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Advances in aphid sustainable control

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To my Family

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Abstract

The need to manage harmful arthropods for crops in a sustainable manner has led to the development of Integrated Pest Management (IPM). This strategy uses all available pest control tools, to reduce as much as possible the use of pesticides. These can cause lethal and sublethal toxicity toward target pests as well as on non-target organisms, e.g., beneficials. The aim of this theses was to estimate the potential of naturally derived substances and chemical insecticides in controlling a key polyphagous aphid pest, Aphis gossypii*, and their side effect on its main parasitoid,* Aphidius colemani. *Because natural enemies deserve attention for the eco-toxicological evaluation, firstly a potential optimized rearing for the coccinellid predator* Harmonia axyridis *was established, then the effects of three synthetic insecticides at sublethal doses were assessed on its functional response to prey density. This predator is also considered an invasive alien species, thus we assessed the relationship between the coccinellid and its main parasitoid* Dinocampus coccinellae *by determining the parasitism and the phylogeography through native and invaded areas. The bioassays revealed that botanical extracts can be promising tools for controlling* A. gossypii *with different selectivity towards the parasitoid. Systemic insecticides at sublethal doses can control effectively the pest and impair differently the physiology and the behaviour of natural enemies. The geographic area influenced the parasitism rate on* H. axyridis. *European and American native coccinellids were highly attacked* by D. coccinellae *independently by the sampled area. Parasitoid phylogenetic analyses highlighted variations in parasitoid COI sequences, while no differences in 16S rRNA region. Altogether these results provide new* *knowledge useful for implementing sustainable IPM programs against aphid pests.*

Keywords
Aphis gossypii; *Aphidius colemani*; biological control; *Dinocampus coccinellae*; essential oils; functional response; *Harmonia axyridis*; invasive species; neonicotinoid; phylogeography; sublethal effects.

Sommario

La necessità di controllare gli artropodi dannosi alle colture in modo sostenibile, e quindi di ridurre l'uso di pesticidi, ha portato allo sviluppo di piani di gestione integrata (IPM), attraverso l’integrazione di tutti gli strumenti di controllo disponibili. Gli insetticidi possono causare tossicità letale e subletale sugli organismi bersaglio e non-bersaglio come i nemici naturali. Il presente lavoro di tesi ha valutato il potenziale insetticida di alcuni insetticidi di sintesi e di origine naturale nei confronti dell’afide Aphis gossypii*, nonchè i loro effetti collaterali sul parassitoide* Aphidius colemani. *Inoltre, poiché i nemici naturali meritano attenzione per le valutazioni eco-tossicologiche, è stato prima stabilito un protocollo di allevamento per il predatore* coccinellide Harmonia axyridis*, quindi sono stati valutati gli effetti di tre insetticidi sintetici a dosi subletali sulla sua risposta funzionale. Considerato che questo predatore è anche considerato una specie invasiva, è stata valutata la relazione tra il coccinellide e il suo principale parassitoide,* Dinocampus coccinellae*, determinando il tasso di parassitizzazione e la sua filogeografia in diverse aree geografiche. I risultati indicano che gli estratti botanici sono strumenti promettenti per il controllo dell’afide e che hanno una diversa selettività nei confronti del parassitoide. Gli insetticidi sistemici a dosi subletali possono controllare efficacemente il fitofago e influenzare in modo diverso la fisiologia e il comportamento dei nemici naturali. L'area geografica ha influenzato il tasso di parassitizzazione di* H. axyridis *e, i coccinellidi nativi europei e americani sono stati parassitizzati indipendentemente dall'area campionata. Le analisi filogenetiche hanno inoltre evidenziato variazioni nelle sequenze COI dei parassitoidi. Complessivamente questi risultati forniscono nuove conoscenze utili per* *l'implementazione di programmi di lotta integrata sostenibile contro gli afidi.*

Parole chiave

Aphis gossypii; Aphidius colemani; controllo biologico; *Dinocampus coccinellae*; effetti subletali; filogeografia; *Harmonia axyridis;* neonicotinoidi; olii essenziali; risposta funzionale; specie invasive.

General introduction

Aphids are a group of small sap-sucking insects that belong to the superfamily Aphidoidea, some of which can be serious agricultural and forestry pests. Aphids mostly occur in the temperate regions of the world and their diversity is more expressed through polyphenism than speciation, this explains the relatively few numbers of aphid species compared to other insect groups (Dixon 2012).

Aphis gossypii Glover (Hemiptera Aphididae) commonly known as the cotton aphid, is a small, spread and adaptable aphid that causes severe plant injury to numerous host plants. The economic damage caused by *A. gossypii* is mainly due to direct feeding, the excretion of honeydew and virus transmission. The cotton aphid has different colours that range from yellow to very dark green. The species has a worldwide distribution and a wide host range (with over 92 plant families) including agricultural and ornamental crops; the species is abundant in the tropics, but it becomes a pest on greenhouse crops in temperate climates (Blackman & Eastop 1984).

This pest attacks plants in vegetative, flowering and fruiting. The plant symptoms caused at the beginning of the infestation consist in yellowing of the leaves, but when aphids increase in density, plant tissues become puckered and curled. The host plant gets generally covered with a black sooty mold which develops on the honeydew excreted by the aphid colony. *Aphis gossypii* is mainly controlled with a wide array of chemicals, in particular with systemic molecules, but persistent problems with the development of resistance to many insecticide classes have been reported (Furk & Hines 1993; Chen et al. 2017).

In the Integrated Pest Management (IPM) context, defined more than fifty years ago (Stern et al. 1959) and currently extended to the wider Integrated Production system (Boller et al. 2011), the control of agricultural pests is reached through the integration of all control tools with particular preference to biological means. Furthermore, the selectivity of pesticide, as the capacity of a substance to act on target pest with lower toxicity on non-target organisms (Stark & Banks 2003), is considered key when selecting chemicals to be included in IPM programmes. Although the use of pesticides results efficacious in the short term period, it does not appear sustainable in the long term because of the multiple unwanted side effects it may cause. Side effects of pesticides concern a plethora of economic and ecological consequences including (i) the outbreak of pest resistance phenomena, (ii) biocenotic structure gaps for the extreme simplification of agro-ecosystem trophic networks (iii) environmental pollution, (iv) ecosystem damages and (v) cost increases for pest management (Siqueira et al. 2001; Stark & Banks 2003; Desneux et al. 2007; Roditakis et al. 2015).

To cope with the aforementioned problems, different countries in the world have introduced more restrictive laws on pesticide trading. For instance, in the context currently provided by the European Directive 2009/128/CE on the sustainable use of pesticides, European Members are obliged to promote phytosanitary control measures by reducing the risks and impacts of pesticide use on human health and the environment, promoting the use of integrated pest management and of alternative approaches or techniques such as organic farming.

For this reason, research is moving towards the study of naturally derived substances, known as biopesticides, which have a safer, selective and sustainable ecotoxicological profile. Biopesticides include microorganisms and/or derived products, entomopathogen nematodes, plant-derived insecticides (i.e., botanicals), pheromones and substances obtained by genetically modified plants (Copping & Menn 2000).

Among plant extracts, plant essential oils (EOs) are complex mixtures of terpenic and aliphatic compounds synthetized by the secondary metabolism of plants that show promising results as insecticidal substances (Regnault-Roger et al. 2012). The potential use for pest control by EO is documented by several studies where lethal, sub-lethal and repellence effects of plant extracts are studied with different methods and their chemical constituents are identified through gas-chromatography analysis (Zapata & Smagghe 2010; Karamaouna et al. 2013; Campolo et al. 2014). The promising relevance of EOs in controlling agricultural pests is basically due to their toxicological properties, such as: (i) high selectivity on mammalians and water organisms, (ii) rapid biodegradation and lack of bioaccumulation phenomena, (iii) multiple modes of actions against insects, and (iv) low risk to develop resistance phenomena (Isman 2000; Regnault-Roger et al. 2012). Moreover, the use of EO-based products is authorized in organic farming, as stated by the Regulation EC N. 889/2008. These compounds show also interesting perspectives in the human and veterinary field for the control of vectors that carry diseases, especially in developing countries where the access to synthetic insecticides is often limited (George et al. 2014). However, the natural origin of these compounds does not often show a safer impact on beneficial arthropods (Biondi et al. 2012). For this reason, the eco-toxicological impact of EO towards pollinators and natural enemies should furtherly investigated (Isman 2000; 2006). Although the scientific community shown an increasing interest for botanicals, only one-third of the publications includes chemical information about the chemical characterization and only a quarter shows the presence of positive control (i.e., a pesticide with known insecticidal activity) in the experimental setup. Therefore, all these published studies appear just preliminary with few applied implications in the field (Isman & Grieneisen 2014).

The use of nanotechnologies for plant protection may help to mitigate the EO drawbacks (e.g., variable toxicity towards plant and target organisms). A growing literature body on the potential entomotoxicity of several nanopesticides has been produced but a lack of studies concerning side effects on beneficial still exists. Therefore, these aspects should be investigated because the ecological services which natural enemies play need to be protected (Jervis & Kidd 1996; Hawkins et al. 1997; Price et al. 2011).

Biological control can be defined as a branch of applied ecology that enhances the role of natural enemies in regulating pest population levels through three main techniques: classical, augmentative and conservation control (Jervis & Kidd 1996; Heimpel & Mills 2017). Among the biological control agents, insect natural enemies (i.e., predators and parasitoids) are strictly involved in the control of crop pests and they are used and marketed for this scope (van Lenteren 2012). A great and worldwide known group of natural enemies are the coccinellid predators (Hodek et al. 2012). Among them, the harlequin ladybird, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae), is a ladybird beetle native to Asia that has been used since the beginning of XX century in biocontrol programs of aphid and coccid pests. However, the intentional introduction of this species in non-native areas has led to an uncontrolled spread, because of its high reproductive rate and the lack of effective natural enemies. The harlequin ladybird rapidly spread in The Americas and Europe and it has been regarded as an invasive organism, due to the controversial side effects it may causes (Roy et al. 2016). The negative impacts of *H. axyridis* regard the competitive displacement of native coccinellids, the possible direct and indirect damages on the grape industry, and the bother to humans caused by the overwinter aggregations and the triggering of allergic reactions (Koch et al. 2003). For the widespread presence and the above-reported concerns, the harlequin ladybird is no more sold by biocontrol suppliers in Europe (van Lenteren 2012); however, the species is still commercialized in North America and Asia for aphid control because the ecological service provided by the coccinellid remains undeniable (Koch et al. 2003).

