Molecular tracking of insect dispersal to verify arthropod predator movement from an alfalfa field to a peach orchard.

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Abstract

Implementation of landscape approaches to conservation biological control programs requires the confirmation of putative sources that contribute to predator colonization of crops. This study aims to confirm predator dispersal from an alfalfa field to a neighboring peach orchard with a DNA mark-capture procedure based on a topical application of a solution of grinded brine shrimp cysts, *Artemia* spp. (Anostraca: Artemiidae), followed by a conventional PCR.

To optimize the marking procedure, a well-known predator present in orchards as well as in arable crops, *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae), was used as a model in this study. In greenhouse trials, the acquisition and the retention time of the *Artemia* markings were determined, either directly by spraying them with the *Artemia* solution or indirectly via residual contact on caged plants after the spray. The topical mark remained detectable on *O. laevigatus* after 6 days, and 50% of the tested predators were positive 3 days after walking on the sprayed plants.

After that, a 25m^2 strip of an alfalfa crop neighboring to a peach orchard was sprayed with the *Artemia* solution just after the alfalfa cuts, and several common predator species were collected using sticky traps placed between both crops. After PCR analysis with the *Artemia* specific primers, 32% of the analyzed predators (coccinellids, anthocorids, chrysopids, and mirids) showed the mark. The results of this study confirm the usefulness of this marking method to monitor dispersal of biological control agents between neighboring crops, in this case alfalfa and peach.

Key words: alfalfa crop, peach orchard, *Artemia* spp. cysts, mark-capture, PCR analysis, predator movement.

1. Introduction

Conservation Biological Control (CBC) represents a sustainable way to enhance naturally occurring Biological Control Agents (BCAs) to control crop pests (Eilenberg et al., 2001). This control strategy is based on the provision of food and shelter to BCAs, and field margins and flower strips are increasingly being used in order to enhance them (Landis et al., 2000; Aguilar-Fenollosa et al., 2011; Amaral et al., 2013; Pollier et al., 2018; Gontijo, 2019). Semi-natural habitats and crops are also important sources of BCAs, and their movement from crop to crop occur especially in agricultural areas with spatial and temporal heterogeneity. An increasing amount of research links landscape composition and configuration with pest and prey abundances in focal crops. Such results help to identify crop and non-crop habitats contributing to higher populations of target insects, particularly of key predators (Haan et al., 2020). However, there is no simple and consistent response of pest or natural enemy abundances to a landscape composition (Karp et al., 2018, Chaplin-Kramer et al., 2019). Samaranayake and Costamagna (2019) indicate the need to study the role that landscape habitats (i.e. crop fields surrounding the target field) play in contributing with BCAs, with studies that evaluate the movement of natural enemies between crops and other habitats. More specifically, landscape approaches to the development of CBC of arthropod pests requires the confirmation of the movement of predators between neighboring crops. Such information is important to implement IPM strategies.

In the Ebro Basin (NE Iberian Peninsula) cropping landscapes that were traditionally dominated by rotation of arable crops (alfalfa, maize and other cereals) have experimented a great increase of orchard production, specially peaches, resulting in a mixed mosaic of arable crops and orchards together with semi-natural habitats (Madeira et al., 2014, Clemente-Orta et al., 2020). According to the Food and Agriculture Organization (FAOSTAT, 2018), Spain was the main peach producer in Europe, with a 30% of the Spanish production concentrated in Catalonia (MAPA, 2019). The coexistence of annual and perennial crops could be advantageous if they share mutual natural enemies which disperse from one to another along the season, searching for refuges and prey. Alfalfa is known to act as a reservoir and source of many insect natural enemies in agricultural landscapes (Samaranayake and Costamagna, 2019; Sisterson et al., 2020). Several important predatory groups have been recorded in alfalfa in the area (*Orius* spp, mirids, nabids and coccinellids), that are shared with other arable crops (Pons et al., 2005, 2009). There are some shared pests with peach too, as the western flower thrips, *Frankliniella occidentalis* (Pergande), that could migrate to the orchards when cutting the alfalfa. Besides, peaches have other pests that cause the application of several pesticides for their control. Among them, the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann; lepidoptera as *Grapholita molesta* Busck and *Anarsia lineatella* Zeller; aphids as *Myzus persicae* (Sulzer) and *Brachycaudus schwartzi* (Börner); and scales as *Diaspidiotus perniciosus* (Comstock) (Avilla et al., 2008).

