minutus* and *O. horvathi*) (B), spider families (Linyphidae and Thomisidae) (C), and the coccinelid *Coccinella septempunctata* (D), collected in the four sampled lettuce plots. (*): specimens collected on the ground.

Prey DNA was detected in 72% of all spiders sampled. From those prey-positive spiders, 95% of them were positive for one prey and 5% for two (Table 3). Prey detection rates for each spider family are presented in Fig. 1 (C), showing a higher predation of Collembola in both spring and summer seasons and in both spider families.

Coccinelid larvae (n=33) were found in spring (Table 3 and Fig. 1 (D)). They were all identified as *C. septempunctata.* Prey DNA was detected in 67% of them and all of them were positive for only one prey species, which mainly was *N. ribisnigri* (86%), and just in some of them was *F. occidentalis* (14%).

Discussion

This study shows an effective method for assessing the strength of the trophic linkages present in the studied agroecosystem and identifying the diversity of predators consuming the target pests. The molecular detection of *N. ribisnigri*, *F. occidentalis* and Collembola has been demonstrated within the main generalist predators found in Mediterranean lettuce plots, which include hoverflies, anthocorids, spiders and coccinelids. Diversification of niches in time and space allows natural enemies to better impact on pests, exerting sufficient pressure on prey communities to control population growth. In this study, a certain niche differentiation among the studied arthropod species has been observed. Predators appeared in the field at different times during the year, some in spring and some others in summer, according also with the target pest species.

When natural hoverfly abundances were estimated, *E. corollae* was the most abundant hoverfly taxa in spring, whereas *S. rueppellii*/*S. scripta* was the most abundant in summer. This coincides with the results obtained in a previous companion study done in the same area (plot 1) (Gomez-Polo et al., 2014b), as well as in pepper greenhouses in Southeast Spain (Pineda and Marcos-Garcia, 2008). Whereas *E. balteatus* and *S. rueppellii*/*S. scripta* were found on the lettuce plant, *E. corollae* was almost exclusively found on the ground, which is probably due to the fact that *E. corollae* overwinters as pupa stage on the ground (Gomez-Polo *et al*., 2014c). When all these hoverfly larvae were analysed by PCR for prey detection, *F. occidentalis* was the most detected prey in spring (even with high abundances of *N. ribisnigri*), as well as in summer, when *F. occidentalis* was the most abundant prey. Even if syrphid larvae had been previously described to prefer aphids (Rojo et al., 2003; Rojo and Marcos-Garcia, 1998), a companion study had also found that those syrphid species fed also on *F. occidentalis* (Gomez-Polo et al., 2014b). *Eupeodes corollae* and *S. rueppellii* have been also described to prey on other thrips, like *Thrips tabaci* Linderman (Thysanoptera: Thripidae) (Sabelis and Van Rijn, 1997). In spring, *E. corollae* and *S. rueppellii*/*S. scripta* were positive for one, two or even three different prey. In summer, only one prey species was

detected, probably because of the decrease of *N. ribisnigri* abundances. This higher proportion of multiprey detection observed within syrphid larvae in spring than other predator species analyzed in this study seems to indicate a higher polyphagy. The prey detection percentage obtained by PCR within syrphids (34%), was much lower that those obtained within *Orius* (61%), coccinelids (67%), or spiders (72%). Then, even if syrphids are more polyphagous, they do not seem to be very efficient as natural enemies because *Orius* and coccinelids have much higher (double) detection percentages. This, together with the fact of being less polyphagous probably makes them more efficient predators for the target pest species of this agroecosystem. However, further studies are needed to demonstrate this hypothesis. On the other hand, spiders have been described to have longer detection periods (Agusti et al., 2003a; Monzó et al., 2010), which could explain these high detection percentages. Anyway, spiders do not seem to play a very important role in controlling the target pest species in the studied agroecosystem in particular. On the other hand, albeit syrphid predation on Collembola was not as high as on *F. occidentalis* or *N. ribisnigri*, it was also observed in both seasons, as also happened in a companion field predation study (Gomez-Polo et al., 2014b). This Collembola consumption indicates a potential effect on the biological control of both target pest species, which can be positive or negative. The positive effect of this consumption could be the maintainance of the syrphid populations in the crop in periods of low abundances of target pest species. The negative effect could be observed by a decrease on the predation of the target pests. In any case, further studies should be conducted to clarify this positive or negative effect on the biological control of *N. ribisnigri* and *F. occidentalis.*

