Development of a PCR-based method to monitor arthropod dispersal in agroecosystems: *Macrolophus pygmaeus* (Hemiptera: Miridae) from banker plants to tomato crops.

Running title: PCR for arthropod dispersal studies

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Abstract

Development of conservation biological control programs requires the identification of sources that contribute to predator colonization of crops. *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) is an efficient polyphagous predator used in biological control programs in vegetable crops in Europe. We have developed a marking method based on spraying with a solution of the brine shrimp *Artemia* spp. (Anostraca: Artemiidae) cysts, followed by a PCR detection of *Artemia* DNA to monitor *M. pygmaeus* dispersal from banker plants to tomato crops. Experiments conducted in climatic chambers show that the topical application of this marking solution on *M. pygmaeus* does not significantly reduce adult longevity and that it is detected up to 6 days after the application. When this *Artemia* solution was applied on *Calendula officinalis* L. banker plants harbouring *M. pygmaeus* and maintained outdoors, *Artemia* DNA was still detected on 62% of the insects after 6 days. The conducted field applications in commercial greenhouses have confirmed the usefulness of this method to monitor *M. pygmaeus* dispersal from banker plants to a newly planted tomato crop. This method can be used to assess arthropod movement, being an interesting molecular approach for further improving future pest management strategies.

Keywords: *Macrolophus pygmaeus*, *Artemia*, PCR analysis, arthropod dispersal, *Calendula officinalis*, tomato crop.

INTRODUCTION

Conservation Biological Control (CBC) of arthropod pests by habitat management is based on maintaining indigenous natural enemies in the agroecosystem by adopting measures to improve their survival and reproduction. For this, it is essential to understand their dispersal patterns, habitat preferences and spatial distribution by tracking their movements (Corbett, 1998; Thomas, 2001). A wide variety of marking techniques, like paints, inks, dyes, powders or rare and trace elements (e.g. rubidium), have been developed over the years to study the dispersal patterns of many arthropods (Lavandero *et al*., 2004a; Madeira & Pons, 2016; Di Lascio *et al*., 2016). They are effective, but most of them require a strong effort to capture and mark all the specimens, and/or they are relatively expensive and time consuming (Akey *et al*., 1991). Arthropod-marking methods using vertebrate-derived immunoglobulin G (IgG) proteins have also been applied directly in the field, avoiding a previous capture and simplifying the process (Jones et al. 2006; Hagler 2019).

Macrolophus pygmaeus (Rambur) (Hemiptera: Miridae) is an efficient generalist predator used in biological control programs to control pests of vegetable crops in Europe. Inoculative releases of this species are currently applied in commercial greenhouse tomato crops to control the whiteflies *Bemisia tabaci* Gennadius and *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae), as well as some lepidopteran pests, as the tomato borer *Tuta absoluta* Meyrick (Alomar *et al*., 2006a; Moreno-Ripoll *et al*.,2012a; Moreno-Ripoll *et al*., 2012b; Moerkens *et al*., 2017; Urbaneja *et al*., 2012). *Macrolophus* predators found in Mediterranean tomato crops were initially identified as *M. caliginosus* Wagner (now *M. melanotoma* (Costa)), but latest taxonomic studies have demonstrated that in fact, they were *M. pygmaeus* (Martinez-Cascales *et al*., 2006; Castañé *et al*., 2013).

In the Mediterranean Basin, abundant naturally occurring populations of *M. pygmaeus* are found on several non-crop host plant refuges from where they move into field and greenhouse vegetable crops (Alomar *et al*., 1994; Alomar *et al*., 2002; Lykouressis *et al*. 2000; Tavella & Goula, 2001; Gabarra *et al*. 2004; Castañé *et al*. 2004; Ingegno *et al*. 2009). Therefore, it is important to identify the relative contribution of these host plant refuges in the colonization of *M. pygmaeus* to crops. The use of PCR-

based approaches, like microsatellites, have been used to determine the genetic structure of *M. pygmaeus* populations (Sanchez *et al*. 2012), as well as to confirm movement of predators between greenhouses and the surrounding vegetation (Streito *et al*. 2017) which had previously been demonstrated with interception traps (Gabarra *et al.,* 2004; Castañé *et al.,* 2004). Detection of consumed plant DNA by PCR has also been used to reveal the movement of a mirid bug between crops (Wang *et al.*, 2017). However, those methods do not specifically target selected refuge plants to be used in CBC programs.