A wide range of marking techniques have been developed to evaluate the dispersal patterns of arthropods (Lavandero et al., 2004; di Lascio et al., 2016; Madeira and Pons, 2016; Jiao et al., 2019; Kenne et al., 2019;

Tavares et al., 2019; Hagler and Machtley, 2020). El Sheikha (2019) reviews the advantages and disadvantages of several tracking techniques. Among them, DNA gut content analyses, that are increasingly being used to identify prey or plant consumption by arthropods of agricultural importance (González-Chang et al., 2016), can also address the movement or dispersal of insects. Examples are the gut content PCR analyses using specific primers of a particular insectary plant (Pumariño et al., 2011; Wang et al., 2017; Hayashi et al., 2020), using universal plant primers and sequencing (Wang et al., 2019; Avanesyan and Lamp, 2020) or the DNA analysis of microbial communities associated with insects (El Sheikha and Menozzi 2019) .

Recently, a new marking method based on spraying plants with an aqueous solution of a grinded aquatic invertebrate (*Artemia* spp. (Anostraca: Artemiidae) that exclusively lives in saline waters, followed by a conventional PCR with specific primers for its DNA detection has been developed (Agustí et al., 2020). In that study, the movement of the mirid bug *Macrolophus pygmaeus* (Rambur) from a banker plant (*Calendula officinalis* L.) to the tomato crop was confirmed under greenhouse conditions. The aim of the present study was to further optimize that procedure and to apply it in open field commercial crops in order to track predator's movement between neighboring crops. This marking method was improved with added laboratory and semi-field experiments using *Orius laevigatus* (Fieber) (Hemiptera: Anthocoridae) as a model. *Orius* spp. are known to be common in several crops in the growing area of Lleida, like alfalfa and maize. *Orius laevigatus* has been found in peach, apple, and pear (Sarasúa et al., 2000; Pons et al., 2005; Albajes et al., 2011). The improved marking method was then applied to confirm predator dispersal from an alfalfa crop to a neighboring peach orchard, and confirms the utility of this technique to identify the sources of beneficial insects that colonize crops.

2. Materials and Methods

The marking solution was prepared by grinding dry *Artemia* spp. cysts (Inve Aquaculture, Inc.), in order to make the *Artemia* DNA more accessible, and mixing them with water at a concentration of 0.1 gr/ml as explained in Agustí et al. (2020), except that Tween-20 (0.02%) was added to the solution as surfactant. The obtained *Artemia* solution was always used in the following 24h.

2.1 Efficiency of the marking on O. laevigatus.

The marking was topically applied to *O. laevigatus* adults in order to know whether it was also effective for marking a smaller predator than *M. pygmaeus* used in Agustí et al. (2020) (*O. laevigatus* 1.4-2.4mm and *M. pygmaeus* 3-6mm). Predators were purchased in Agrobío S.L. (Almería, Spain). To improve PCR detection, the addition of Tween-20 (0, 1 µl and 2.5 µl) to the marking solution was compared. PCR analysis were also tested just after spraying *O. laevigatus* dead adults with the *Artemia* solution, and after 24h of the spray (n=4-10 and n=14 adults, respectively) (Table 1). In addition, two different concentrations of the *Artemia* solution (0.1 gr/ml and 0.01 gr/ml) were tested (n=34 and n=20 adults, respectively). All individuals were analyzed by conventional PCR, using the specific pair of primers of *Artemia* spp., as described in Agustí et al. (2020). Each specimen was DNA extracted using the Speedtools Tissue DNA Extraction Kit (Biotools, CA, USA) following the manufacturer protocol and using the whole body of the insect. The obtained DNA was eluted in 100 µl of elution buffer provided by the manufacturer and stored at -20 ºC. A negative extraction control was added to each set of DNA extractions.

2.2. Semi-field trials to study the extent of the marking

Acquisition and retention time of the DNA marker was tested after spraying the *Artemia* solution on alfalfa plants containing stationary (dead) or freely roaming (alive) individuals.