The molecular analisis of *Orius* specimens found in the studied plots showed that *O. laevigatus*, *O. niger* and *O. majusculus* were the most abundant species, while *O. albidepennis, O. minutus* and *O. horvathi* were present in lower numbers. Unlike syrphids, which were present mainly in spring, the presence of *Orius* in the sampled plots was only observed in summer, when *F. occidentalis* abundance was also higher. Molecular prey detection analysis showed the well-known *Orius* affinity for thrips as prey (Arnó et al., 2008; Riudavets and Castañé, 1998), as they were almost the only prey detected within the *Orius* specimens captured on lettuces. Therefore, it is expected that these predators exert a more significant control on this pest species than on *N. ribisnigri*. On the other hand, because Collembolla were detected in very low percentages, the non-pest prey effect (positive or negative) may be despised in this case.

Spiders have been described to play an important role as biological control agents (Birkhofer et al., 2008; Greenstone and Shufran, 2003; Harwood et al., 2004; Sunderland et al., 1999). The spiders collected in the studied plots belonged to Linyphidae and Thomisidae families, both very abundant in agroecosystems (Agusti et al., 2003a; Gonzalez et al., 2009; Schmidt and Tscharntke, 2005). Collembola was the most detected prey within spiders in both spring and summer, and both on lettuce and on the ground. This agrees with other studies, where Collembola were considered a major source of prey to linyphiid spiders in arable fields (Agusti et al., 2003a; Gavish-Regev et al., 2009; Harwood et al., 2003; Marcussen et al., 1999; Opatovsky et al., 2012; Piñol et al., 2014). In the present study, spiders also preyed on *F. occidentalis* in lower percentage, but no spider was positive for *N. ribisnigri*. The most reasonable explanation is that *N. ribisnigri* colonies are present in the lettuce hearts (Liu and McCreight, 2006), thus they are less accessible for spiders, basically because it is very difficult that they fall down to the ground, where they would be more accessible for those predators. It is also possible that *N. ribisnigri* has low nutritional value (Toft and Wise, 1999), being not attractive to spiders. This has been described in a previous study of linyphiids, where some biological parameters rapidly declined when they were fed on single-species diets of three common cereal aphids (Bilde and Toft, 1998). In other studies, spiders have been described to be active predators of aphids in orchards (Bumroongsook et al., 1992; de Roince et al., 2013; Hartfield, 1997;; Wyss et al., 1995) and in cereal fields (Birkhofer et al., 2008; Gavish-Regev et al., 2009; Greenstone, 2001; Harwood et al., 2001; Holland et al., 2004; Oelbermann and Scheu, 2009; Schmidt et al., 2004). However, in the studied agroecosystem the role of the spiders as effective predators for *F. occidentalis* and *N. ribisnigri* should be ignored, as it seems of limited importance.

Coccinella septempunctata larvae were found exclusively in spring, when *N. ribisnigri* was very abundant in the plot. Coccinellids are well known to be efficient biological control agents of aphids (Neved and Hodek 2012; Singh and Singh, 1994). In the present study they showed the highest predation percentages on *N. ribisnigri*. Some *F. occidentalis* predation was also observed, which has been also reported by another study (Deligeorgidis *et al.,* 2005) on cucumber and tomato in greenhouses. Therefore, the role of coccinellids as natural enemies seems important in the studied agroecosystem to control *N. ribisnigri*.