The aim of this study is to develop a new PCR-based method to study dispersal patterns between habitats. The proposed marking method consists in spraying refuge banker plants hosting the target insects with an aqueous solution of grinded arthropods not found in terrestrial agroecosystems, followed by a conventional PCR for its DNA detection. The use of banker plants that allow the conservation and reproduction of predators is a viable strategy to keep them in the greenhouse during crop-free periods and to enhance early establishment on the new crops (Arnó *et al*. 2018; Payton Miller & Rebek, 2018). One of the main hosts plants of *M. pygmaeus* is the common marigold, *Calendula officinalis* L., which is widely distributed (Tavella & Goula, 2001; Alomar *et al.,* 2006b), and it has been proposed as a companion plant for this predator (Lambion *et al.,* 2014; Balzan, 2017; Messelink *et al*., 2014).

In this study, we have used *Artemia* spp. (Anostraca: Artemiidae) for insect-marking, which is a small shrimp that lives in saline waters exclusively. This aquatic arthropod species produces dormant embryos (cysts), which are commonly used as food source in fish larval rearing, as well as in mass rearing of insect predators, such as *M. pygmaeus* (Castañé *et al.,* 2006; Vandekerkhove *et al*., 2009). The novelty and usefulness of this kind of marking method to identify the source of predators that colonize greenhouse tomato crops is tested in this study by spraying established populations of *M. pygmaeus* on *C. officinalis* banker plants located in commercial tomato greenhouses. It is expected that this new approach, reliable and environmentally safe, based on the DNA detection of an organism non-present in an agroecosystem, would be useful for further improving current CBC programs. The identification of key predator refuges in adjacent non-crop habitats will contribute to the optimization of managed wildflower strips close to the crops, as stated by Gontijo (2019).

MATERIALS AND METHODS

Insects

All *M. pygmaeus* used in the laboratory experiments were reared at IRTA's facilities (Cabrils, Spain) as described in previous studies (Agustí & Gabarra, 2009a; Agustí & Gabarra, 2009b). They were maintained on tobacco plants (*Nicotiana tabacum* L., cv. Brasilian blend) under controlled conditions at 25 ± 1 ºC, 70 ± 20% RH and with a L16:D8 photoperiod. They were fed exclusively with *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs. Colonies were renewed every year with introductions of new tomato field-collected insects from the same area. All the following laboratory experiments were performed under the same controlled conditions.

Marking solution preparation and application

A solution prepared with dry commercial *Artemia* spp. cysts (Inve Aquaculture, Inc.) at a concentration of 0.1 g/ml of distilled water was used for marking plants and insect specimens. For this, dry cysts were grinded for 60 seconds in a standard coffee grinder in order to make the *Artemia* DNA more accessible. Then, the broken cysts were hydrated with distilled water for 30 min, filtered with an 8 threads/mm mesh and brought to the final volume with distilled water. The obtained aqueous solution was maintained in a refrigerator and used in the following 24h.

The marking process consisted in spraying the solution directly to the *M. pygmaeus* specimens used in the experiments with a regular handheld sprayer, or to the plants containing *M. pygmaeus* with a compressed air sprayer (Matabi Berry 5, Goizper Spraying, Spain). Spraying was done from a distance of 10 cm to the insect or plant until run-off (ca. 0.25 liter/plant on average).

PCR detection of marked specimens: primer design and specificity

Each *M. pygmaeus* was DNA extracted using the Speedtools Tissue DNA Extraction Kit (Biotools, CA, USA) following the manufacturer protocol. The whole body was used in all cases, and 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. Then, each *M. pygmaeus* was analyzed by conventional PCR using a pair of *Artemia*-specific primers designed from the mitochondrial Cytochrome Oxidase I (COI) region using the same procedure described in a previous study (Agustí *et al*., 2003). Each *M. pygmaeus* specimen was tested up to 3 times and considered positive if *Artemia* DNA was detected in at least one of them, as conducted in previous studies (Gomez-Polo *et al*., 2015, 2016; Moreno-Ripoll *et al*., 2012a).