A first trial aimed to verify the effectiveness and persistence of the *Artemia* mark when spraying stationary *O. laevigatus* placed at two heights within the alfalfa plant canopy. The trial was arranged in a randomized complete block design consisting of three glasshouse compartments (4x6 m), each one with 10 closely placed pots (5L capacity) containing 4-5 alfalfa plants each. All plants were ca. 50 cm height. *O. laevigatus* cadavers (killed by freezing) were glued with their dorsum facing up on the upper side and on the lower side of a yellow sticky label (9 cm long and 2 cm wide). Nine individuals were glued on each side, in three sections of three individuals, one section for each sample date. Two labels were attached horizontally at two heights (at 25cm and 40cm from the top of the plant) on a wooden stick that was placed in the middle of each pot (Fig. 1). Overall, 1080 predators were exposed. Twenty-seven pots (9 per compartment) were sprayed with the *Artemia* solution (0.1 gr / ml plus the surfactant Tween-20 at 0.02% until run-off) with a commercial backpack sprayer (Matabi Super Green 16L, Goizper Spraying, Spain). Three other pots (one from each compartment) were sprayed only with water in another compartment and afterwards each one placed in each of the three compartments, as controls. The effectiveness of the sprays was assessed with water sensitive spray cards placed below the labels. Twelve hours after spraying, the outer section of all labels was cut and predators were individualized in 1.5 ml centrifuge tubes, labelled and frozen at -20ºC until PCR analysis. Similarly, the middle and the inner sections of each label were cut 3 and 6 days later, respectively. For the analysis, one adult was randomly chosen from the three corresponding to each sample date section.

A second trial aimed to verify the acquisition of the mark by alive *O. laevigatus* adults when walking on dry residues of the *Artemia* solution after spraying the alfalfa canopy. For this, eight pots of alfalfa were placed in a greenhouse compartment. Two of these pots were sprayed with water outside the compartment (control pots) and the other six were sprayed with the *Artemia* solution, as done in the previous test. When plants were dry, each pot was then covered with a fine mesh (eight threads/mm) sleeve cage, and 10 live adult *O. laevigatus* were released on each cage. The insects could roam freely on the alfalfa for 12 hours or 3 days. After each of those times, all insects of three sprayed pots and one control pot were collected. Each collected insect was individually placed in a clean 1.5 ml centrifuge tube and frozen at -20 ºC for further PCR analysis.

The results from the first trial were analyzed with a generalized linear model (GLM) assuming a binomial distribution and logit function. The initial model included the proportion of marked individuals as dependent variable, and the factors height (middle, bottom), side (upper, lower), and time (12h, 3d, 6d) as well as all their interactions as predictors. Akaike's information criterion (AIC) by multi-model inference using the 'MuMIn' package (Bartoń, 2018), and analysis of deviance (with Chi-squared test) were used to compare fitted models and test the significance of predictor terms (Burnham and Anderson, 2002; Hastie and Pregibon, 1992). To ensure there was no violation of the normality and homoscedasticity assumptions, model residuals were graphically inspected with Q-Q plot, and a residual versus fitted values plot (Zuur et al., 2010; Crawley, 2013). For the second trial, the proportion of marked individuals obtained at two different times (12h and 3d) were analyzed with a test of equal or given proportions using the prop.test function (Newcombe, 1998). All data were analyzed with R version 3.5.1) (R Development Core Team, 2018).

2.3. Field effectiveness of the marking method and PCR detection

The effectiveness of the DNA mark was finally tested under open-field conditions in a commercial alfalfa field (1.3 ha) adjacent to an organic peach orchard (2 ha) located in Vilanova de Segrià, Lleida, Spain (41º43'3"N, 0º37'7"E). Alfalfa plants were about 50 cm high at the time of study, which is when the crop was ready to be cut. As in other studies (Madeira and Pons, 2016), a strip of the alfalfa field (2.5 m width x 10 m long, and 2-4 m from the peach orchard margin) was sprayed with 8L of the *Artemia* solution 2h before being cut. The spray was done with a knapsack sprayer (Matabi Super Green 16L, Goizper Spraying, Spain). Effectiveness of the spray was assessed with water sensitive spray cards. After the spray, 10 (1^{st} cut), and 20 (2^{nd} and 3^{rd} cut) unfolded Pherocon® Unbaited AM Yellow Sticky Traps (Trécé Inc., OK, USA) were placed between the alfalfa and the peach orchard. Traps, separated ca. 2 m between them, were placed at 60 and 80cm from the ground, in order to catch insects flying at different heights. Sticky traps were collected 24h (1^{st} and 2^{nd} cuts) or 3h (3^{rd} cut) after being placed, and they were stored at 4ºC in a portable cooler. Once in the laboratory, predators collected on the sticky traps were picked up carefully, individualized in order to avoid cross-contamination, and stored at -20ºC. Finally, they were all analyzed by PCR for the topical presence of *Artemia* DNA. The experiment was repeated three times, at the time of the cuttings of the alfalfa field (6^{th} of July, 6^{th} of August, and 6^{th} of September).

Both crops were sampled during the experiments in order to determine key predators present in them. The alfalfa field was sampled before the spray with a sweep net. The branches of the peach trees that were facing the sprayed alfalfa strip were vigorously shaken in order to remove most of the predators present, both before the alfalfa cutting and after traps were removed.