An effective CBC program should be designed according to the interactions present at the community level. Other important factors like prey preference (Bilde and Toft, 1998),

availability of alternative prey (Harwood et al., 2004; Lester and Harmsen, 2002) and the mobility of both prey and predators (Rosenheim and Corbett, 2003) could also affect the impact that a given predator species has on a particular target pest.The interaction pathways reported here are of significant value in the future for mitigating the effect of *N. ribisnigri* and *F.occidentalis* damages in Mediterranean lettuce crops.

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General discussion

Based on the first main objective stated in this Thesis, molecular methods for the identification of the main NE found in Mediterranean lettuce crops (syrphids. and *Orius* spp) have been developed in **Chapters 1** and **2**. These molecular methods have been used to identify field-collected syrphids (**Chapters 1** and **3**) and *Orius* spp. (**Chapters 2** and **4**). After that, based on the second main objective, the trophic interactions among the main pest species, non-pest prey and generalist arthropod predators, like hoverflies (**Chapters 3 and 5**), *Orius* spp. (**Chapters 4 and 5**), and spiders and coccinellids (**Chapter 5**) were evaluated under field conditions. Other trophic interactions, like IGP have also been demonstrated to be present in this agroecosystem (**Chapters 3** and **4**).

Molecular identification of natural enemies

In **Chapters 1** and **2**, two accurate, quick and cheap PCR-based diagnostic techniques have been set up to identify field-collected syrphid and *Orius* specimens, respectively. In the case of syrphid species, species-specific primers were needed. They were designed from the mitochondrial COI region to identify the five most common hoverfly taxa of Mediterranean vegetable crops (*E. balteatus*, *S. pyrastri*, *E. corollae*, *M. auricollis* and *S. scripta*/*S. rueppellii)* and used in a multiplex PCR (**Chapter 1)**. It is known that this molecular method enables a simultaneous amplification of many target species in just one reaction by using several pairs of specific primers all together in the same PCR reaction. Multiplex PCR is a challenging application that requires more optimization than Singleplex PCR, being a very cost and time-saving technique to easily and rapidly identify species (King et al., 2011; Traugott et al., 2006). The choice of the COI region for the design of specific primers was not arbitrary. The Consortium for the Barcode of Life (htpp://www.barcoding.si.edu/) has exploited the COI features and has proposed a worldwide initiative in which all know species are "bar coded" by DNA sequences from COI region (Hebert et al., 2003). This region is proving highly effective in identifying birds, fishes and many other animal groups, like arthropods (e.g., Aliabadian et al., 2013; Keskin and Atar, 2013; Krishnamurthy and Francis, 2012; Langor and Sperling, 1995; Sengupta and Homechaudhuri, 2013; Yang et al., 2012). However, in other groups of organisms, like in plants and fungi, COI does not perform well as DNA barcode because it is too invariable (Chase et al., 2007; Dentinger et al., 2011; Seifert, 2009). In these cases other regions, like ITS-1 and 2, have been recognized as the "official" barcode

marker for fungi (Chase et al., 2007; Selvaraj et al., 2013; Shneyer, 2009) and two genes located on the chloroplast genome, *rbcL* and *matK*, have been recognized as the "official" barcodes for plants (Hollingsworth et al., 2011). In diet studies, highly conserved PCR primers have usually been designed from these chloroplast regions for plant DNA detection (Avanesyan, 2014; Jurado-Rivera et al., 2009; Matheson et al., 2007; Taberlet et al., 2007; Wallinger et al., 2013), but the ITS 1-2 regions have also been used (Pumariño et al., 2011).