Therefore, *H. axyridis* can be an effective biocontrol agent whose ecological role should be protected and enhanced on IPM programs. On the contrary, the species can be seen as a model organism which deserves attention to better understand the invasion process phenomenon of alien species (Mack et al. 2000; Brown et al. 2011). Moreover, the acquisition of the role and the dynamics of trophic networks can provide useful indications to better understand complex biocenotic relationships that occur in ecological communities and this knowledge can be used to develop suitable control strategies (Wajnberg et al. 2008).

The Asian ladybeetle is naturally parasitized by *Dinocampus coccinellae* (Schrank) (Hymenoptera: Braconidae), a worldwide distributed parasitoid of coccinellid beetles (Ceryngier et al. 2018). Field data suggest that *H. axyridis* is a marginal host for *D. coccinellae* in the new colonization areas, supporting thus the enemy release hypothesis to explain the harlequin ladybird invasion success (Berkvens et al. 2010; Ceryngier et al. 2018). Conversely, a meta-analysis showed higher parasitism rates of *D. coccinellae* on *H. axyridis* compared to invasive populations sampled across the world, suggesting an ongoing adaptation (Knapp et al. 2019). These findings suggest that more studies are needed to determine the relationship between the coccinellid species and its natural enemy within the territories invaded over different ages. On the other hand, the genetic structure of a parasitoid is closely related to its host because variations in the host range and the geographical host distribution can determine variations in parasitoid populations (Althoff & Thompson 1999). Information on the genetic structure of *D. coccinellae* may highlight potential factors involved in the invasion process of the harlequin ladybird worldwide.

Within this context, the main aim of the present Ph.D. thesis was providing new knowledge bases for the control of the cotton aphid *A. gossypii* in IPM programmes, though the use of plant extract and the evaluation of side effect on biocontrol control agents. This aim was carried out through the assessment of target and non-target activity of nanoformulated essential oils on *A. gossypii* and its parasitoid *A. colemani* (**chapter I**). The target pest was chosen because it is a pest in several greenhouse crops. The natural enemy was chosen because it is routinely used in augmentative biological control programs to suppress different aphids, and it is currently and largely marketed by several biofactories (van Lenteren 2012). Because synthetic pesticides are currently the main control tool against sap-sucking insects and pesticide can exert different effect even at sublethal doses, the effect of three commonly applied systemic insecticides was assessed in term of efficacy (baseline toxicity) against the target pest *A. gossypii* and in term sublethal effects on the parasitoid *A. colemani* (**chapter II**). The following step was to assess the impact on natural enemies of commonly used pesticides in organic and/or conventional agricultural cropping systems, including insecticides that have been increasingly used to control aphid pest.

To continue the evaluation of the side effects of pesticides on the behaviour of aphid natural enemies, the predator *H. axyridis* was chosen because it is a key natural enemy of this pest. However, before performing side effect evaluation on the behaviour of the harlequin ladybird, different alternative food regimes were tested for the laboratory culture of *H. axyridis*. Four different diets were used to rear in laboratory conditions to assess the nutritional requirements and the cost-effectiveness **(chapter III)**. The side effects of systemic insecticides at sublethal doses previously estimated for the target pest were thus assessed on the harlequin ladybird, by studying the acute toxicity and the sublethal effect on the behavioural functional response of this predator **(chapter IV)**.

Finally, the parasitism rate of *H. axyridis* by *D. coccinellae* through different areas colonized in diverse ages during the harlequin ladybird invasion process was assessed. Indigenous and exotic ladybeetle were collected to assess the parasitism by *D. coccinellae* in the native range of *H. axyridis* (China), as well as areas where *H. axyridis* invaded over the last 100 years (Minnesota), more recently (Chile) and very recently (Sicily, Italy). We also assessed the phylogenetic relationship of *D. coccinellae* populations among this geographical range and their hosts by using generic mitochondrial markers to discover the genetic structure at larger scale of this widespread parasitoid and the possible linkage to its hosts (**chapter V**).

CHAPTER I.

Target and non-target effects of eight nanoformulated essential oils on the cotton aphid and its main parasitoid

Abstract

Among botanical extracts, plant essential oils (EOs) represent a promising tool for the sustainable control of agricultural pests thanks to their low risk for pest resistance development and non-target impact. However, EOs are not fully included in Integrated Pest Management (IPM) programs because they might be phytotoxic and show variable effects on target pests. These drawbacks can be mitigated by employing novel formulations. Here, we assessed in laboratory conditions the contact toxicity of eight nanoformulated EOs belonging to four different botanical families (i.e., Apiaceae, Asteraceae, Liliaceae and Lamiaceae) against the cotton aphid, *Aphis gossypii* (Hemiptera: Aphididae), by spraying infested cucurbit plants. Then, we evaluated the acute residual toxicity of these substances by exposing adults of the parasitoid *Aphidius colemani* (Hymenoptera: Braconidae) to EO Lethal Concentrations (LC) 90%, earlier estimated for the target pest. Anise, fennel, artemisia, garlic, lavender, peppermint, sage and rosemary EOs caused significant mortality to the pest and their calculated LCs varied significantly. The tested formulations also showed different selectivity towards the parasitoid. Our findings suggest that EO-based nanoemulsions can be used as eco-friendly control tool against the cotton aphid. Nevertheless, the non-target effects of nano-EOs on natural enemies used in biological control should be carefully evaluated before their inclusion in IPM programs.

Keywords
Botanicals, melon aphid, toxicology, Integrated Pest Management, biological control

1.1. Introduction

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is considered one of the main destructive pests of both field and greenhouse crops worldwide because the species is extremely polyphagous with a broad host range and a wide switching ability. This pest causes direct plant injury through sucking phloem sap and indirect damages by the transmission of plant pathogen viruses (Satar et al. 1999; Campolo et al. 2014a). *Aphis gossypii* might be considered an invasive species and it has a global distribution resulting abundant particularly in the tropics (Ebert & Cartwright, 1997). Because of the huge economic losses caused in both agricultural and ornamentals crops, the species has been controlled over the years with different chemicals including carbamates, pyrethroids, neonicotinoids and insect growth regulators (Wang et al. 2017; Yuan et al. 2017). Nevertheless, the routine and extensive application of synthetic insecticides has led to the development of resistance phenomena to insecticides belonging to several chemical classes which make difficult the control of this organism (Furk et al. 1993; Chen et al. 2017). Besides, the application of synthetic chemicals is fully recognized to be deleterious against multiple non-target organisms, including beneficial arthropods, such as natural enemies and pollinators (Desneux et al. 2007). Indeed, numerous studies fully recognize the positive impact of natural enemies in controlling the cotton aphid in multiple crops (Han et al. 2014; Yang et al. 2017). Among these, the koinobiont endoparasitoid *Aphidius colemani* Viereck (Hymenoptera: Braconidae) is widely exploited via conservative and augmentative biological control (Frank 2010; van Lenteren 2012). For example, this species is routinely used in biological control programs in greenhouse against aphid pests including *A. gossypii* and the green peach aphid, *Myzus persicae* (Hemiptera: Aphididae) (Zamani et al. 2006). For these reasons, the research of sustainable and eco-friendly alternatives to control the cotton aphid is necessary. Currently, botanical insecticides are considered a promising tool for the sustainable control of several plant and fruit pests (Isman 2000, 2006; Benelli et al. 2013). Among botanicals, plant-derived essential oils show unique features such as the multiple modes of actions that help in insect resistance phenomena management, the rapid biodegradability and low risks against mammals (Regnault-Roger et al. 2012; Hatt et al. 2019).

Despite these strengths, EOs present several constraints and unsolved challenges. The major EO's drawbacks are mainly represented by the huge variability in their chemical composition, the hydrophobic nature which makes difficult their solubility in water, the different stability and variable degradation patterns (Pavela & Benelli 2016). These features led to several technical problems, such as the changing in toxicity against target pests, phytotoxic effect and the rapid degradation in the environment, limiting thus their potential inclusion in Integrated Pest Management (IPM) programs (Campolo et al. 2014a). However, these changes can be minimized through the development of new carrier formulations (Athanassiou et al. 2018). In particular, the inclusion of plant essential oils into nanostructured formulations may prevent degradation phenomena, enhance chemical stability and maintain the minimum effective rate of application (Campolo et al. 2017; Athanassiou et al. 2018; Hashem et al. 2018; Satehi et al. 2018).

A plethora of studies have demonstrated that EOs are a good source of active substances against aphids by contact and fumigation (Chaieb et al. 2018; Pavela 2018; Benelli et al. 2019 and see Ikbal & Pavela 2019), but EO risk assessment towards non-target organisms has been scarcely documented, especially for natural enemies that are routinely used in biological control programs (Pavela & Benelli 2016). Concerning plant extracted EOs, few studies have been addressed towards the cotton aphid in contact toxicity trials, including *Azadirachta indica* A. Juss. (Meliacee), *Cinnamomum camphora* (L.) J. Presl (Lauraceae), *Eucalyptus camaldulensis* Dehnh. (Myrtaceae) and *Lavandula angustifolia* Mill. (Lamiaceae) (Ebrahimi et al. 2013; Jiang et al. 2016). Likewise, to the best of our knowledge the non-target effect of plant extracts on *A. colemani* was solely investigated for a chenopodium-based extract through residual contact on glass (Bostanian et al. 2005). Moreover, the latter methodology used for EO risk-assessment towards natural enemies has been recognized to be unrealistic because toxicological bioassays on glass may mislead the estimation (Desneux et al. 2004). For this reason, a field-realistic approach through the use of plant substrate appears the best way to assess both target and non-target activity of EOs, as already conceived for synthetic pesticides (Biondi et al. 2012a).

In this context, we evaluated in laboratory conditions the contact toxicity of eight nanoformulated essential oils derived from different Mediterranean aromatic plants on the cotton aphid by estimating the curve response. Then, we assessed the lethal toxicity of EO nanoemulsions by exposing adults of the parasitoid *A. colemani* residues of the EO Lethal Concentrations 90% (LC_{90}), previously calculated for the pest, *A. gossypii*. The results are then discussed in the framework of the potential inclusion of nanocarried EOs for the sustainable integrated control of *A. gossypii*.

1.2. Materials and Methods

1.2.1. Biological materials

The colony of *A. gossypii* was initiated from infested zucchini plants [*Cucurbita pepo* L. (Cucurbitaceae)] collected in an organic protected crop located in Ragusa (Sicily, Italy) in 2015. The insect colony was maintained inside ventilated propylene boxes (56 x 39 x 42 cm) infesting the same host plants [zucchini potted plants (cv 'Bianca di Trieste'), 15 cm in high] that were renewed twice a week and kept at standard laboratory conditions, as follows: 24 ± 2 °C, 50 ± 10% RH, 14L:10D and 10.000 lux through led lights. The rearing of *A. colemani* was established from newly emerged adults commercially provided by Bioplanet (Cesena, Italy) in AphidiPAK500, using *A. gossypii* as host. The aphid colony used for rearing the parasitoid was maintained at the same environmental conditions as described above. Plants used for insect rearing and the experimental trials were grown in greenhouse conditions in 2L pots, following the best agricultural practices and excluding any pesticide treatment to avoid unwanted side effects on the investigated biological system.