Only adults from major aerial predator groups (Heteroptera, Coccinellidae and Neuroptera) were finally collected. Those predators were identified to family and species level when possible using taxonomic keys, except the *Orius*, which were identified using a molecular method previously developed (Gomez-Polo et al., 2013).

3. Results

3.1. Efficiency of the marking on O. laevigatus.

When testing the effect of adding Tween-20 as a booster in the PCR reactions of sprayed *O. laevigatus*, all tested individuals sprayed with the highest *Artemia* concentration (0.1 gr/ml) at t=0 were amplified, regardless whether Tween-20 was added or not (Table 1). However, with the lowest *Artemia* concentration (0.01 gr/ml), higher amplification percentages were obtained with the highest amount of Tween-20. Based on that, we tested the highest *Artemia* concentration (0.1 gr/ml) together with the highest Tween-20 amount (2.5 µl) after 24h of spraying, obtaining a 100% amplification (Table 1). From this results, this methodology was used in the following semi-field and field trials. No phytotoxic effects were observed on the alfalfa plants after being sprayed with the *Artemia* solution in any case.

3.2. Semi-field trials to study the extent of the marking

The first trial, conducted to verify the effectiveness and persistence of the *Artemia* mark on *O. laevigatus* placed at two different heights within the alfalfa plant canopy, indicated significant differences only regarding the sides of the labels, with a higher number of marked individuals (77-96 %) on the upper side (Fig. 2, Fig. 3, Table 1). Therefore, the efficiency of the spray in marking those predators was not affected by their location in the plant canopy (either 25cm or 40cm from the top of the plant), nor by the time lapse after spraying (12 h, 3 days or 6 days). Water sensitive spray cards also indicated that the sprays done with the knapsack sprayer had an effective coverage of the plant. The lowest percentages of marked insects were obtained on the lower sides of the labels, 3 and 6 days after the spray (ca. 15%).

The second trial conducted to verify the acquisition of the mark by adults of *O. laevigatus* when freely walking on dry residues of the *Artemia* solution after spraying the alfalfa canopy showed that they were able to self-mark in that way. From the 30 released adults, 80% of them were marked 12h after the spray. After 3 days 56.6 % were still marked. Although there was a major reduction in the efficiency of the mark, differences were not significative (Chi= 2.7728, df = 1, P-value = 0.09588). None of the control insects showed PCR amplification.

3.3. Field effectiveness of the marking method and PCR detection

When the effectiveness of the mark was tested in open-field, several predator species were captured on the yellow sticky traps. In total, 102 adult predators were collected in the sticky traps: 35 in the first cut (34 %), 47 in the second cut (45 %) and 21 in the third cut (21%), which belonged to the families Coccinellidae (61%), Anthocoridae (21%), Chrysopidae (12%), and Miridae (6%). Overall, 33 of them (32%) scored positive by PCR for *Artemia* DNA (Table 3), indicating that they had dispersed from the sprayed alfalfa strip to the peach orchard after the alfalfa cuttings.

From the three samplings conducted in the alfalfa field before the sprays, 372 predators were collected, comprising Coccinellidae (*Coccinella septempunctata* L., *Hippodamia variegata* (Goeze), *Propilea* sp., *Scymnus* sp., *Hyperaspis campestris* Herbst, *H. reppensis* (Herbst)), Cantharidae, Anthocoridae (*O. majusculus* (Reuter), *O. laevigatus*, *O. minutus* L., *O. niger* (Wolff), *Anthocoris nemoralis* (Fabricius)), Lygeidae (*Nysius* sp.), Miridae and Aeolothripidae. From the intensive sampling of peach trees facing the sprayed alfalfa, 110 predators were collected. Seventy-five of them were collected before the spray: Coccinellidae (*Propilea* sp., *Oenopia conglobata* L., *H. variegata*, *Stethorus* sp., *Scymnus* sp.), Anthocoridae (*O. albidipennis* (Reuter), *O. minutus*, *A. nemoralis*), Lygeidae (*Nysius* sp.), Dermaptera, Chrysopidae and Syrphidae; and 35 after the spray, thus indicating that they may have moved from the nearby alfalfa: Coccinellidae (*O. conglobata*, *H. variegata*, *Stethorus* sp., *Scymnus* sp.), Anthocoridae (*O. majusculus*, *O. laevigatus*, *O. minutus*, *A. nemoralis*), Lygeidae (*Nysius* sp.), Miridae and Dermaptera.