A previously developed molecular tool was used for the discrimination of *Orius* species (Hinomoto *et al*., 2004) (**Chapter 2**). These authors found that the variation in the length of the entire ITS-1 region, which was amplified using a couple of general primers, allowed the discrimination of two *Orius* species from Japan. For this reason, the same primers were tested in this Thesis for the discrimination of seven Mediterranean *Orius* species: *O. majusculus*, *O. laevigatus*, *O. albidipennis*, *O. niger*, *O. minutus*, *O. horvathi* and *O. laticollis.* Because this method was able to discriminate between these species, the development of *Orius* species-specific primers was not necessary.

Trophic interaction studies

Advantages and disadvantages of the molecular techniques used

Molecular gut-content analysis is an excellent tool for identifying potential biological control agents and evaluating their role within the ecosystem studied. In this Thesis, molecular gut-content analysis allowed us to study dietary choice in natural field conditions, which means avoiding laboratory artefacts. Generalist predators were analyzed by conventional PCR and real time PCR in **Chapters 3, 4** and **5**, and by real-time PCR and Ion Torrent PGM in **Chapters 3** and **4**. These PCR-based technologies used here have advantages and disadvantages, show in Table 1, and depending on the final goal of the study one should be more suitable that the other.

For example, one of the main benefits of conventional PCR is the reduced cost of analysis compared with real-time PCR or Ion Torrent PGM. In the case of Ion Torrent PGM, Table 1 shows the price of analyzing the samples of this Thesis (€700 for 7 pools with 14 to 45 samples per pool), but it is worth pointing out that it is possible to include more samples in each pool and/or to include more pools in a run, marking each pool with a different sequence (tag) to be recognized in the following bioinformatic analysis. In this case, the cost per sample would decrease considerably.

Table 1- Advantages and disadvantages of the PCR-based technologies used in this Thesis: conventional PCR, real-time PCR and Ion Torrent PGM.

	Conventional PCR	**Real-time PCR**	**Ion Torrent PGM**
Cost analysis (€/sample)	0.5-0.8	1.5	5
Cost equipment (€)	4000	12000	55000
Time of analysis	2.8 min /sample	1.8 min/sample	0.8min/sample
Bioinformatics (Data analyses)	No	No	Yes
Sensitivity	Good sensitivity	High sensitivity	Very high sensitivity
Primers	Specific	Specific	Universal

Regarding the cost of the equipment, conventional PCR is the cheapest, followed by real-time PCR, both of them being present in many laboratories. Ion Torrent PGM requires a massive investment even if it is the cheapest NGS platform nowadays (see Introduction). For this reason and because it is a very recent technology, it is still not a common equipment in the laboratories. However, because of its reduced size and maintenance, together with its reduced cost compared with other NGS platforms (Quail et al., 2012), this equipment is starting to be present in many institutions.

The time of analysis of conventional PCR is a little longer than real-time PCR due to the gel electrophoresis, which it is avoided in the real-time PCR. Analysis time with Ion Torrent PGM is shorter (particularly if a large number of samples are present in the same run). Nevertheless, NGS data analysis requires bioinformatic skills, computational resources and additional time (not included in Table 1), unlike conventional or real-time PCR, lengthening the process slightly.

It is well known that real-time PCR is more sensitive than conventional PCR, as has been shown in this Thesis (**Chapters 3** and **4)** in both laboratory-fed and field-collected predators. However, neither conventional nor real-time PCR can compete with the higher sensitivity of NGS, in this case using the Ion Torrent PGM platform. On the other hand, it is also important to point out that real-time PCR allows DNA quantification, which is not possible with conventional PCR. However, quantification of ingested DNA has not been conducted in this Thesis because one of the main problems in trophic studies is the inability to distinguish whether DNA quantification is reflecting either the real quantity of ingested prey DNA or the post-ingestion elapsed time. Ion Torrent PGM is being considered for quantifying predation. Preliminary studies seem to indicate

that this is not possible due to the impossibility of the universal primers of amplifying all species and with the same intensity (Piñol et al., 2014a).