For the design of these *Artemia*-specific primers the following sequences of *Artemia* species and populations from the GenBank database (www.ncbi.nlm.nih.gov) were used: EU543474 (*A. salina* from Italy), EU543450 (*A. salina* from Spain), EU543473 (*A. salina* from Cyprus), EU543485 (*A. salina* from SouthAfrica), EF615583 (*A. franciscana*), DQ119646 (*A. franciscana*), GU248378 (*A. franciscana*), HM998997 (*A. parthenogenetica*), HM998995 (*A. parthenogenetica*), HM998993 (*A. parthenogenetica*), EF615589 (*A. tibetiana*), EF615586 (*A. tibetiana*). Besides of the sequence of *M. pygmaeus/melanotoma* (FM210178; Pasquer *et al*., 2009), the sequence of another polyphagous predator also used in CBC programs in the Mediterranean area (*Orius laevigatus* L. (Hemiptera: Anthocoridae); FM210187) was used as well. All these sequences were aligned using CLUSTALW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) and compared for the design of an *Artemia*-specific pair of primers.

All PCR reaction volumes (25 µl) contained 4µl of DNA extract, 0.65U of Taq DNA polymerase (Invitrogen, CA, USA), 0.2 mM dNTPs (Promega Biotech Corporation, WI, USA), 0.6 µM of each primer and 7 mM MgCl2 in 10× manufacturer's buffer. Amplifications were conducted in a 2720 thermocycler (Applied Biosystems, CA, USA), where the samples were subjected to 94 ºC for 2 min, followed by 35 cycles of 94 ºC for 30 s, 66 ºC for 30 s, and 72 ºC for 45 s and a final extension of 72 ºC 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.

The designed *Artemia*-specific primer pair was tested by conventional PCR with 2-5 individuals of the most common arthropod species present in vegetable crops in the area of study, which could be potentially ingested by *M. pygmaeus*, as well as with some other predator species present in the same area of study. The arthropod species included

in this specificity test were: the pests *Macrosiphum euphorbiae* Thomas and *Myzus persicae* Sulzer (Hemiptera: Aphididae); *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae); *Bemisia tabaci* Gennadius and *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae); *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae); *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae); and *Tetranychus urticae* (Koch) (Acari: Tetranychidae); and the predators *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae), *O. laevigatus, Macrolophus costalis* (Hemiptera: Miridae), *M. melanotoma* and *M. pygmaeus*.

Characterization of the marking method

Several tests were conducted in order to confirm the usefulness and safety of the *Artemia* solution as a marker for dispersal studies. First of all, in order to test whether the *Artemia* solution had phytotoxic effects, four shoots of *C. officinalis* were soaked for 30 seconds in three different concentrations of the *Artemia* solution: 1) the regular solution (0.1 g/ml); 2) a 1:2 dilution of the regular solution; and 3) a 1:4 dilution of the regular solution in distilled water. Distilled water was also tested as a negative control. Then, the treated shoots were visually checked for any sign of phytotoxicity, like changes in leaf colour, turgidity, injuries or blemishes after 3, 5, 7, 10 and 25 days.

To test whether the spray with the *Artemia* solution would reduce the survival of *M. pygmaeus*, 36 one-day old adults were placed on two tobacco plants in acrylic cages (20 cm diameter x 40 cm height) and sprayed with the *Artemia* solution. After that, *E. kuehniella* eggs were added as food and dead and alive insects were counted every 3 days until all died. Every 15 days the remaining insects were transferred to new unsprayed plants in order to avoid the presence of newly emerged nymphs of the following generation. The longevity of 29 non-marked adults was also tested as a control using a similar setting, but without spraying the plants. Longevity results were pooled, and mean longevities obtained for sprayed and non-sprayed insects were compared by Student's *t*-test.