1.2.2. EO composition and nanoemulsion preparation

For the experiment, we used eight commercial EOs provided by Esperis s.p.a. (Milano, Italy) extracted with cold press technique from the following aromatic plants: anise [*Pimpinella anisum* L. (Apiaceae)], artemisia [*Artemisia vulgaris* L. (Asteraceae)], fennel [*Foeniculum vulgare* Mill. (Apiaceae)], garlic [*Allium sativum* L. (Liliaceae)], lavender [*Lavandula angustifolia* Miller. (Lamiaceae)], peppermint [*Mentha piperita* L. (Lamiaceae)], rosemary [*Rosmarinus officinalis* L. (Lamiaceae)] and sage [*Salvia officinalis* L. (Lamiaceae)]. The same EOs were characterized by Campolo et al. (in prep.) by GC-MS and GC/FID. According to the chemical characterization, anise EO consisted of 8 compounds with anethole being the most abundant (89.96%). Artemisia EO resulted in 16 compounds, among which the major constituents were α-thujone (45.74%) and camphor (24.29%). For fennel EO were detected 14 compounds, with being the most abundant cis-anethole (42.53%), limonene (32.33%) and fenchone (10.35%). More than 80 volatile compounds were detected for Garlic EO. The volatile fraction contained 90% of sulfurized groups, and the major constituents were diallyl disulfide (33.58%), diallyl triulfide (21.23%) and diallyl tetrasulfide (14.05%). Lavender EO was composed of 12 compounds, including mainly linalool (41.13%), linalyle formiate (36.05%) and camphor (7.20%). Peppermint EO included 16 compounds, among which the most representative were alcoholic and monoterpenic fractions, with menthol (41.05%) and menthone (26.84%), respectively. Among the 12 compounds detected for rosemary EO, eucalyptol (55.27%) was the major constituent, followed by camphor (12.45%) and α-pinene (12.38%). For sage EO, α-thujone, α-pinene and sabinene were represented in similar proportions (20.37%, 18.97% and 18.87%, respectively), followed by camphor (12.50%) among a total of 17 detected compounds. For the complete EO chemical characterization see Campolo et al. (in prep.).

Nanoemulsions were prepared using the self-emulsifying sonication process and included the 15% of EO, the 5% of a tensioactive and 80% of distilled water. For the complete preparation of nanoparticled formulation procedure see Giunti et al. (2020).

1.2.3. Baseline toxicity on Aphis gossypii

The concentration-mortality relationship of the nanoformulated EOs on the cotton aphid was assessed by exposing coetaneous *A. gossypii* adults to serial dilutions of each formulation. The emulsions were prepared using distilled water mixing the formulations by using an agitator for 15 minutes at 300 rpm.

Newly-molted aphid adult cohorts (24±12 h old) were obtained by isolating 2-3rd instar nymphs on fresh zucchini pots approximately one week before the trial and kept at standard environmental conditions as described above. For the bioassay, a fresh 2-week old zucchini plant with two expanded leaves, previously infested with twenty coetaneous young adults (that represented a single replicate), was sprayed with a 2L hand sprayer (Dea 2000 Volpi®, Italy) and let dry in laboratory conditions under a fume-hood. Sprayed aphid-infested plants were kept in ventilated cylindrical arenas at standard environmental conditions. The aphid mortality was assessed under a stereomicroscope 48 hours after the treatment. The aphids were considered dead when they did not show any movement under the stimulus of a soft paintbrush or they were shaking and not able to walk for twice the distance of their body length (Yousaf et al. 2018). A negative control with distilled water was also included to mimic the zero concentration. Moreover, a control including the emulsifier at the concentration used to prepare the nanoemulsions (i.e. Tween 80 at 5%) was also included in the trials. Between 5 and 10 replicates were carried out per each concentration, tested formulation and controls. The LC_{50} and LC_{90} towards the target pest were estimated through the probit analysis (see *Data analysis*).

1.2.4. Lethal toxicity on Aphidius colemani

The acute toxicity of the eight nanoformulated EOs against the adult stage of *A. colemani* was assessed through a residual contact toxicity bioassay. For this trial, we sprayed zucchini plants with the LC_{90}s previously estimated for the target pest (see *Results*). This exposure setting was chosen in order to simulate the field scenario in which artificial parasitoid releases are used carried out in combination (soon after) with EOs applications (Desneux et al. 2004) at the concentration useful for controlling most of the pest population. Newly emerged parasitoid adults (24±12 h old) were obtained from coetaneous mummies which were isolated from the colony into ventilated Petri dishes. Per each replicate, ten adults (5 females and 5 males) were exposed to 1-h old dry residues on zucchini plants. Honey droplets were provided in the wall of a two cup-system arena (modified from Biondi et al. 2012a). The mortality of adult parasitoids was recorded after 2 days of exposure under the stereomicroscope. Parasitoid adults were considered dead when did not move after being touched with a fine paintbrush (Jam & Saber 2018). An untreated control (i.e. distilled water) and a treated control (i.e. spinosad, LASER™, Dow AgroSciences Ltd, at the highest field recommend rate, 0.75 L x ha^{-1}) were included in the bioassay. Spinosad was chosen because active by contact and because it has been shown to be highly toxic toward Hymenoptera parasitoids (Biondi et al. 2012b). By contrast, we did not include a control with the emulsifier used to prepare the nanoemulsions (i.e. Tween 80 at 5%) because preliminary trials caused a mortality comparable to the untreated control, and also because we consider the nanoemulsioned EOs as a ready to use formulation, in which is not possible to discriminate between each single compound. Per each treatment, the experiment was replicated ten times.

1.2.5. Data analysis

Probit analysis was performed in order to estimate the LC_{50}

and LC_{90} of the EOs against *A. gossypii* through direct contact. Dose-mortality relationships were considered valid (i.e. they fitted the observed data) when there was absence of significant deviation between the observed and the expected data (Finney et al 1947). For the mortality of the parasitoids, the homogeneity and normality of variance of the dependent factors were checked through Levene and Shapiro-Wilk tests and the dataset was arcsin transformed whenever needed. Data met the ANOVA assumptions and thus were analyzed through a General Linear Model (GLM) with a one-way ANOVA. LSD post hoc test ($P<0.05$) was used to highlight ex-post the differences among the treatments which resulted significant. Data analysis was performed in IBM® SPSS® Statistics for Macintosh, Version 23.0.0.0 (IBM Corp. Released 2015. Armonk, NY: IBM Corp.).

1.3. Results

1.3.1. Baseline toxicity on Aphis gossypii

Probit regression analysis of the concentration–mortality relationship was fitted to the observed data for all the tested nano-formulations, as indicated by the absence of significant deviation between the observed and the expected data (Table 1). All estimated LCs were thus considered valid. Mortality in all control groups (i.e. distilled water and Tween 80 at 5%) was always lower than 10% (data not shown). The majority of tested EOs showed LC_{50} values below 1 $\mu L \times mL^{-1}$ (1000 ppm), except for rosemary EO which slightly exceeded this value (Table 1). The estimated LC_{50} values were ranked as follows lavender<peppermint<garlic<fennel<artemisia<anise<sage< rosemary. While, the ranking of the estimated LC_{90} values slightly differed from LC_{50}: fennel<peppermint<anise< garlic<lavender<artemisia <rosemary< sage. The lowest

LC_{90}s were observed for fennel and peppermint, while the highest value for sage and rosemary nanoemulsions (Table 1).

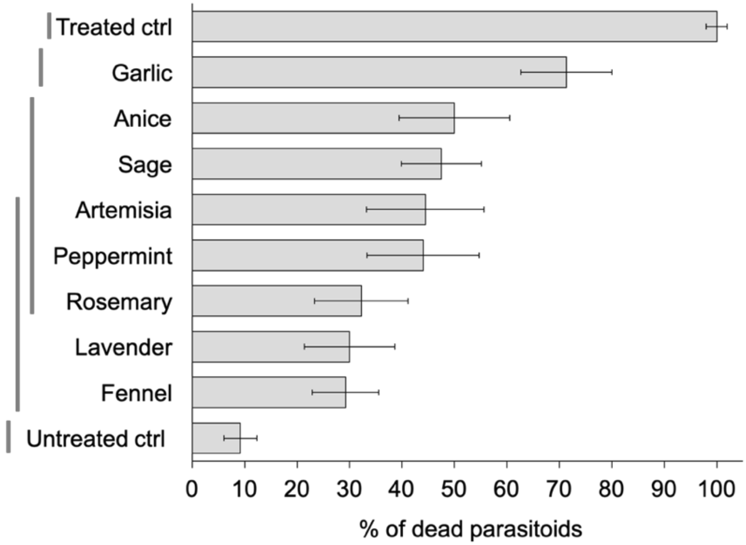
1.3.2. Lethal toxicity on Aphidius colemani

The LC_{90}s of the tested nanoemulsioned essential oils estimated for the target pest caused significant mortality of the adult parasitoids exposed to 1h-old residues compared to the untreated control (F= 9.703, d.f.=9; 109, *P* <0.001). Among the formulations, the nanoemulsion of garlic EO caused the highest significant mortality (71.33%±8.67), followed by anise, sage, artemisia and peppermint which were not significantly different from each other and caused on average the 50% of mortality (Fig. 1). Rosemary, Lavender and fennel EOs caused on average the 30% of mortality, resulting thus the less harmful compounds. Noteworthy was the difference in the higher mortality recorded for the treated control (i.e. spinosad) in comparison with garlic EO, and between the lowest mortality registered for the untreated control (<10%) and the rest of EOs (Fig. 1).

Table 1. Biotoxicity of nanoemulsioned essential oils by contact exposure against *Aphis gossypii* adults feeding on zucchini plants. Estimated lethal concentrations that kill 50% (LC_{50}) and 90% (LC_{90}) of the tested insect population, respectively. Concentration-mortality relationships were considered valid when no significant deviation between the observed and the expected data existed at the $P > 0.05$ level. CL, confidence limits; SE, standard error; χ2 chi-square testing goodness of fit of concentration–mortality response; df, degrees of freedom.