4. Discussion

Predator movement into crops is crucial to ensure pest control. The present study successfully validates a mark-capture method for dispersal studies, based on spraying a putative source habitat with a solution of the shrimp *Artemia* spp. and detecting its DNA by conventional PCR with specific primers, as previously proposed by Agustí et al., (2020). Our findings demonstrate that spraying such a DNA solution from a species that is not naturally present in the agroecosystem, is able to effectively mark several predator species within a range of very different insect families in an open-field environment.

When spraying stationary insects, more than 77% of those placed on the upper side of the labels were still marked after 6 days in a greenhouse, even when they were located at the bottom of the alfalfa plant canopy, indicating that the backpack sprayer provided a uniform coverage of the plant. The water sensitive spray cards confirmed this. Less individuals were marked on the lower side of the labels and the mark was also lost quicker. For this reason, it is of a great importance to try to spray both surfaces of the leaves when conducting this kind of marking experiments, in order to ensure a correct spray coverage and to reduce untreated parts of the leaves. In addition, at least 50% of those predators that could roam freely on previously sprayed plants were able to self-mark up to 3 days after the spray due to the contact with the residues, indicating that, under field conditions, it is more likely that more insects than those directly sprayed would be able to acquire the mark. On the other hand, it is also possible that some predators are self-marked by feeding on unbroken hydrated cysts of *Artemia*, even if it is expected to show a weak detection by this way, as already stated by Agustí et al. (2020). It is well known that *Artemia* cysts are accepted as prey by some predators, since they are used as supplemental food to sustain populations of several species when establishing in greenhouse crops (Castañé et al., 2006; Labbé et al., 2018; Seko et al., 2019). The fact that in our study the mark could last up to 6 days indicates that this technique is suitable to track local short-term dispersal into fields.

Exposure to direct sunlight in some parts of the plant canopy has been argued to be a cause for degradation of protein markers (Hagler et al., 2014) and it could also be the case with DNA. Nevertheless, in the greenhouse trial the mark persisted for 6 days on those insects located on the upper side of the labels with a high detection percentage (around 80% in both cases: top and middle height in the plant canopy), which was in principle more exposed to the UV light than the lower side of the label. Agustí et al. (2020) reported a similar persistence (6 days) of the *Artemia* solution when sprayed on the whitefly predator *M. pygmaeus* in tomato greenhouses during spring. In the present study, marked insects were also recovered from sticky traps placed in the ecotone between alfalfa and peach. In this case, sprays were conducted during summer months. The study area is a continental interior that features warm to hot dry summers, classified as a cold semi-arid climate (type BSk, Kottek et al., 2006). During the field trial days, mean temperatures and irradiation levels were high (33.6ºC; 22-30 MJ/m^2). Nevertheless, DNA persistence under those conditions was enough to ensure the marking of the insects.

This DNA mark–capture technique proved useful for uniquely tagging the predators inhabiting the alfalfa crop. Overall, 32% (n = 33 out of 102) of all the focal predators captured on the sticky traps showed the DNA mark. As expected, most of the trapped species (except *Stethorus punctillum* (Weise) and *O. albidipennis*) were also captured when sampling the alfalfa crop. In addition, most of those species (*P. quatordecimpunctata*, *H. variegata*, *Stethorus* sp., *Scymnus* sp., *O. albidipennis* and *O. minutus*) were also captured when sampling the peach orchard before the alfalfa cut, indicating that they are also part of the predator complex present in peach. After the alfalfa cuts, some predator species (*H. variegata*, *Scymnus*, *O. majusculus*, *O. laevigatus* and *O. minutus*) were collected again in the peach orchard, indicating that the trap captures confirmed the immigration of common predators into the orchard. Those traps captured five coccinellid species and all of them were represented in the marked individuals. Most of them are aphidophagous, except *S. punctillum* that prey on mites. They are cosmopolitan and commonly found in arable crops (alfalfa and maize) as well as in orchards (de la Poza et al., 2005; Miñarro et al., 2005; Pons et al., 2005, 2009; Dib et al., 2010; Michaud, 2012; Markó et al., 2013; Zhou et al., 2014) which form the crop mosaic in the study area. They all are present in the Iberian Peninsula (Benhadi-Marin et al., 2011). Even if few surveys have been done in peach in the study area, *C. septempunctata* has been cited to be present

(Celada, 2000), as well as in apple, where *Stethorus* and *P. quatuordecimpunctata* are common (Happe et al., 2019). *Coccinella septempunctata* and *P. quatuordecimpunctata* are important BCAs of many important aphid pests, but they can also survive feeding on other alternative prey (e.g. scales, psyllids, lepidopteran eggs or mites) and even on plant materials (e.g. pollen and fruits), moving between trees and herbaceous plants along the season (Hodek and Michaud, 2008; Omkar, 2011; Papachristos et al., 2015). The small *Scymnus* species are still poorly known, but recent papers address their importance also as aphid biocontrol agents (Sebastião et al., 2015). *Stethorus* species have been cited as predators of spider mites (Rott and Ponsonby, 2000; Ragkou et al., 2004), and *S. punctillum* has been also identified as an important predator in peach orchards (Ivancich, 1974).