In order to discard differences of the marking due to the gender, 35 females and 25 males of *M. pygmaeus* (7-14 days old) were used. They were placed in an acrylic cage (20cm diameter x 40cm height) on *C. officinalis* plants and sprayed with the *Artemia*

solution. Once they were dry, they were analyzed with the *Artemia*-specific primers in order to determine the proportion of marked individuals of each gender.

Another experiment was conducted to determine whether the *Artemia* DNA detection was possible after the imaginal molting. For this, 10 5th-instar nymphs of *M. pygmaeus* were first sprayed with the *Artemia* solution and then maintained on green bean pods (*Phaseolus vulgaris L.*) with *E. kuehniella* eggs as food. After 24h those nymphs that had molted to adults were analyzed by PCR in order to determine the percentage of adults marked after molting.

In order to evaluate the persistence of the topical mark in the climatic chamber and under field conditions, *M. pygmaeus* adults were placed on two *C. officinalis* plants, sprayed with the *Artemia* solution and then put in an acrylic cage each (49 x 39 x 47 cm) with *E. kuehniella* eggs as food. One cage was kept inside the climatic chamber (25 ± 1 ºC and L16:D8 photoperiod) and the other one was placed outdoors (23.5 ± 3.9ºC and L16:D8 photoperiod; July conditions in our area). After that, *M. pygmaeus* collected just after spraying with the *Artemia* solution (t=0) and after 3 and 6 days (n=20 *M. pygmaeus*/time) from the cage maintained in the climatic chamber were analyzed by PCR. Regarding the cage maintained outdoors, PCR analyses were conducted with *M. pygmaeus* collected after 1, 3 and 6 days after spraying (n=10 *M. pygmaeus*/time).

Because some of the *Artemia* cysts in the *Artemia* solution might be still unbroken even after grinding and filtering, the capability of *M. pygmaeus* for self-marking by the ingestion on those unbroken cysts was also tested. For this, 10 7-days old *M. pygmaeus* females were starved for 48h, individually placed in small Petri dishes (2.5 cm diameter), and offered 20 hydrated *Artemia* cysts over a period of 90 min. Afterwards, those predators which had fed 5-9 *Artemia* cysts were immediately (t=0) frozen at –20°C for subsequent PCR analysis. Additionally, 10 predators were also starved for 48h and frozen to be tested as negative controls.

Marking and dispersal evaluation in commercial tomato greenhouses

The field persistence of the *Artemia* marking and the dispersal of *M. pygmaeus* from *C. officinalis* banker plants to a newly planted tomato crop was evaluated in 3 commercial greenhouses (A, B and C) located in El Maresme area (Barcelona, Spain). In order to

ensure that the *C. officinalis* plants harbour well-established *M. pygmaeus* populations, those potted *C. officinalis* plants were previously placed in summer tomato greenhouses before the final harvest and left to be naturally infested by *M. pygmaeus* for three months after the tomato harvest, that is to say until the next tomato crop was planted in the following February. Those *C. officinalis* plants were placed as follows: 5 patches of 4 pots in greenhouse A (1800 m²), 7 patches of 4 pots in greenhouse B (1130 m²), and 1 patch of 7 pots in greenhouse C (920 m²). When more than one patch was set-up, they were separated from each other in order to minimize the overlap of individuals dispersing from each patch. Patches were placed in the middle of the greenhouses and separated from each other in order to minimize the possible colonization from outside and cross contamination between patches.

In order to determine the persistence of the *Artemia* marking under greenhouse conditions, all *C. officinalis* plants of greenhouse A were sprayed with the *Artemia* solution few days after the tomato planting. All sprays were carefully conducted by surrounding the *C. officinalis* patches with a 1.5m high plastic fence to avoid spraying the closest tomato plants and to minimize *M. pygmaeus* escapes towards them. Afterwards, 20 *M. pygmaeus* were collected from the sprayed plants at t = 0 (just after 2h, when they were dry), and after 3 and 6 days and analyzed by PCR with the *Artemia*-specific primers.