Essential oil	Slope ± SE	X^2 (df)	P	Lethal concentration (µL x mL^{-1})	95% CL
Anise	2.922±0.473	2.260 (3)	0.520	LC_{50}=0.832	0.635 - 1.091
				LC_{90}=2.282	1.629 - 4.019
Artemisia	2.005±0.304	1.596 (4)	0.810	LC_{50}=0.748	0.542 - 1.065
				LC_{90}=3.258	2.034 - 7.159
Fennel	3.791±0.683	3.714 (3)	0.294	LC_{50}=0.636	0.514 - 0.775
				LC_{90}=1.385	1.073 - 2.198
Garlic	1.498±0.268	6.320 (3)	0.097	LC_{50}=0.607	0.248 - 5.162
				LC_{90}=2.421	0.916 - 4.112
Lavender	1.709±0.241	5.308 (5)	0.379	LC_{50}=0.459	0.322 - 0.666
				LC_{90}=2.581	1.552 - 5.827
Peppermint	2.347±0.339	3.399 (4)	0.493	LC_{50}=0.549	0.410 - 0.744
				LC_{90}=1.933	1.309 - 3.591
Rosemary	2.150±0.375	3.577(3)	0.311	LC_{50}=1.074	0.779 - 1.539
				LC_{90}=4.235	2.628 - 10.208
Sage	1.800±0.287	3.788 (4)	0.435	LC_{50}=0.891	0.630 - 1.344
				LC_{90}=4.590	2.637 - 12.461

Figure 1. Mean % (±SE) mortality of *Aphidius colemani* newly emerged adults exposed to different nanoemulsioned EOs at LC_{90}s estimated for *Aphis gossypii* in residual contact toxicity bioassays. Means for treatment subtended by vertical lines do not differ at $P > 0.05$ (GLM, LSD test). Treated ctrl: Laser™ Dow AgroSciences (a.i. Spinosad) at the highest field rate (75mL x hL^{-1}).



1.4. Discussion

Our work demonstrated that nanoformulated essential oils of several Mediterranean plants can be considered as a promising tool for controlling *A. gossypii*. The results showed quantitative differences in the EO insecticidal activity which linearly augmented with increasing concentrations. The majority of tested EOs had LC_{50} values below 1000 ppm, being thus a promising tool for the effective field applications against the cotton aphid.

The recent review of Ikbal & Pavela (2019) highlighted the contact toxicity of numerous EOs against aphid pests. Similarly, as in our studied EOs, *Artemisia absinthium* and *A. sieberi*, *Lavandula angustifolia*, *Mentha piperita*, *Pimpinella anisum*, *Rosmarinus officinalis* and *Salvia officinalis* were tested as crude extracts against different aphid pests by direct contact (i.e., topical application). However, among them, only lavender EO was tested towards *A. gossypii* (Ebrahimi et al. 2013). In the concentration-mortality bioassay conducted for *P. anisum* EO against *A. gossypii* a LC_{50} equal to a 0.849 µL x mL^{-1} was determined. Similarly, *Pimpinella anisum* EO showed very high toxicity against *M. persicae* LD_{50}= 0.03 µL x mL^{-1} (Al-Antary et al. 2017); although the same result was confirmed neither for the same target species (LD_{50}= 4.3 µL x mL^{-1}) (Benelli et al. 2018), nor for *Lipaphis pseudobrassicae* (Davis) (Hemiptera: Aphididae)(LC_{50}= 3.20 µL x mL^{-1}) (Sampson et al. 2005). The LC_{50} of *Artemisia absinthium* e *A. sieber* were 6.9 and 6.16 µL x mL^{-1}, respectively compared to *A. vulgaris* included in our study, which value was 0.791 µL x mL^{-1} and thus it can be considered more toxic towards the target pests. According Sampson et al. (2005) fennel EO showed a LD_{50}=9.90 µL x mL^{-1}against *L. pseudobrassicae* in an hour of exposure time, but the estimated lethal concentration significantly decreased for *M. persicae* (Pavela 2018).

For nanoformulated garlic EO we estimated a LC_{50} equal to 0.568 µL x mL^{-1}, suggesting thus the high toxicity of this compound. Mousa et al. (2013) found that garlic EO at the rate of 30 µL x mL^{-1}caused 71.78% of mortality against *Aphis craccivora* Koch (Hemiptera: Aphididae). Similarly, the authors compared the bioactivity of eucaliptus and garlic EOs with two organaophosphates and their efficacy against two beans pest and two non-target beneficial arthropods, demonstrating that garlic EO reduced significantly aphid population of 90.98% while eucalyptus EO caused a reduction of 80.66%. Mousa et al. (2013) also found that the tested EOs caused a fluctuation on the population density of a spider and *Orius* sp. (Hemiptera: Anthocoridae). Garlic extract had been also reported for lethal and sublethal activity against several species of insect and mite pests (Prowse et al. 2006; Herrick & Cloyd 2017; Akyazi 2018).

Lavender angustifolia EO tested on *A. gossypii* showed a LC_{50} equal to a 0.517 µL x mL^{-1}, that is quite lower compared the values obtained testing this extract against *L. pseudobrassicae, A. gossypii* and *M. persicae* in which was 7.50, 13.73 and 20.00 µL x mL^{-1}, respectively (Sampson et al. 2005; Machial 2010; Ebrahimi et al. 2013). Therefore, we can suppose that the high efficacy of our EO-based nanoemulsions is likely due to their peculiar formulation that increased the bio-toxicity of the EOs.

In this study, the nanoformulation of *Mentha piperita* EO was shown to be the most effective against *A. gossypii* at low concentration with an estimated LC_{50}=0.606 µL x mL^{-1}. The high toxicity of peppermint was also proved towards *M. persicae* (LC_{50}=0.04 µL x mL^{-1}) (Al-Antary & Belghasem et al. 2017), but not towards *L. pseudobrassicae* (LC_{50}= 8.80 µL x mL^{-1}) (Sampson et al. 2005). However, similar value to our findings was found for *M. pulegium* EO towards *M. persicae* (LD_{50}=0.88 µL x mL^{-1}) (Behi et al. 2017).

According to our findings, the EO of *R. officinalis* exhibited a moderate toxicity by contact against other target sap-sucking pests. For instance, the median lethal concentrantions calculated for *L. pseudobrassicae* and *Macrosiphum rosae* were respectively 7.40 and 57.57 µL x mL^{-1} (Sampson et al. 2005; Modarres Najafabadi et al. 2018). Sage EO seemed not very toxic towards *Metopolophium dirhodum* (Walk.) (Hemiptera: Aphididae) with an estimated LC_{50}=15.20 µL x mL^{-1} whether compared to our results obtained on *A. gossypii* (LC_{50}=0.99 µL x mL^{-1}) (Sánchez Chopa & Descamps 2012). Differences in insecticidal activity by essential oils can be due to multiple reasons. The chemical composition and the broad spectrum of biological activity for essential oils vary with plant age, plant tissues or organs used in the extraction process, the type of extraction, besides the species and the age of the target pest organism (Regnault-Roger et al. 2012).

Plant essential oils also act against insects in multiple ways, interfering with basic physiological and behavioural functions (Pavela & Benelli 2016). Firstly, EOs are good penetrants because of the disruption of lipid bilayers cells. Moreover, since volatile monoterpenes are involved in the transmission signals from plants to insects, they can affect odorant bindings proteins. Lastly, monoterpenes as major EO constituents are neurotoxic to insects, acting towards different kinds of receptors, including inhibition of P450 cytochromes, GABA, octopaminergic system and acetylcholinesterase (Rajendran & Sriranjini 2008). On the other hand, EOs can also act as a repellent, feeding deterrents as well as a growth and/or reproduction inhibitor (Pushpanathan et al. 2006; Campolo et al. 2014b). More recently, Hashem et al. (2018) demonstrated that *P.*

anisum essential oil nanoemulsion affected the midgut cells of *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) at morphological and histological level. Therefore, assessment at physiological level of new developed EO-based formulations appears necessary.

Besides that, our experiments were not carried out to correlate the different toxicity results towards the target pest within the chemical classes of the EO major constituents. Nevertheless, the assessment of the different molecules involved as active substances can be a further step of this research product. Future investigations are thus required to elucidate the target specificity of EO mode of action to understand the main physiological pathway involved in EO toxicity towards the cotton aphid by giving helpful information concerning the pest resistance management.

Our study demonstrated that nanoformulated EOs tested at LC_{90} earlier estimated for the target pest caused lethal toxicity towards the parasitoid *A. colemani* in the residual trial. In particular, garlic EO caused the highest mortality (70%) against the parasitoid, showing thus its topical and residual contact toxicity towards the pest and its biocontrol agent. Rosemary, Lavender and Peppermint EOs caused on average the 30% of mortality, respectively. To the best of our knowledge, no previous research papers were published on the side effect of garlic EO on *A. colemani* and this may represent a novelty in the research. The toxicity of garlic extracts was proved against *Rhipicephalus annulatus* Say (Ixodida: Ixodidae) with both aqueous and ethanol solutions at different concentration (Aboelhadid et al. 2013). By contrast, sulfur volatiles extracted from different *Allium* spp. did not affect the behavior of *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) an ecto-parasitoid of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), suggesting that such compound could be integrated into *D. citri* management programs (Mann et al. 2011).

The non-target effect of EOs against beneficial arthropods was investigated by Bostanian et al. (2005). Testing the insecticidal efficacy of a natural based extract of *Chenopodium ambrosioides* L. (Amaranthaceae) (UDA-245®), the authors found no toxicity towards *A. colemani* 1d after exposing topically the organism to biopesticide at the recommended label dose (i.e. 5 g x L^{-1}) and at the double. Nevertheless, 48 hours after the exposure, both the concentrations caused a significant lethal effect on the parasitoid wasp compared to the control. By contrast, a residual contact toxicity bioassay caused mortality neither at 24h nor at 48 hours after the exposure to both concentrations. Although UDA-245 based on an essential oil extract from *C. ambrosioides* at 0.5% was quite effective in controlling the green peach aphid and the whitefly *Trialeurodes vaporariorium* (Westwood) (Hemiptera: Aleyrodidae), the formulation was considered safe against *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) in a contact toxicity bioassay (Chiasson et al. 2004).

In our study several EOs were harmful against the parasitoid through residual contact, causing high mortality such us the case of garlic and anise. Although our experiment was conceived to mimic a hypothetic field scenario in where natural enemies are likely to be exposed to pesticide application, the underestimation of lethal effect is possible because the topical exposure route can often result in more toxic than residual exposure (Bostanian et al. 2005).