Probably the most important and interesting characteristic of Ion Torrent PGM is its ability to obtain DNA sequences of all prey species present in the predator gut using universal primers, whereas conventional and real-time PCR require the development of a pair of specific primers for each prey species. This would be of a limited utility for generalist predators in some cases, because each prey species has to be tested in different specific PCRs (one per prey species) or in several Multiplex PCRs, which would be almost impossible in an environment with very high biodiversity. As shown in **Chapters 3** and **4,** NGS provides a more efficient mean of rapidly gathering a mass of information on the dietary ranges of generalist predators (Pompanon et al., 2012). Notwithstanding this, as previously mentioned, it is also common that universal primers are not able to amplify some species. That is to say, some potential prey can be excluded, as happened in **Chapters 3** and **4** with the invertebrate universal primers used (ZBJ-ArtF1c and ZBJ-ArtR2c). As has been demonstrated, these primers amplified a wide range of insect and spider orders, but some other insect species were not amplified, including *N. ribisnigri* and *F. occidentalis*. The lack of amplification of some species is not exclusive to this pair of primers. All universal primers present a certain lack of amplification of some species. This is an important issue that was recently discussed in Piñol et al. (2014b), where it is argued that no universal primer is truly universal when confronted with a complex of species. Something to consider in future studies would be the combination of two or more sets of universal arthropod or invertebrate primers, which would amplify a wider range of prey species.

In dietary studies, the result of the PCR amplification with universal primers is usually dominated by predator DNA rather than by prey DNA. This fact produces a lot of non-informative predator reads, reducing the sequencing power of NGS. To decrease predator DNA amplification, the ideal system lies in combining universal primers and a blocking primer, namely a primer that specifically reduces or even blocks the amplification of the predator DNA. Such a blocking primer must be specifically designed to target predator DNA, as done in **Chapters 3** and **4.** However, even if these specific blocking primers could be beneficial preferentially binding with predator sequences and limiting their amplification, they could also have a detrimental blocking effect with some prey species, particularly if prey and predators are closely related. In this Thesis**,** most of the sequences obtained (>99%) using Ion Torrent PGM still belonged to the predator itself. When blocking primers were designed, conventional PCR followed by gel electrophoresis showed that the target species were blocked at the concentration used, but when Ion Torrent analyses were conducted these blocking primers were not efficient enough. This could be due to the higher sensitivity of the Ion Torrent PGM with respect to conventional PCR. Considering this, a higher concentration of

blocking primer is probably needed when using Ion Torrent PGM or another NGS platform. This issue is now being studied and will shortly be submitted for its publication (Piñol et al. 2014b).

Prey-predator interactions in Mediterranean lettuce crops

This Thesis showed that generalist predators, like syrphids, *Orius* spp, coccinelids and spiders colonized Mediterranean lettuce crops (**Chapters 1**, **2** and **5**). These predator communities coexist occupying different niches depending on the time of the year (coccinellids and syrphids were more abundant in spring; *Orius* in summer; spiders equally present in both seasons) and on the soil or the plant species where they live (lettuce; *L. maritima*) (**Chapters 3**, **4** and **5**). On the other hand, even if some trophic interactions were already known (see Fig. 6 in the Introduction), some other previously undiagnosed trophic interactions present in this agroecosystem have been revealed (Fig. 8), which enriches the understanding of the community structure. This is the first study that shows arthropod predator-prey trophic interactions in Mediterranean lettuce crops as naturally occur in field conditions. For example, syrphids are known to be voracious aphid predators (including *N. ribisnigri*). They can also feed on thrips, although it was not known whether they prey on *F. occidentalis* (see General Introduction, section 2.2.1). In this Thesis their predation on *F. occidentalis*, as well as on springtails, it has been demonstrated for the first time. It was already known that *Orius* prey on *F. occidentalis,* as well as on aphids (see General Introduction, section 2.2.2), but it was unknown whether they prey on *N. ribisnigri* in particular. In the assays conducted in this Thesis, *Orius* preyed mainly on *F. occidentalis*, and predation of *Orius* on springtails and on *N. ribisnigri* has been demonstrated for the first time. Coccinellids are well-known predators of *N. ribisnigri*, as well as on *F. occidentalis* (see General Introduction, section 2.2.3). In this Thesis, coccinellid predation on *N. ribisnigri* and *F. occidentalis* was also demonstrated, but this was not the case of Collembola. Finally, spiders are known to be predators of aphids (but there is not any study of spider predation on *N. ribisnigri*), *F. occidentalis* and collembolans (see General Introduction, section 2.2.4). In this thesis, collembolans were highly detected within spiders, as well as *F. occidentalis* in lower proportions, but no spider was positive for *N. ribisnigri*. It is known that spiders prefer mobile prey, and in the case of aphids they are much slower than springtails or thrips. On the other hand, due to the fact that *N. ribisnigri* colonize lettuce hearts (Liu and McCreight, 2006; and also observed in the present Thesis), they are less accessible for spiders because they do not fall down onto the ground, where they would be more available for them.