In order to verify the usefulness of this marking method to study the dispersal of *M. pygmaeus* to the newly planted tomato crop, all *C. officinalis* patches of greenhouse B were sprayed 3 times at 3-day interval (Fig. 1). In order to determine whether more frequent sprays of the calendula plants would increase the percentage of marked individuals, the calendula patch of greenhouse C was sprayed 3 times at 1-day intervals (Fig. 1). Before the first spray of the *C. officinalis* plants with the *Artemia* solution, all *M. pygmaeus* present on the newly transplanted tomato plants were manually removed. After spraying, neighbouring tomato plants were sampled (5 plants per row on the 6 rows closest to each of the 7 *C. officinalis* groups of greenhouse B; 11 plants per row of the 8 rows closest to the only *C. officinalis* group of greenhouse C). The sampling distances in both greenhouses differed due to the different tomato plant spacing, and covered ca. 2.5 meters on either side of the calendula patches. All *M. pygmaeus* from these tomato plants were collected three days after each spray in greenhouse B and one day after the last banker plant spraying in greenhouse C (Fig. 1). *Macrolophus. pygmaeus* adults were also collected from the sprayed *C. officinalis* plants following the last spray in both

greenhouses; specifically, at the 9th day in greenhouse B and at the 3rd day in greenhouse C (Fig. 1). Additional random samples were also collected in other tomato plants close to the openings of greenhouses B and C to confirm that there was no colonization from outside and the lack of dispersion of *M. pygmaeus* further away. In order to avoid any risk of cross-contamination between insect specimens, the laboratory tips and the mesh filters of the mouth aspirator were replaced for each captured specimen. After collection, all mirid bugs were individually placed in 1.5 ml tubes and stored at -20ºC until PCR analysis with the *Artemia*-specific primers.

RESULTS

PCR Detection of marked specimens: primer design and specificity

A pair of degenerated *Artemia*-specific primers was designed (ARTF2: 5´-CYTCHGCYATTGCYCATGCYGGRCCTT-3´ and ARTR3: 5´-GYAYVCGRTCRAYRGAYATYGMYKGRG-3´) to amplify a fragment of 146 bp of *Artemia* spp. These primers together with the developed PCR protocol showed a successful amplification of the commercial *Artemia* cysts. Additionally, when these primers were tested for cross-amplification against the most common tomato pests and predators species (8 pests and 5 predators) present in our area, none of them was amplified in any case, showing a high specificity for our crop setting.

Characterization of the marking method

All *C. officinalis* shoots soaked in the three different concentrations of the *Artemia* marking solution (0.1 g/ml; 1:2 dilution and 1:4 dilution) had the same appearance as the controls, which were soaked in distilled water, after 3, 5, 7, 10 and 25 days after soaking. No changes in leaf colour, turgidity, injuries or blemishes were observed.

Spraying with the *Artemia* solution did not significantly reduce the mean longevity of the *M. pygmaeus* adults when compared to control adults sprayed with water (15.9 ± 1.48 and 20.9 ± 2.46 days respectively) ($t = 1.73$; $df = 47.07$; $P = 0.089$).

When *M. pygmaeus* males and females sprayed with the *Artemia* solution were analyzed by PCR, all of them (100%) gave a positive detection, showing no differences with respect the marking efficacy on both genders. On the other hand, no *M. pygmaeus* adult showed *Artemia* DNA amplification after being sprayed with the *Artemia* solution as 5th-nymphal instar, indicating the loss of the marking after the imaginal molting.

PCR analyses of *M. pygmaeus* sprayed with the *Artemia* solution and maintained in the climatic chamber showed that they were still all marked even after 6 days (100% detection). On the other hand, the detection of the sprayed adults from the cage placed outdoors decreased with time, changing from 100% after 1 day, to 90% after 3 days and 62% after 6 days.

When 10 *M. pygmaeus* females that had fed the contents of 5-9 *Artemia* cysts were tested by PCR just after eating (t=0), a faint band was obtained in 4 of them indicating that the *Artemia* DNA could be also detected by ingestion, even if this detection may be lost with time. All control starved predators were negative when tested by PCR with the *Artemia*-specific primers.