Asadi et al. (2018) investigated the effect of *R. officinalis e S. officinalis* EOs on the demographic traits of the ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) via fumigation. The authors found higher toxicity for rosemary

EO comparing to sage EO, with a LC_{50} of 4.15 µL x L^{-1} and 18.36 µL x L^{-1} respectively. By contrast, in our experiment *S. officinalis* EO was more toxic than *R. officinalis*, with an adult parasitoid mortality of 50% and 30%, respectively. According to Yi et al. (2016), *L. angustifolia*-derived EO caused contact toxicity by topical application against the endoparasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae). Different percentages of mortality were recorded, namely 17%, 87%, 93% at 1, 2, 3 g x L^{-1} of EO, respectively. While *L. angustifolia* EO caused the 100% mortality both at 4 and 5 g x L^{-1}. This topical contact toxicity can be compared with the results obtained in our bioassays, in which nanoparticled *L. latifolia* EO caused 30% of mortality towards *A. colemani.* Conversely, essential oils extracted from *Origanum vulgare* and *Thymus vulgaris* were selective for *Trissolcus basalis* (Hymenoptera: Scelionidae) females, egg-parasitoid of the green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae), in term of contact and residual toxicity, as well as for sublethal effects after 1 week (González et al. 2013). These changes might be owing to the considered species, but also to the used methodology and the chemical compounds contained in the EO.

Our laboratory results suggest that different nanoformulations, such as lavender and fennel EO, could be considered to control *A. gossypii* together with the parasitoid *A. colemani*, in a potential IPM program. Further experiments in laboratory and field conditions are required to assess the toxicity in a more extended period, including also the behavioral and physiological sublethal effects that could directly impact the demographic parasitoid dynamics and its biological control services (Biondi et al. 2013). In the same context, the assessment of different-age residual toxicity appears necessary.

Besides that, complementary further researches in semi-field and field conditions will be useful in order to understand how environmental factors may affect the degradation of EO active components and consequently their bioactivity on target and non-target organisms since different biodegradation and persistency time for EOs have been reported (Ikbal & Pavela 2019). Worth of noting is also the research concerning the effect of EO at sublethal doses, this is well known for conventional pesticides and it was already demonstrated for some EO-based compound (González et al. 2013). Moreover, because the synergistic interactions between constituents of essential oils have been reported for insect pests (Tak et al. 2015), the interaction of single constituents and/or different essential oils belonging to different chemical families can be also investigated and properly exploited in the field (Isman 2017).

In conclusion, this study provided solid scientific bases on the biotoxicity of several EOs extracted from different Mediterranean aromatic plants against one of the major key-pest of agricultural crops. We also highlighted that such extracts, although in nanoformulations, are not always safe towards non-target organisms, such the beneficial *A. colemani*. Our findings extend the potential of essential oils for the control of the cotton aphid and provide useful information for the development of integrated control programs. This could implement the alternative strategies for the sustainable control of aphid crop pests, through the reduction of the commonly used broad-spectrum insecticides.

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CHAPTER II.

Efficacy and sublethal effects of systemic insecticides on *Aphis gossypii* and its parasitoid *Aphidius colemani*

Abstract

Systemic insecticides are used to control agricultural pests globally and their non-target impact at non-lethal doses on beneficial arthropods has been recognized. We assessed the baseline toxicity of imidacloprid, thiamethoxam and sulfoxaflor-based insecticides on the polyphagous aphid pest, *Aphis gossypii* (Hemiptera: Aphididae), and their non-target effects on its main parasitoid, *Aphidius colemani* (Hymenoptera: Braconidae), evaluated by residual contact exposure to the median Lethal Concentration (LC_{50}), LC_{20} and LC_1 of all three tested insecticides, earlier estimated for the target pest. The results showed that the LC_{50}s for the aphid were 6.4×10^{-3}, 5×10^{-3}, 2.9×10^{-2} times lower compared to the label concentrations of imidacloprid, thiamethoxam and sulfoxaflor, respectively. LC_{50}s of thiamethoxam caused the highest mortality rate followed by sulfoxaflor, while imidacloprid had the lowest impact. Our findings highlight the importance of case-specific evaluation to optimize pesticide applications in Integrated Pest Management packages taking into account the ecological services provided by biological control agents.

Keywords

Neonicotinoids, sulfoxaflor, melon aphid, toxicology, biological control

2.1. Introduction

The control of agricultural sap-feeding insect pests and associated transmitted plant viruses mainly relies on the application of insecticides with systemic properties (Jeschke et al. 2011; Simon-Delso et al. 2015). Systemic insecticides are mostly represented by neonicotinoids which act in the insect central nervous system towards nicotinic acetylcholine (nACh) and gamma-aminobutyric acid (GABA) receptors, respectively (Simon-Delso et al. 2015). The popularity of the widespread use of systemic insecticides is largely due to their chemical and physical properties, such as the capacity to be systemically translocated over plant vessels, the high toxicity towards invertebrates associated to long persistence and water solubility, lower binding efficiency to vertebrate receptors which makes these compounds selective for vertebrates (Simon-Delso et al. 2015; Sparks & Nauen 2015). Nevertheless, these properties dramatically increase the possibility of environmental contamination and exposure of non-target organisms (Desneux et al. 2007). Environmental contamination by systemic insecticides occurs via multiple routes including accumulation in cultivated soils and soil water, runoff into waterways, direct uptake by non-target plants and, dust generated during drilling of coated seeds, and via a plethora of multitrophic interactions among plant and pollinators and among plant, pests and natural enemies (He et al. 2012; Sanchez Bayo 2016; Mohammed et al. 2018; Calvo-Agudo 2019; Sâmia et al. 2019). Such environmental fate provides thus a number of acute and chronic routes of exposure to non-target organisms (Desneux et al. 2007; Bonmatin et al. 2015). A large number of studies give evidence on the adverse impact of systemic insecticides towards beneficial arthropods, with particular regards to pollinators, posing serious risks on the ecosystem services that they provide (Cloyd & Bethke 2011). For these reasons, following the European Food Safety Authority updated risk assessment report for three neonicotinoids (i.e. imidacloprid, clothianidin and thiamethoxam), the European Union banned the field use of these active substances in 2018; however, their application in protected crops is still authorized (Jactel et al. 2019).

The need to manage harmful crop pests in a sustainable manner, both from an environmental and human health viewpoint, is a fundamental requirement of Integrated Pest Management (IPM). Therefore, the risk assessment of the side effects of pesticides is always needed in this context (Radcliffe et al. 2009). The evaluation of the non-target effects of pesticides on beneficial organisms generally includes both lethal and sublethal effects (Desneux et al. 2007). Sublethal effects are physiological and/or behavioural effects on individuals that survive exposure to a toxic compound at a low concentration. The physiological changes caused by the exposure to sublethal concentrations of insecticides can affect multiple biological traits (e.g. developmental rate, longevity, fecundity, and fertility) (Biondi et al. 2013). Exposure to, or feeding on, sublethal or low concentrations of systemic insecticides can reduce growth, survival, and reproduction in many hemipteran insect pests (He et al., 2013). By contrast, exposure of various insects to plants treated with systemic insecticides can have also a stimulatory effect, i.e. hormesis, on the insect fecundity (Yu et al. 2010; Qu et al. 2015; and see Guedes et al. 2016). Behavioural changes caused by exposure to sublethal concentrations of an insecticide can result in reduced feeding or searching behaviour (Wang et al. 2008). Low insecticide concentrations in field crops often arise after insecticide application, depending on the frequency of application and the rate of degradation by abiotic factors (He et al. 2012; Yao et al. 2015). In insecticide-treated crops, insects are therefore likely to be exposed to sublethal concentrations.

The cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is considered one of the main destructive agricultural pests worldwide because of the extreme polyphagia, the wide switching ability and the transmission of agriculturally relevant plant viruses (Ebert & Cartwright 1997). The cotton aphid has been controlled over the years with different chemicals including both most conventional and relatively new compounds such us neonicotinoid and sulfoxamine insecticides (Babcock et al. 2011). *Aphidius colemani* Viereck (Hymenoptera: Braconidae) is a parasitoid routinely marketed for biological control programs to suppress different aphid pests, and the cotton aphid is one of its main hosts (Stara et al. 2011).

Here, we studied the effects of three systemic insecticides, imidacloprid, thiamethoxam and sulfoxaflor (Afidane® 200 SL, Actara® 25 WG and Transform® WG, respectively), on the key polyphagous aphid pest, *A. gossypii*, and on its main parasitoid, *A. colemani*. Through laboratory bioassays, we first estimated the baseline toxicity of the three insecticides. Then, the mortality of *A. colemani* adults was evaluated by residual contact exposure to the median lethal concentrations (LC_{50}), LC_{20} and LC_1 of the insecticides, earlier estimated for the target pest. Further tests on measuring the fertility (sublethal effects on surviving adults) of surviving females expressed as the number of mummies produced and the percentage of adult emergences were conducted. Our work may help in determining the use of systemic insecticides for the control of the cotton aphid taking well into account the ecological services provided by beneficial organisms in the crops to be sprayed.

2.2. Materials and Methods

2.2.1. Biological materials

The colony of *A. gossypii* was initiated from infested zucchini plants collected in organic greenhouses in Southeast Sicily (Italy) in 2015. The insect colony was maintained inside ventilated propylene boxes (56 x 39 x42 cm) on zucchini plants (cv “Bianca di Trieste”) that were renewed twice a week and kept at standard laboratory conditions as follow: 24 ± 2 °C, 50 ± 10% RH, 14L:10D and 10,000 lux led lamps located on top of rearing cages.

Zucchini plants used for insect rearing and the experiment were grown in greenhouse conditions in 2L pots, following the good agricultural practices and avoiding any pesticide treatment. For the experiments, newly-molted adults (24±12 h old) were obtained by isolating 3rd instar nymph on fresh zucchini pots (15 cm in height) one week before the bioassays in controlled environmental conditions as reported above. The rearing of *A. colemani* was established from newly emerged adults provided by Bioplanet (Cesena, Italy) in AphidiPAK500, using the cotton aphid as host. The host colony was maintained in the same conditions as described above. Newly emerged adults (24±12 h old) used in the experiment were obtained from coetaneous mummies which were isolated from the parasitized colony into ventilated Petri dishes. Honey droplets were daily provided to the parasitoids.

2.2.2. Systemic insecticides

We tested three commercial systemic insecticides Afidane® 200 SL Chimiberg, Italy, (a.i. imidacloprid), Actara® 25 WG, Syngenta, Italy, (a.i. thiamethoxam) and Transform® WG, Dow AgroSciences, USA, (a.i. sulfoxaflor). Imidacloprid and thiamethoxam belong to the chemical class of neonicotinoids and act in the nACh system (Simon-Delso et al. 2015). Sulfoxaflor belongs to the chemical class of sulfoximine and acts similarly to neonicotinoids, however, it shows differences in the insect SAR (Systemic Acquired Resistance), in particular over the cytochrome P450 mono-oxygenase (Sparks & Nauen 2015). The tested commercial formulations are authorized for the control of the cotton aphid on various crops (including zucchini crop) and they were prepared following their label guidelines.