Four species of *Orius* were also captured in the sticky traps and all of them had some marked individuals. *Orius* spp. are well known predators of thrips, while they can also feed on other pests including aphids, mites, whiteflies and lepidopteran eggs (Riudavets, 1995; Arnó et al., 2008; Atakan, 2010; Bán et al., 2010; Gomez-Polo et al., 2016), some of which can be important pests in peach orchards. All four species are common in several weeds and crops, including orchards (Brown and Schmitt, 2001; Bosco and Tavella, 2013; Pehlivan and Atakan, 2020). More specifically, *O. laevigatus* and *O. niger* have been recorded in peach orchards in the area of study (Avilla et al., 2008; Aparicio, 2019) and *O. minutus* also in peach orchards in France (Remaudière and Leclant, 1971). *Orius niger*, *O. minutus*, *O. majusculus* can be abundant in alfalfa (Pons et al., 2009; Bán et al., 2010) and *O. niger* plays a major role in controlling hemipteran pests in maize (Albajes et al., 2011). *Orius albidipennis* is an efficient predator of the thrips *F. occidentalis* (Blaeser et al., 2004), an important pest of peaches and nectarines. Other predators found in alfalfa in the area of study, as *A. nemoralis, O. majusculus* and *Nysius* sp. (Heteroptera: Lygaeidae) (Pons et al., 2005; Scaccini and Furlan, 2019), were not captured on the sticky traps, but they were collected in the sampling conducted on peach before and after the alfalfa cutting.

The fact that the same predators were collected both in the alfalfa field and the peach orchard indicate that both crops share a similar predator complex and highlights the importance of neighboring crops as a source of predators as BCAs. Our results confirm the contribution of the alfalfa field as a source of predators in the peach orchard, and that repetitive cuts of the alfalfa provided an influx of predators that should contribute to control peach pests. However, not only the alfalfa cuts trigger the dispersal of predators from alfalfa to the adjacent crops. In the area of study, a bidirectional movement of coccinellids (*C. septempunctata, P.*

quatuordecimpunctata, H. variegata) and anthocorids (*O. majusculus, O. niger*) has been documented between arable crops (di Lascio et al., 2016; Madeira et al., 2014, 2019), and the same can also be expected between orchards and arable crops. Predator abundance in apple orchards (*Orius* spp.) seems to depend on the proportion of extensive arable crops over the landscape (Whalon and Croft, 1986), which is also true for other orchards (Markó et al., 2017). Conversely, intensive pesticide applications in orchards has been related with a reduction of *C. septempunctata* abundance in neighboring maize fields (Clemente-Orta et al., 2020). Our results indicate that conserving beneficial fauna in alfalfa favors key predators in fruit orchards. The development of sustainable pest control practices together with a reduction in intensive pesticide applications in fruit orchards should therefore enhance the biological control functions in surrounding arable crops in such mixed landscapes (Markó et al., 2017; Clemente-Orta et al., 2020).

5. Conclusion.

This study proofs the efficacy of a novel DNA topical marking method to identify putative sources of predators colonizing crops and to study dispersal of arthropod species of agronomic interest under natural conditions. Spraying different habitats with different DNA solutions could provide unique tags (El-Sheikha and Menozzi 2019), which should make possible to trace captured insects to their sources and produce more accurate food webs of key predators. Such method offers prospects to be integrated with other molecular approaches in order to improve pest management strategies. For example, the DNA extraction of each predator can be further used to identify the ingested prey, thus determining the contribution of each predator species to the biological control of selected target crop pests (Moreno-Ripoll et al., 2012), or confirm the consumption of plant resources by omnivorous predators (Pumariño et al., 2011; Wang et al., 2017).).

Declaration of Competing Interest

All authors have seen and agree with the contents of the manuscript and declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ivan Batuecas: Conceptualization; Data curation; Formal analysis; Methodology; Validation; Visualization; Writing - original draft; Writing - review & editing. **Nuria Agustí**: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Validation; Writing - original draft; Writing - review & editing. **Cristina Castañe**: Conceptualization; Funding acquisition; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Oscar Alomar**: Conceptualization; Funding acquisition; Investigation; Methodology; Project administration; Validation; Writing - original draft; Writing - review & editing.