Marking and dispersal evaluation in commercial tomato greenhouses

PCR analysis with the *Artemia*-specific primers showed that 70% of the *M. pygmaeus* adults collected on *C. officinalis* in greenhouse A just after spraying with the *Artemia* solution (t=0) were marked. This percentage decreased to 50% and 10%, 3 and 6 days after spraying, respectively (n=20/time). In greenhouses B and C, 55% of the *M. pygmaeus* adults collected on *C. officinalis* (n = 20/greenhouse) at the end of each trial were marked (Table 1, Figure 1). *M. pygmaeus* dispersed to the crop at an average rate of 0.38 adults per tomato plant per three days (Table 1).

When all *M. pygmaeus* collected on the newly transplanted tomato plants in greenhouses B and C were analyzed by PCR, the percentage of marked individuals in greenhouse B after repeated sprays of the *C. officinalis* plants increased with time from 4.3 to 30.8% (Table 1). In greenhouse C, after 3 sprays in 3 consecutive days, 12% of the *M. pygmaeus* collected on tomato plants were marked. No *M. pygmaeus* was recorded on tomato plants close to openings of the greenhouse, indicating that all collected predators came from the calendula banker plants.

DISCUSSION

This study demonstrates the usefulness of a topical marking method made from an aqueous solution of an arthropod not present in terrestrial agroecosystems followed by a conventional PCR method for its DNA detection to study arthropod dispersal patterns. In this case, an *Artemia* spp. solution has been used as topical marker for tracking *M. pygmaeus* movement from *C. officinalis* to a tomato crop, showing that these predators effectively move from their refuge plants to the newly planted tomato crop. At the same time, the use of banker plants to maintain predators in the greenhouses during crop-free periods is also demonstrated, as well as their fast colonization of tomato plants after planting the new crop.

When this *Artemia* solution was tested on *C. officinalis,* no phytotoxic effects were detected, confirming its safety, at least on this plant species. On the other hand, when the efficacy of the marking method was evaluated on *M. pygmaeus*, *Artemia* DNA was detected on all adults tested, and without significantly reducing their longevity. Nevertheless, this mark was not detected on any adult emerged from sprayed 5th-instar nymphs, indicating that it is lost with molting. Even if protein markers have been described to show variable detection rates after molting (Hagler & Jackson, 2001), other marking techniques, like paints and dyes are also lost when the insects molt (Lavandero *et al.,* 2004b). This, which can be negative because it reduces the number of marked specimens, could be useful as the identification of putative predator sources will be only based on newly sprayed adults dispersing into the crop.

Because the *Artemia* solution used in this study was sprayed directly on the plant, it may be possible that *M. pygmaeus* was also marked by feeding on potential unbroken hydrated cysts, as demonstrated previously (Castañé *et al.,* 2006). Our results indicate that this is possible, however it has been previously demonstrated that this predator degrades ingested DNA within the following few hours of consumption (Pumariño *et al.,* 2011). Therefore, it is expected that such a low self-mark would only be added to the topical marking not affecting the dispersal detection of *M. pygmaeus*.

Under laboratory conditions, all tested mirid bugs were still marked for 6 days after spraying the *Artemia* solution, which is a relatively long detection period compared

with other methods, and similar to those obtained by protein immunomarking. When protein markers were tested in the laboratory on *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), egg albumin persisted on all flies for 7 days after the application, decreasing to 94% after 14 days; milk casein was detected on 65% of specimens on day 1 declining to 50% on day 3, after which it remained relatively constant (49% after 14 days); and soy trypsin detection decreased quickly after 1 day to less than 10% after 14 days (Klick *et al.,* 2014). Comparing these results with the percentages obtained here when analysing sprayed *M. pygmaeus* adults from the cage placed outdoors, it is observed that detection also decreased with time from 100% after 24h, to 90% after 3 days and 62% after 6 days, showing efficacies in the same range. It is also observed that this outdoor persistence was lower than that maintained in the climatic chamber (62% and 100%, respectively). This was expected due to the well-known effect of the ultraviolet (UV) radiation on the DNA degradation, even used as a DNA decontaminating agent (Sarkar & Sommer, 1990; Ou *et al*., 1991; Thacker *et al*., 2006), which was not emitted by the climatic chamber lights (Sylvania Luxline Plus F58W/840 Cool White De Luxe, Germany). Exposure to direct sunlight in parts of the plant canopy has also been argued to be a cause for degradation of protein markers (Hagler *et al*., 2014). The other climatic conditions (temperature and RH) were very similar.