2.2.3. Toxicological bioassays

2.2.3.1. Baseline toxicity on Aphis gossypii

All the experiments were carried out at the Department of Agriculture, Food and Environment of the University of Catania. The concentration-mortality response of systemic insecticides was evaluated by exposing adults of *A. gossypii* from 5 to 7 serial dilutions of each formulation in a solution of distilled water and Tween 80 at 0.01%. For each insecticide, the range of tested concentrations was established according to preliminary trials aimed at identifying the minimum dose necessary to cause the total mortality of the tested individuals and the maximum dose that does not cause significant mortality of the treated insects.

For this bioassay, twenty coetaneous young adults feeding on a fresh zucchini plant (that represented a single replicate) were sprayed with a 2L hand sprayer (Dea 2000 Volpi®, Italy) and let dry in laboratory conditions. The aphid mortality was assessed under a stereomicroscope 48 hours after the exposure. Among the treatments, an untreated control with distilled water was also included. The insecticidal solutions were prepared using a magnetic agitator for 10 minutes at 300 rpm.

2.2.3.2. Lethal toxicity on Aphidius colemani

The residual contact toxicity of three LCs (i.e., LC_{50}, LC_{20} and LC_1) of the three systemic insecticides previously estimated for the target pest (see the result section) was assessed on adults of the parasitoid *A. colemani*. Zucchini pots were sprayed with the insecticidal solutions until runoff from a distance of 0.5 m through the hand sprayer and were left to dry for 1hour at laboratory conditions.

Five couples (5 females and 5 males) of newly emerged adults (<48 hours old) were exposed to 1-hour old dry residues on treated zucchini plants inside a two superposed cup arena (Biondi et al. 2012a). Honey drops and a water source were provided into the two cup-system arena. The mortality was recorded after 2 days of exposure. Parasitoid wasps were considered dead when they did not react after being touched with a paintbrush. Zucchini plants sprayed with water were used in the untreated control. Eight replicates were carried out per each treatment.

2.2.3.3. Sublethal toxicity on Aphidius colemani *reproduction*

The sublethal effects of the three systemic insecticides at their LC_{50}, LC_{20} and LC_1 previously calculated for the target pest were assessed on the fertility of the survived females from the previous bioassay. Briefly, each survived female was paired with a newly emerged and untreated male and exposed for 24 h to 20 aphid nymphs (3rd stage), previously transferred with a paintbrush to a fresh and untreated zucchini plants into the two-cup system arena. Honey droplets were supplied inside the arena and the parasitoid couple was removed after 24 hours. For the whole duration of the experiment, the arenas were maintained at standard environmental conditions, as described above. The number of developed aphid mummies (i.e., parasitoid pupae) and of emerged parasitoid adults was counted every two days approximately for 7 days after the beginning of the experiment. Eight replicates were carried out per each treatment. The toxicological bioassays were performed under standardized environmental conditions in a climatic chamber (Refrigerated incubator model IRE-475, Raypa® R. Espinar, s.l. Spain) kept at 25 ± 1°C, 60 ± 5% R.H. and 14L:10D h photoperiod.

2.5. Data analysis

The homogeneity and normality of variance of the dependent variables were checked through Levene and Shapiro-Wilk tests and the dataset was transformed whenever needed. Probit analysis was performed in order to estimate the lethal concentrations of the systemic insecticides against *A. gossypii* through direct contact. Values were considered significantly different whether their 95% fiducial limits did not overlap (Finney et al 1947).

The percentage survival of parasitoid pupae was estimated as: 100 x (number of emerged parasitoids / number of developed mummies). For lethal and sublethal effect assessment, we tested the effect of pesticide (factor *pesticide*), concentration (factor *concentration*) and the potential interaction of these two factors (*pesticide* x *concentration*) on the mortality of the exposed parasitoid adults, the number of developed mummies (i.e. parasitoid pupae) and the proportion of the newly emerged parasitoids by carrying out a factorial ANOVA. Additional one-way ANOVA followed by Least Significant Difference (LSD) *post hoc* test (*P* <0.05) was used for multiple mean comparisons among the treatments. Data analysis was performed in IBM® SPSS® Statistics for Macintosh, Version 23.0.0.0 (IBM Corp. Released 2015. Armonk, NY: IBM Corp.).

2.3. Results

2.3.1. Toxicological bioassays

2.3.1.1. Baseline toxicity on Aphis gossypii

The LC_{50}, LC_{20} and LC_1 for imidacloprid, thiamethoxam and sulfoxaflor were estimated (Table 1). Overall, thiamethoxam was the most toxic insecticide showing the lowest LC_{50}, LC_{20} and LC_1 values (i.e. the concentration of active ingredient able to control the 50, 20 and 1% of the tested population, respectively). Whereas, the highest values for LC_{50}, LC_{20} were found for imidacloprid and the highest LC_1 was estimated for sulfoxaflor. All the estimated lethal concentrations had lower values compared to the field dose (ppm) (i.e. label dose), and the LC/label dose ratio ranged from 2.8×10^{-2} for LC_{50} of sulfoxaflor to 8.7×10^{-6} for the thiamethoxam LC_1 (more details in Table 1).

2.3.1.2. Lethal toxicity on Aphidius colemani

The mortality of the parasitoid exposed to chemical residues on plants was significantly affected by the pesticide ($F_{3,80} = 5.462$; $P < 0.001$), concentration ($F_{3,80} = 11.789$; $P < 0.001$) and their interaction ($F_{9,80} = 5.025$; $P < 0.001$). A mortality rate of 8.75±5.15% was recorded for the untreated control. The LC_{20}s and LC_1s caused significantly lower mortality than caused by the LC_{50}s, except in the case of imidacloprid, for which we did not record significant differences in parasitoid survival following exposure to different concentrations and compared to the untreated control (Fig. 1). The LC_{50} of thiamethoxam was the most harmful against the parasitoid, with an average mortality of 53.75±15.46%, compared to sulfoxaflor, with a mean mortality rate of 28.75±4.40%, and imidacloprid LC_{50} with 17.50±3.66% (Fig. 1). Regarding the exposure to LC_1, thiamethoxam reported the lowest average mortality with 5.75±1.83%. Conversely, imidacloprid at LC_1 induced mortality of 17.5±3.13%.

2.3.1.3. Sublethal toxicity on Aphidius colemani *reproduction*

The systemic pesticides at sublethal concentrations affected neither the number of developed mummies (Fig. 2), nor the number of emerged parasitoids (Fig. 3). The statistical analysis revealed no significant effect of the factors pesticide, concentration and their interaction respectively on the number of mummies ($F_{3,80} = 0.702$; $P = 0.499$; $F_{3,80} = 1.002$; $P = 0.373$; $F_{3, 80} = 1.168$; $P = 0.333$) and on the proportion of emerged adults ($F_{3,80} = 0.932$; P =0.504; $F_{3,80} = 1.705$; $P = 0.190$; $F_{3,80} = 0.853$; $P = 0.504$).

Overall, following the parasitoid exposure to all treatments and untreated control, the total number of developed parasitoid mummies was on average 5.75±1.82 per parasitoid female (Fig. 2). The highest number was recorded for thiamethoxam at LC_{50} with 8.50±2.59 mummies per female, while the lowest value of formed mummies was recorded for imidacloprid at LC_{50} with 3.00±0.60 mummies.

The percentage of survival of parasitoid pupae (i.e. number of emerged parasitoids) was on average 81.58±3.30%, with the highest mean value recorded for sulfoxaflor LC_{20} and the lowest for imidacloprid $LC_{1.}$

Table 1. Baseline toxicity by contact exposure of three systemic insecticides against *Aphis gossypii* adults feeding on zucchini plants. SE, standard error; χ^2, chi-square testing goodness of fit of concentration–mortality response; df, degrees of freedom. LC/FR, ratio between Lethal Concentration and maximum Field Rate reported in the formulation label against the target pest.

Insecticide	**Tradename**	**% a.i.**	**Field Rate (a.i. ppm)**	**Slope ± SE**	**χ2 (df)**	***P***	**Lethal Concentration (a.i. ppm)**	**95% Confidence Limits (a.i. ppm)**	**LC/FR**
Imidacloprid	Afidane® 200 SL	17.7	500	1.055±0.153	8.336 (4)	0.080	$LC_1 = 0.020$	0.000-0.177	$3.929 \cdot 10^{-5}$
							$LC_{20} = 0.503$	0.021-1.816	$1.005 \cdot 10^{-3}$
							$LC_{50} = 3.186$	0.679-14.301	$6.372 \cdot 10^{-3}$
Thiamethoxam	Actara® 25 WG	25	200	0.636±0.107	4.408 (5)	0.492	$LC_1 = 0.002$	0.000-0.016	$8.750 \cdot 10^{-6}$
							$LC_{20} = 0.386$	0.065-1.098	$1.928 \cdot 10^{-3}$
							$LC_{50} = 1.000$	3.405-19.767	$5.000 \cdot 10^{-3}$
Sulfoxaflor	Transform® WG	50	70	1.276±0.223	5.625 (4)	0.229	$LC_1 = 0.028$	0.003-0.087	$3.929 \cdot 10^{-4}$
							$LC_{20} = 0.397$	0.146-0.714	$5.671 \cdot 10^{-3}$
							$LC_{50} = 2.000$	1.076-3.033	$2.857 \cdot 10^{-2}$

Figure 1. Mean (±SE) mortality of systemic insecticides towards *Aphis gossypii* adult following 2-day exposure on treated plants to dry residues of sublethal concentration of systemic insecticides. Bars for treatment subtended by the same letters did not differ at $P < 0.05$ (GLM, LSD test).