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Figure and Table captions

Figure 1. Alfalfa pot with the two labels attached to a stick at two different heights in the plant canopy, and detail of a label. Dead *Orius laevigatus* were glued on both sides and on three sections of the label, each section to be cut after 12h, 3 days and 6 days after spraying.

Figure 2. Percentage of *Orius laevigatus* individuals scoring positive by PCR for the presence of *Artemia* DNA. Dead adult insects were glued on both sides (upper/lower) of labels placed at two heights (25 cm (middle) and 40 cm (bottom) from the top) within the canopy of alfalfa. Plants were sprayed with the *Artemia* DNA solution, and predators were collected after 12h, 3 days and 6 days.

Figure 3. Agarose gel electrophoresis of amplified DNA from *Orius laeviatus* specimens tested in the semi-field trial by PCR using the *Artemia*-specific primers ARTF2 and ARTR3 (146 bp). Lane 1: 100bp molecular-size marker; lane 2: PCR negative control; lanes 3 to 11, *O. laevigatus* placed on the lower side of the labels; lanes 9 to 20, *O. laevigatus* placed on the upper side of the labels.

Table 1. Percentage (%) of PCR amplification of the *O. laevigatus* adults sprayed with two different concentrations (0.1 and 0.01 gr/ml) of the *Artemia* solution, regarding the time elapsed after the spray (h) and the amount of Tween-20 added in the PCR reactions (µl). The number of *O. laevigatus* tested in each case is also indicated (n).

Table 2. Statistical parameters of the percentage of marked *O. laevigatus* adults by the *Artemia* solution after different times after spraying (12h, 3 days, 6 days) on two yellow sticky labels placed at different hights (middle= 25cm, bottom=40cm) from the top of the plant and in both sides of the label (upper, lower).

Table 3. Number of field-collected predators scoring positive by conventional PCR for the topical presence of *Artemia* DNA from the total number tested (N).

Table 1

Time (h)	**Tween-20 (µl)**	**n**	**1gr/ml (%)**	**0.01gr/ml (%)**
0	0	4	100	0
0	1	6	100	66.7
0	2.5	10	100	100
24	2.5	14	100	-

Table 2

Factors	Degrees of freedom	Deviance	Residual Degrees of freedom	Residual Deviance	Pr(>Chi)
Time (12h, 3d, 6d)	2	5.041	9	129.135	0.08041
Height (middle, bottom)	1	2.937	8	126.198	0.08659
Side (upper, lower)	1	119.695	7	6.503	< 2e-16 ***

Table 3

Family	Species	N	Nº Positives
Coccinellidae		**45**	**20**
	Propylea quatuordecimpunctata L.	21	8
	Coccinella septempunctata L.	1	0
	Hippodamia variegata Goeze	4	0
	Stethorus punctillum (Weise)	11	8
	Scymnus sp.	2	1
	Unidentified	6	3
Anthocoridae		**34**	**7**
	Orius niger Wolff	22	4
	Orius laevigatus (Fieber)	3	1
	Orius albidipennis Say	6	1
	Orius minutus L.	3	1
Miridae		**11**	**4**
Chrysopidae		**12**	**2**
TOTAL		**102**	**33**