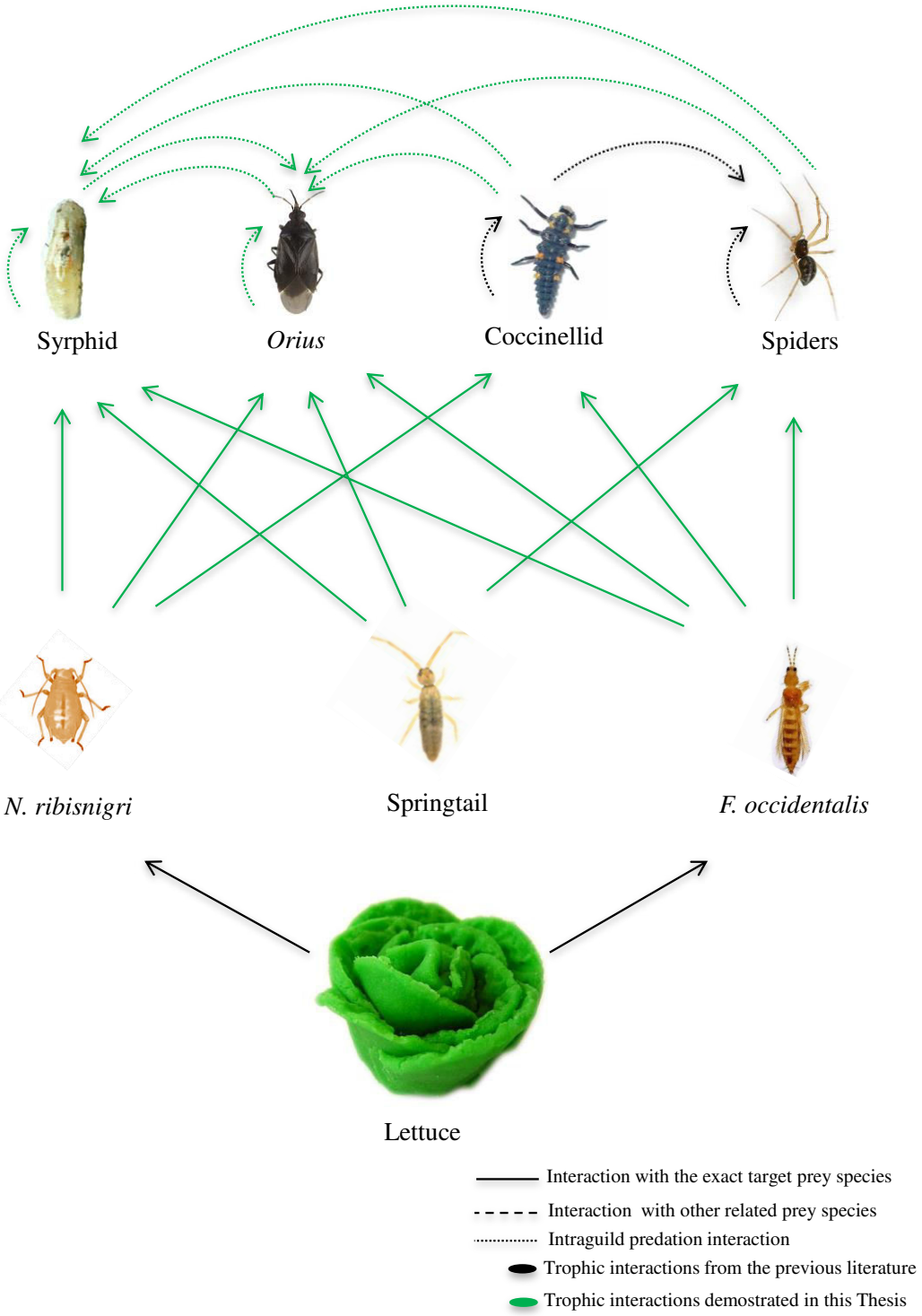

Fig .8.- Trophic interactions between the main predators (*Orius* spp., syrphids, coccidellids and spiders), pests (*N. ribisnigri* and *F. occidentalis*) and non-pest prey (springtails) present in Mediterranean lettuce crops.

When hoverfly larvae were molecularly analyzed, they were found to be the most polyphagous predators, because of preying on *N. ribisnigri*, *F. occidentalis*, as well as on Collembola. *Orius* spp. preyed mainly on *F. occidentalis*, whereas coccinelid larvae preyed mainly on *N. ribisnigri*. Spiders preyed mainly on Collembola, the non-pest prey. Other "less expected" interactions, like IGP, were shown when using Ion Torrent PGM (**Chapters 3 and 4**). These analyses showed for the first time the evidence that *E. balteatus* may feed on syrphids of the genus *Sphaerophoria* and *O. majusculus* may feed on *O. laevigatus* in natural field conditions. Also, *E. balteatus* and and *O. majusculus* may feed on each other, and both species may also feed on spiders and coccinellids. 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. New CBC programs, should consider these interactions, and those already existing should then be re-evaluated. The fact that other NE, particularly some parasitoids, like some Cecidomyiids and the braconid *A. colemani,* were also detected within the guts of *E. balteatus* in the NGS analyses (Chapter 3) could also have a consequence on the biological control of *N. ribisnigri,* which should also be further investigated.

Future perspectives

Molecular diagnostics provide invaluable tools for detecting trophic links within the tangled network of interactions found in diverse communities. They can help to inform us about network structures that can lead to positive outcomes, like pest control. Newly developed technologies, like NGS are