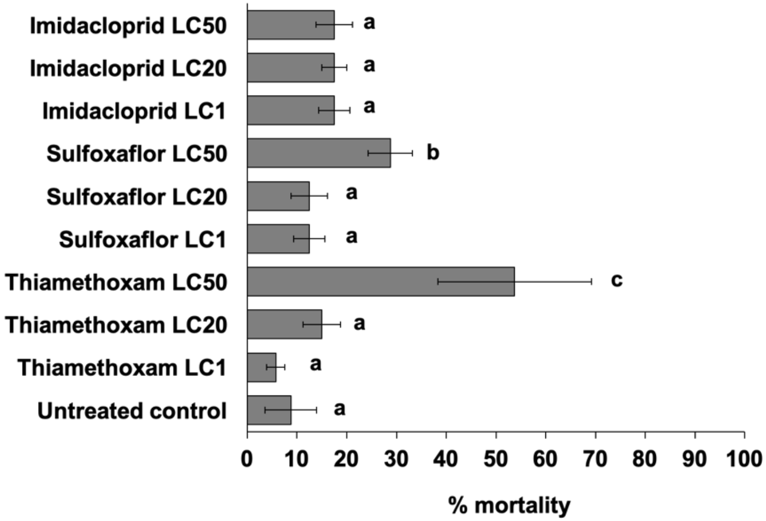


Figure 2. Mean (±SE) number of successfully developed *Aphis gossypii* mummies following exposure to one *Aphidius colemani* female previously exposed for two days to residues of sublethal concentration of systemic insecticides estimated for *Aphis gossypii* on plants.

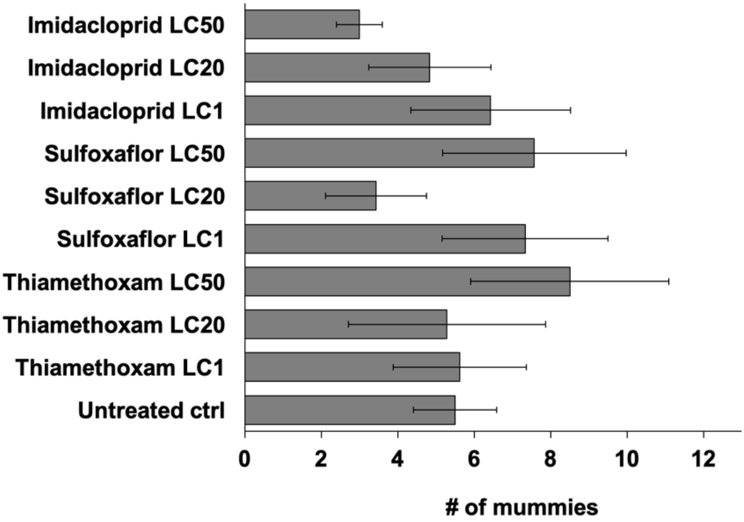
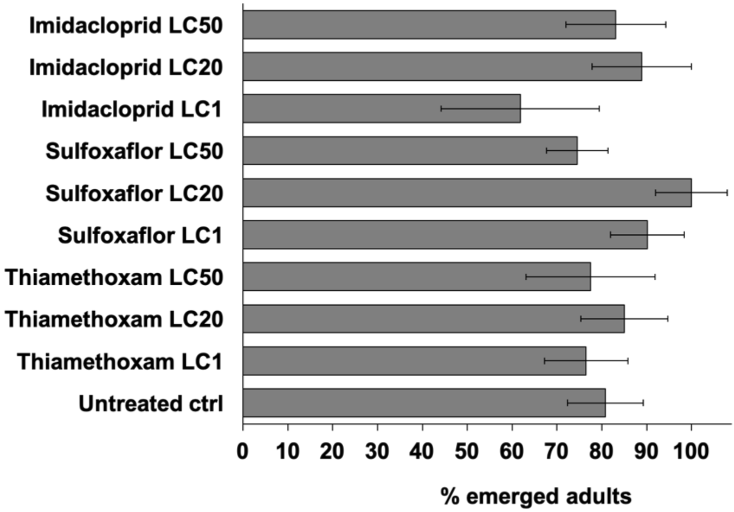


Figure 3. Mean (±SE) percentage of *Aphis gossypii* mummies from which successfully emerged *Aphidius colemani* adults. Aphid mummies derived from the exposure of *A. colemani* females to dry residues of sublethal concentration of systemic insecticides estimated for *A. gossypii* on zucchini plants.



2.4. Discussion

The results showed that the three systemic insecticides can be very effective in controlling the cotton aphid, because the LC_{50}s were 0.0064, 0.0050, 0.0285 times lower than the label doses for imidacloprid, thiamethoxam and sulfoxaflor, respectively. These findings can be very promising for the effective use of systemic insecticides in the IPM framework, even though field validation is still needed. Firstly, because we obtained these results at standardized environmental conditions and we used a laboratory strain of the target species. Secondly, different aphid pest populations could exhibit different degrees of susceptibility to these insecticides in the field, since potential pest resistance phenomena may occur, as has been already reported in different locations for several crops (Gerami 2012; Wei et al. 2017; Wu et al. 2018). Moreover, experiments evaluating sublethal effects on survived aphids (e.g. reproduction) and during longer periods are needed because systemic insecticides can have both sublethal and transgenerational impact on aphid populations, such as in the case of *Aphis gossypii* exposed to the neonicotinoid nitenpyram (Wang et al. 2017).

Regarding the non-target effects on the parasitoid, and in particular the lethal toxicity results on *A. colemani*, we have proved that thiamethoxam at LC_{50} was the most harmful active ingredient towards the parasitoid, with an average mortality rate of 53.75%. By contrast, sulfoxaflor at LC_{50} caused 28.75% of mortality, followed by imidacloprid at LC_{50} (17.50%) that can be considered the safest compound among the tested insecticides. Whereas, in the sublethal toxicity bioassays on the parasitoid fertility the mean number of total mummies was 5.65 per female, and ranged between 3.00 and 8.50 for imidacloprid LC_{50} and thiamethoxam LC_{50}, respectively. The fertility of females which survived to the exposure of thiamethoxam LC_{50} residues on plants was high but, a high number of mummies was not followed by a high emergence rate, with an average recorded of 77.50%. Conversely, the highest emergence rate was observed for the progeny of females exposed to sulfoxaflor at LC_{20}. By contrast, imidacloprid LC_1 caused the lowest percentage emergence rate with 61.81%. Nevertheless, these results were not significantly different compared to the untreated control.

Previous studies have examined the lethal and sublethal effects on *A. colemani* of two synthetic insecticides and a bioinsecticide, namely imidacloprid, lambda-cyhalothrin and spinosad (D'Ávila et al. 2018). Concentration-mortality curves showed that the parasitoid was almost 20 times more susceptible to spinosad in comparison to conventional insecticides, and this result is consistent with most of the other studies on spinosad toxicity regarding Hymenoptera (Biondi et al. 2012b). Moreover, increased concentrations of imidacloprid and lambda-cyhalothrin negatively affected the longevity and reduced the total progeny of the parasitoid, but not as significantly as spinosad did (D'Ávila et al. 2018). In addition, a recent study showed how LC_{25} of imidacloprid impaired the functional response to host density for the braconid aphid parasitoid *Lysiphlebus fabarum* (Marshall) (Jam and Saber 2019).

Other studies have been conducted on the systemic insecticide non-target impact on aphid natural enemies. The sublethal effect of imidacloprid at LC_{10} and LC_5 (calculated for the target species) was assessed towards the coccinellid *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) (Xiao et al. 2016). According to these findings, the insecticide at the two sublethal concentrations led to a reduction of the longevity by 23.97% and 28.68%, while the fecundity was reduced by 52.81% and 56.09% respectively, in comparison to the control. The progeny of exposed individuals (F1 generation) showed a slower development and the oviposition period was also reduced. The fertility of the F1 generation decreased by 17.88, 44.03 and 51.69% when exposed to 1% and 10% of the LC_5, respectively. The results of the demographic growth estimates showed that the intrinsic rate of increase (rm) and the net reproduction rate (R_0) were lower in *C. septempunctata* populations that had been exposed to sublethal concentrations of imidacloprid (Xiao et al. 2016). Rahmani and Bandani (2013) demonstrated that thiamethoxam applications have not significant impact on the development time of the third stage of *Hippodamia variegata* Goeze (Coleoptera: Coccinellidae), an important predator of aphid and psyllid pests. However, the fourth nymphal stage, the pupae and the pre-position period were negatively impaired.

A further study evaluated the three active ingredients, on *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae), an important parasitoid of the tobacco whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae), and in laboratory tests showed over the 98% of mortality after 6 hours of the treatment at field dose (Wang et al. 2019). Kramarz and Stark (2003) evaluated the effects of a heavy metal (i.e. cadmium) in combination with the insecticide imidacloprid on the population growth rate of *Aphidius ervi* Haliday (Hymenoptera: Braconidae). Cadmium, alone or in combination with imidacloprid, had a negative impact on the beneficial insect because it reduced the growth rate of the parasitoid population by 77%. The sublethal toxicity of imidacloprid was studied on females of *Aphidius gifuensis* Ashmead (Hymenoptera: Braconidae) and a significant reduction of different biological parameters was found at sublethal doses including the reduction of both the parasitization capacity and the longevity (Kang et al. 2018).

Another study evaluated the sublethal effects of thiamethoxam and pirimicarb on an important predator, *Macrolophus pygmaeus* Rambur (Hemiptera: Miridae), and it was found that both the active substances affected insect demographic parameters (Rahmani 2016). Other studies conducted on *Podisus nigrispinus* Dallas (Hemiptera: Pentatomidae), a predator used for the control of *Gonipterus platensis* Marelli (Coleoptera: Curculionidae), showed an increase in the development time and a reduction in the weight when the insect fed over plants and prey treated with thiamethoxam (Torres et al. 2003).

A further study analyzed the lethal and sublethal effects of six insecticides towards several natural enemies used in the IPM context. With regards to sulfoxaflor, slight toxicity has been demonstrated on adults of *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and high toxicity against the fourth-age larvae of the coccinellid *Adalia bipunctata* L. (Coleoptera: Coccinellidae), two important aphid predators used in integrated management strategies. In particular, the most harmful compounds were sulfoxaflor and deltamethrin with cumulative mortality on the larval stage equal to 100%. Therefore, the authors suggested that the use of these active ingredients should be carefully evaluated when one of the two biological control agents is released and in general in the protection of beneficial arthropods (Garzón et al. 2015).

A similar conclusion was recently achieved by Taning et al. (2019) when analyzing the foraging behaviour of the bumble bee *B. terrestris* orally exposed to field-realistic concentrations of sulfoxaflor.