Figure 1

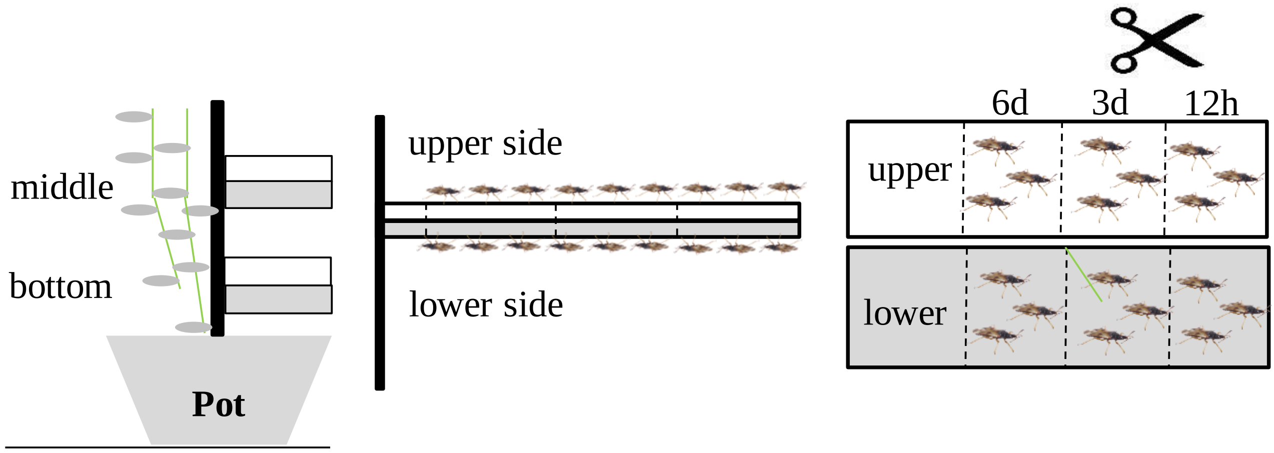
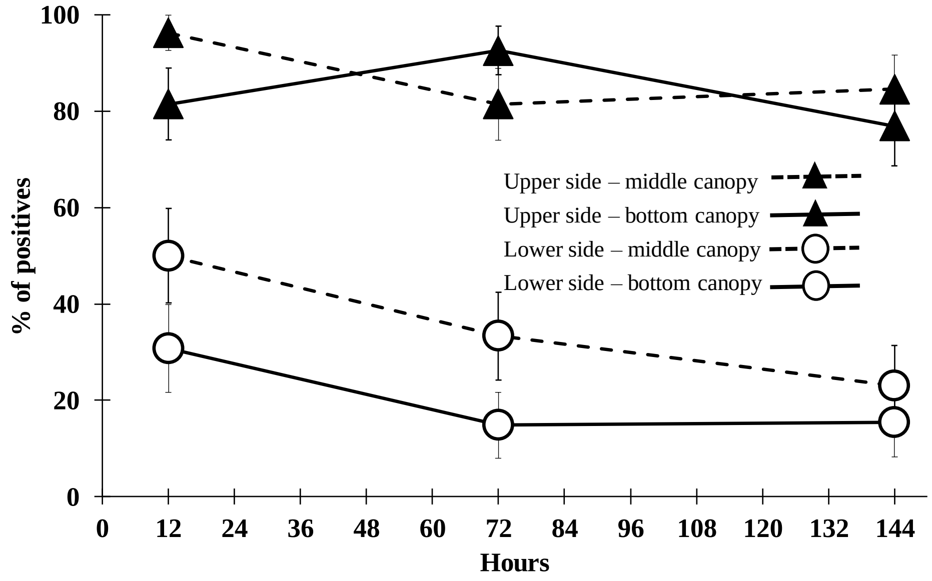
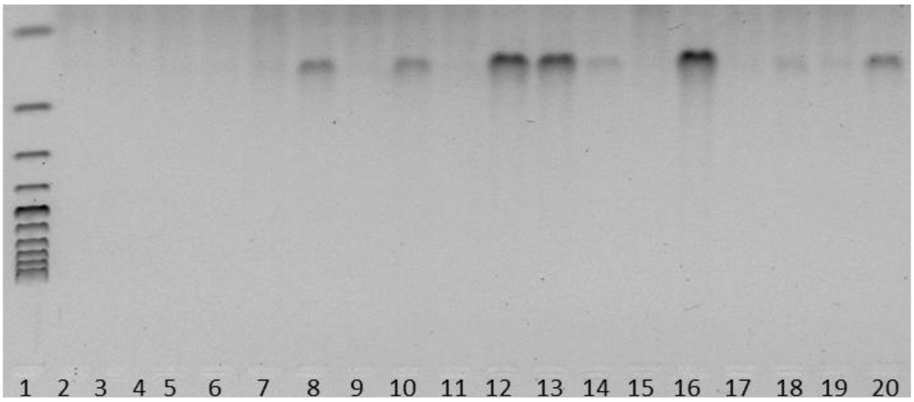

middle
bottom
Pot
upper side
lower side
6d
3d
12h
upper
lower

Figure 2



% of positives
100
80
60
40
20
0
0
12
24
36
48
60
72
84
96
108
120
132
144
Hours
Upper side – middle canopy
Upper side – bottom canopy
Lower side – middle canopy
Lower side – bottom canopy

Figure 3



Highlights

Conservation BC programs require confirmation of predator sources

We optimize a DNA mark-capture procedure to confirm the dispersal of predators

Orius laevigatus was marked for 6 days in the laboratory and in semi-field conditions

In the field, 32 predators were marked on sticky traps placed between crops

Such DNA mark-recaptured procedure has the potential to tag insect source habitats