producing a "genomic revolution" facilitating such work prodigiously. Ecologists have just started to use NGS technologies for diet studies of herbivores (e.g. Kowalczyk et al., (2011)), as well as of carnivores (e.g. Deagle et al. (2009); Shehzad et al. (2012)), sequencing fast and at reasonable costs thanks to the continuous refinement of high-throughput sequencing technologies.

Several NGS platforms from different manufacturers are available (see the General Introduction), but Ion Torrent PGM has a more affordable price than other platforms (the equipment costs up to 9 times less), offering the first reasonably priced high-throughput sequencing platform (Quail et al., 2012), as well as more affordable analysis costs (at least 5 times cheaper) than other platforms (Loman et al., 2012). On the other hand, as sequencing facilities improve, more sequence data are becoming available in public databases, like GenBank or BOLD. Conventional or real-time PCR will continue to be used in the future but, depending on the purpose of the study, they would be replaced by NGS analysis, particularly by Ion Torrent. In food web studies, in those cases when we do not have information about the species present in the ecosystem, or in ecosystems

with a very high diversity, such as those in tropical environments, it is unrealistic to identify all potential prey using specific primers. Therefore, the first step may be a NGS analysis to have an initial idea about the potential prey present in the area of study. Then, it would be more convenient (in terms of economy) to develop specific primers for the most common prey species followed by a conventional or real-time PCR-based study. The NGS evolution will clearly keep revolutionizing the study of trophic interactions in the years to come revitalizing research in ecology and environmental DNA.