When detection percentages of *M. pygmaeus* on *C. officinalis* maintained in a cage outdoors were compared with those maintained in the greenhouse A, lower percentages were obtained in the greenhouse (70%, 50% and 10% after 1, 3 and 6 days, respectively). There would be three main reasons for this lower detection in the greenhouse. While the *C. officinalis* plants placed in the cage were small, the *C. officinalis* banker plants placed in the greenhouse had been growing during winter, providing more shelter for *M. pygmaeus,* so that the spray might not reach all predators. The importance of plant canopy growth has also been considered as a factor for a different efficiency of protein markers (Lucia *et al*., 2018). Also, while those *M. pygmaeus* in the outdoor cages were only adults, in the greenhouse there was a well-established population with adults and nymphs. As a result, those individuals sprayed when they were nymphs, may have lost the marking when molted to adults, further decreasing the percentage of marked adults on the plant. Finally, the lower detection obtained in the greenhouse was also due to the movement of the marked *M. pygmaeus* adults to the new tomato crop.

Based on these results, in order to confirm the effectiveness of the marking method to study dispersal and to provide *M. pygmaeus* adults with sufficient time to disperse to the crop, we sampled the tomato plants three days after spraying the calendula plants. In greenhouse B, marked *M. pygmaeus* adults were recovered from the tomato plants in each of the three consecutive sprays, confirming the usefulness of this method to study dispersal. The percentage of marked predators increased with time (4.3%, 18.8% and 30.8%), presumably because recently molted adults were freshly marked again.

In greenhouse C, after spraying the calendula patches 3 times on a daily basis (Fig. 1), 12% of the *M. pygmaeus* recovered from the tomato plants were marked. This is higher than the 4.3% obtained after just one spray, as expected because such frequent consecutive sprays on the same patch marked those previously unmarked adults, as well as the newly moulted adults. However, considering that spraying at 3 day intervals also provides a good estimate of predator dispersal, there is a risk that repeated sprays may disturb too much the adults. In both greenhouses, half of the *M. pygmaeus* collected on the calendula banker plants were marked after the last spray, indicating that with such levels of marked adults at the source it is possible to verify dispersal.

The efficacy of some protein markers (egg albumin and milk casein) has been also evaluated in an alfalfa field on several arthropod species (Sivakoff *et al*., 2012). In that study, the field was sprayed with both proteins and arthropods were collected 24h later. Serological analysis showed that the efficacy on five arthropod species ranged from 32% to 100% for egg albumin and from 15% to 83% for milk casein. Our results are within the range of variation of proportions of marked individuals obtained after applying protein solutions to source refuges obtained in recent papers (Lucia *et al*., 2018; Irvin *et al*., 2018; Leach *et al*., 2018).The observed rates of 0.38 adults per tomato plant (Table 1) represent a continuous flow of approximately 1.6 adults / m^2 every three days, in the ranges of recommended commercial preventive releases (between 0.25 and 0.5 adults / m^2, repeated 2 to 4 times at 1 to 2 weeks intervals) (Moerkens *et al*., 2017). *Macrolophus pygmaeus* should be released as soon as possible after planting in order to ensure fast establishment and control of pest populations (Arnó *et al*., 2018). Our results confirm the usefulness of using banker plants in order to conserve predators during crop-free periods and ensure an early and quick crop colonization soon after transplant. *Calendula officinalis* has also been shown to contribute to the establishment of other predators in greenhouses (Zhao *et al*., 2017). Such open rearing units ensure predator persistence in the crop at low pest

populations (Messelink *et al*., 2014). The dispersal of natural enemies must be considered when choosing the optimal spatial arrangement of banker plants in a greenhouse. To exploit the dispersal capacities of *M. pygmaeus*, these results indicate that calendula banker plants could be separated 5m between rows. In order to maximize the establishment, plants might not be placed in patches, but evenly spaced along the rows.