Conclusions

I. The most common generalist predators found in the lettuce crops studied were *Orius*, hoverflies, coccinellids and spiders, which were potential candidates to be used in CBC programs to control *Nasonovia ribisnigri* and *Frankliniella occidentalis*, the most common pest species found in the lettuce crops studied.

II. A multiplex PCR tool was developed, which allows the identification of the five most common hoverfly taxa present in Mediterranean vegetable crops. This tool allows the correct identification of all development stages, included the predatory larvae.

III. *Eupeodes corollae*, *Episyrpus balteatus*, and *Sphaerophoria scripta*/*Sphaerophoria rueppellii* are the most common hoverfly species present in the lettuce plots sampled, also being potential candidates to be used in CBC programs.

IV. A molecular tool was developed for the discrimination of the seven most common *Orius* species present in Mediterranean vegetable crops, allowing the detection of all development stages, included nymphs.

V. In the plots sampled, *Orius laevigatus*, *O. majusculus* and *O. niger* are the most common *Orius* species found on lettuces, as well as on the *Lobularia maritima* border, being the main *Orius* candidates to be used in CBC programs.

VI. Under semi-field conditions (field cages with altered prey and predator abundances), when predation on *N. ribisnigri* and *F. occidentalis* was studied by conventional PCR, both *Orius* and syrphids showed higher predation percentages on *N. ribisnigri* than on *F. occidentalis* in spring. In summer, syrphids prey more on *N. ribisnigri* and *Orius* on *F. occidentalis*.

VII. In real field conditions, *N. ribisnigri* abundance is higher in spring and *F. occidentalis* abundance is higher in summer. Regarding the predators, coccinellids are only found in spring, syrphids mainly in spring, but also in summer, and *Orius* are only found in summer. With these abundances, coccinellids are the most efficient predators of *N. ribisnigri* in spring; syrphids are the most efficient predators of *F. occidentalis* in spring; and *Orius* are the most efficient control agents of *F. occidentalis* in summer.

VIII. The lower syrphid predation rates on *N. ribisnigri* and *F. occidentalis* make them the least efficient predators of both target pest species compared with *Orius* and coccinellids. On the other hand, they are found to be the most polyphagous predators because of their higher multiprey predation.

IX. The higher spider predation rates detected on Collembola, together with their lower predation on *N. ribisnigri* and *F. occidentalis* make them the least important predators to control both pest species compared with *Orius*, coccinellids and syrphids.

X. The consumption of Collembola, the alternative prey, found in *Orius* and syrphids suggest that they can either play an important role in the maintenance of these generalist predator populations in the lettuce crops studied, or interfere with the control of *N. ribisnigri* and *F. occidentalis*. Further studies should be conducted to clarify this issue.

XI. When NGS analyses were conducted to study other potential trophic interactions present in the agroecosystem studied, some intraguild interactions were shown. The interaction between *O. majusculus* and *E. balteatus*, as well as with other natural enemies could have either positive or negative effects on the biological control of *N. ribisnigri* and *F. occidentalis,* which need a further attention in future studies to develop CBC programs of both pest species.

XII. The molecular methods used in this Thesis are very effective in showing the prey-predator interactions present in the agroecosystem studied. Real-time PCR is more sensitive than conventional PCR, but other advantages, like its lower equipment and analysis cost, makes conventional PCR the most used technique in this kind of studies at the moment. NGS allows the detection of "hidden" trophic relationships, which cannot be demonstrated by conventional or real-time PCR. This new method may be the most common method to be used in future ecological studies, like prey-predator trophic interactions.

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