The PCR-based methodology developed in the present study is simple and effective, which makes it a promising marking alternative method to study movement of arthropod species. It is also fast and highly sensitive because is able to amplify very small amounts of *Artemia* DNA on the marked insects. We have already used this methodology in a field study to assess predator movement from alfalfa to a peach orchard with success (unpublished data). The reliability of this method is based on the fact that insects can only acquire this mark when sprayed, because *Artemia* is not present in terrestrial ecosystems. *Artemia* spp. dry cysts are commercially available, and the *Artemia* solution prepared as described here is easy to prepare and to apply on the plants, being also environmentally safe. Further investigations should be conducted in order to study the ability of the studied arthropods to self-mark with the *Artemia* solution by walking on a previously sprayed plant (with *Artemia* residues) (Jones *et al.,* 2006). Although the cost of this preliminary marking method using *Artemia* cysts as explained here would be around 15€/liter nowadays, further development should decrease the cost as it happened with other methods. Reducing the concentration of the *Artemia* cysts in the marking solution in order to decrease costs should be further investigated.

The use of such a molecular approach to identify putative sources of predators colonizing crops may be advantageous as it can be integrated with other approaches within the same study. For example, the same DNA extraction can be also used to identify ingested prey, thus determining their contribution to the biological control of crop pests (Moreno-Ripoll *et al.,* 2012a) and it could be also used for the analysis of ingested plant DNA to verify the movement of insects between crops (Pumariño *et al*., 2011; Wang *et al*., 2017). The equipment needed for DNA analysis is modest (a regular termocycler and an electrophoresis equipment), and an available routine equipment in most entomology laboratories.

In conclusion, this study developed a method for arthropod marking with a solution of a species not present in terrestrial ecosystems followed by a PCR-based

detection method of its DNA to study arthropod dispersal in agroecosystems. This method can be used to assess intercrop movement, as well as among crop and non-crop habitats. Our findings herein provide direct evidence of the movement of *M. pygmaeus* adults from *C. officinalis* banker plants to a newly planted tomato crop, but this methodology can be used to study dispersion of other arthropod species of agronomical interest under natural conditions, which could improve future pest management strategies.

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611

612 **Table 1.** Percentages of PCR detection of *Artemia* DNA when analyzing *Macrolophus*
613 *pygmaeus* collected in greenhouses B and C. In the table is also indicated the plant species
614 where those *M. pygmaeus* were collected on; the number of sprays conducted on the *C.*
615 *officinalis* that hosted *M. pygmaeus*; the number of days elapsed since the 1st spray; and
616 the number of *M. pygmaeus* analyzed by PCR. *Calendula officinalis* plants were sprayed
617 3 times every 3 days (greenhouse B) and every day (greenhouse C).

Greenhouse	Plant	Number of sprays	Number of days after 1st spray	Number of analysed *M. pygmaeus*	% PCR detection	*M. pygmaeus* departure rate (1)
B	*C. officinalis*	3	9	20	55.0	-
	Tomato	1	3	70	4.3	0.33
	Tomato	2	6	69	18.8	0.33
	Tomato	3	9	65	30.8	0.31
C	*C. officinalis*	3	3	20	55.0	-
	Tomato	3	3	50	12.0	0.57

618 (1) *M. pygmaeus* departure rate has been calculated dividing the number of collected predators on tomato
619 by the number of sampled tomato plants on each greenhouse. At an average planting of 4 tomatoes/m^2, the
620 resulting mean rate of 0.38 adults/plant, is 1.6 adults/m^2 every three days.

621

Figure 1. Diagram of the sprays conducted in greenhouses B and C with the *Artemia* solution over time. The moments in which *Macrolophus pygmaeus* were collected either from *Calendula officinalis* or from adjacent tomato plants are also indicated.