The toxicity of imidacloprid and fipronil was evaluated towards pollinator insects, in particular, the effect to a lethal dose (LD_{50}) and sublethal dose (1×500^{-1} of LD_{50}) of these two substances was assessed on the locomotor activity of the common honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). The results showed high toxicity to bees due to alterations caused to the locomotor functions of pollinators

(Bovi et al. 2018). The continuous decline of bees has become a very sensitive issue for the scientific community worldwide. This phenomenon has been partly attributed to the use of neonicotinoids, whose toxicity has been demonstrated against many pollinator insects, including honey bees, especially when applied in proximity to flowering crops (Collison et al. 2018). Gauthier et al. (2018) demonstrated that the exposure to low levels of neonicotinoids altered the retinoid-carotenoid system in bees, impairing their health. Research has also assessed the effects of thiamethoxam by exposing a colony of the bumble bee, *Bombus terrestris* L. (Hymenoptera: Apidae) to a wide range of concentrations up to 98 μg x Kg^{-1}. At the highest dose, the results showed that both the longevity of the adult hornets and the size of the brood (eggs and larvae) decreased. Otherwise, no sublethal effects were found in the case of dosage at lower levels (between 1 and 11 μg x Kg^{-1}) which correspond to the levels commonly found in the nectars of the treated crops (Laycock et al. 2014). Recently, Calvo-Agudo et al. (2019) showed how imidacloprid and thiamethoxam can be found on the honeydew produced by Hemiptera pests feeding on plants treated with these two compounds; more importantly, lethal and sublethal consequences for the Encyrtid parasitoid, *Anagyrus pseudococci* (Girault), and the hoverfly, *Sphaerophoria rueppellii* Zetterstedt were demonstrated. Despite the need of eco-friendly non-chemical biological control strategies, e.g., biopesticides (Campolo et al. 2017; Ikbal and Pavela 2019), synthetic insecticides still play a fundamental role in the management of harmful insect pests (Asplen et al. 2015; Mansour et al. 2018; Simon and Peccoud 2018). However, these toxicants can negatively affect non-target organisms such as natural enemies (i.e., predators and parasitoids) and pollinators, even at sublethal concentrations.

On the other hand, each agro-ecosystem has peculiar characteristics that should be taken into account for the planning of pest control strategies; therefore, it is necessary to refer to the specific agricultural context (Radcliffe et al. 2009). Nevertheless, the goal is always to reduce the use of pesticides to allow the establishment of beneficials by maintaining the phytophagous pest populations within tolerance thresholds (Higley 1996; Furlan 2014). For these reasons, in the IPM context, the study of interactions that take place between chemical inputs and entomofauna is necessary for understanding how different formulations impact the non-target organisms. These studies can be used to improve the selectivity of plant protection products currently marketed worldwide with the aim of enhancing the formulations by reducing their environmental impact. The research carried out in this work has revealed that there are more selective active ingredients than others. We have highlighted that imidacloprid has shown a lower impact, in terms of mortality of adults exposed to the active ingredient and reduction of the fertility of females of the *A. colemani* parasitoid, compared to the active ingredient thiamethoxam. The goal is, therefore, to increase the activity of this kind of research to provide farmers, technicians and operators in the agriculture industry with the possibility of being able to inform regarding the use of agrochemicals, with the aim of reducing reliance on those products with a negative impact and improving the environmental sustainability of agro-ecosystems.

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CHAPTER III.
Potential diet regimens for laboratory rearing of the harlequin ladybird

Abstract

Harmonia axyridis Pallas (Coleoptera: Coccinellidae) is an invasive generalist predator and it is considered a biological model to address several questions over different research areas. Laboratory colonies of the harlequin ladybird are often maintained on costly natural prey, and alternative dietary regimens are required to optimize the rearing of this species. We studied the influence of four different food regimens on the developmental and reproductive life traits of *H. axyridis*, including the body size and the demographic growth parameters. The tested diets were: *Ephestia kuehniella* Zeller eggs, a commercial mix of *E. kuehniella* and *Artemia* sp. cysts, a liver-based artificial diet and the natural prey, *Aphis gossypii* Glover. Larvae developed successfully and over the shortest time on moth eggs, while an opposite trend was found for the coccinellids reared upon the liver-based diet. The survivorship of the ladybird beetles fed on its natural prey and the mixture of lepidopteran eggs with crustacean cysts was similar. The tested food regimes differently affected the investigated life parameters of the harlequin ladybird. Our outcomes can contribute to optimizing the experimental rearing of this important predatory ladybird beetle.

Keywords: factitious food, insect rearing, *Ephestia kuehniella*, life table, cotton aphid

3.1. Introduction

Insect rearing has a wide range of aims concerning to the activity by which the production is required. Insect species reared for research applications in biological sciences involved a plethora of fields, from the most theoretical (e.g. evolutionary ecology) to the closely applied (e.g. ecotoxicology). Insect mass-rearing is also routinely conducted for agricultural applications in pest management programs (e.g. biological control applications and sterile insect technique), but also for pharmaceutical, animal food and commodity productions (Parker 2005; Rockwood et al. 2011; Van Huis 2013; Henry et al. 2015). Moreover, insect rearing can also involve population restoring of rare and threatened species besides the recreational and educational uses (Hogue 1987; Matthews et al. 1997).

The most important requirement of an insect rearing is the continuity of insect production, because the maintenance of the balance between the involved trophic levels may result challenging because of discontinuity constraints (Vanderzant 1974; Singh 1977). These aspects become particularly relevant when the natural diet is seasonally unavailable or difficult to maintain (e.g. prey or host for predators and parasitoids) (Thompson 1999). To overcome this aspect, insect colonies are generally fed with factitious food that may represent a viable alternative to natural food (Reinecke 2013; Cohen 2015). However, a substitute food should have at least equal characteristics of the natural diet to ensure the survival and the reproductive capacity of the species as well as its behaviour (Singh 1983). Among the other required properties, an alternative diet should be also economically convenient, do not present any risks for human health, be easily prepared and stored for a long time without changing in the composition (Burton 1970; Singh 1977; 1983).

Coccinellid predators represent an important ecological group for the control of sap-sucking insect pests; for this reason, they have received huge attention in the biological control applications in several agricultural crops (Hodek et al. 2012). The harlequin ladybird *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae) is native to Asia and since the last century has been introduced in several countries for the control of aphid and coccid pests (Koch 2003). However, during the past decade, the species had led growing concerns because of the multiple side effects it may cause and more recently was regarded as an alien invasive organism (Roy et al. 2016). The negative impacts of *H. axyridis* mostly concern the environmental risks (i.e., competitive displacement of native coccinellids) and the bother of human activities, which includes the wine fault ladybug taint (LBT), the phytophagy over ripening fruit, the overwinter aggregations and the triggering of allergic reactions (Koch 2003, 2004; Albright et al. 2006; Pickering et al. 2005; Mirande et al. 2015). For these reasons, the commercial production of the harlequin ladybird for biocontrol applications was suspended in Europe (van Lenteren 2012) but, the species is still routinely applied in China for Integrated Pest Management programs (Wang & Shen 2002).

Despite of the negative impacts, *H. axyridis* has received a growing interest as biological model to study the invasion process as alien species (Lombaert et al. 2010; Brown et al. 2011; Raak-van den Berg et al. 2017), to investigate its trophic interactions with native coccinellids (Hoogendoorn & Heimpel 2002; Burgio et al. 2008; Cabrera et al. 2018), or simply for investigating various aspects of its bio-ecology (Legrand et al. 2019; Ovchinnikov et al. 2019). More recently, the species has been also chosen as a non-target organism for the risk assessment of pesticides and transgenic crops as well (Cabrera et al. 2017; Meissle & Romeis 2017;

Wang et al. 2018). Therefore, the positive role of *H. axyridis* as predator in natural and agricultural contexts has been evaluated and further investigations are suggested to solely promote its positive effects as beneficial organism (Riddick 2017). In this framework, the coccinellid is receiving growing attention and its laboratory rearing appears still necessary for the above reported aims.

Over the years, numerous studies have investigated potential alternative food in optimizing the rearing of *H. axyridis*, including natural prey (i.e., aphids), lepidopteran eggs, *Artemia* sp. cysts, pollen and their combination, besides different protein-based diets (Hongo & Obayashi 1997; Abdel-Salam & Abdel-Baky 2001; Dong et al. 2001; Tsaganou et al. 2004; Sighinolfi et al. 2008). To the best of our knowledge, the combination of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs and *Artemia* spp. cysts were not tested for the rearing of the species. On the other hand, artificial diets that include animal proteins generally give scarce rearing performances (Sighinolfi et al. 2008). Therefore, research on substitute food that satisfies both the nutritional requirements and the cost-effectiveness for the laboratory culture of *H. axyridis* appears still necessary and should be empirically evaluated.

The aim of our study was thus to compare alternative food regimens to sustain the survival and the reproduction of *H. axyridis*. Life-history traits of the coccinellid predator were assessed under standardized laboratory conditions. Our research can contribute to implementing knowledge about the rearing of the harlequin ladybird and its optimization for further research perspectives.

3.2. Materials and methods

3.2.1 Insect culture

Laboratory colony of *H. axyrids* f. *succinea,* was established with fifty adults collected during a field survey on citrus orchards conducted in Sicily (Italy) during the spring season in 2016. Coccinellids were morphologically identified with the key proposed by McCornack (2007). The specimens were reared for three generations on zucchini pots infested by *Aphis gossypii* Glover (Hemiptera: Aphididae) inside a fine mesh net insect cage. Plant pots were infested in a separate cage and weekly provided to the colony with a sugary source of honey-water solution (1:1). Zucchini seedlings were grown in 1L pot at greenhouse conditions following the common agricultural practices and excluding any pesticide treatment. The rearing and the performed experiments were kept at standard environmental conditions inside a climatic chamber (Refrigerated incubator model IRE-475, Raypa® R. Espinar, s.l. Spain) held at 25 ± 1°C, 65 ± 5% R.H. and 14L:10D h photoperiod.

3.2.2. Food regimens

We investigated the effect of four different diet regimens on the developmental and reproductive parameters of the harlequin ladybird. The food we tested were: (i) *E. kuheniella* devitalized frozen eggs (EE) purchased from Bioplanet (Cesena, Italy); (ii) a mixture 1: 5 of *E. kuheniella* and *Artemia* sp. cysts commercially traded as Entofood® (EF) (Koppert, The Netherlands); (iii) a meridic artificial diet (AD) with a slight modification following the recipe of Haramboure *et al.* (2016) (see Table 1 for the composition), and (iv) the cotton aphid, *A. gossypii* (AG), as natural living prey that was also used as rearing prey for the colony. The devitalized *E. kuehniella* eggs and Entofood were stored at –20°C until the experimental use.

3.2.3. Effect of diet regimens on juveniles

In the first experiment, *H. axyridis* coetaneous fresh egg batches (≤12 hours) were transferred with a wet and soft paintbrush from the culture to a Petri dish (6 cm diameter), to allow the hatching. Newly hatchlings were individually transferred to an aired plastic box (2 x 2 x 2 cm), in which food was daily offered *ad libitum* on a filter paper disc. For all treatments, a water-honey (1:1) source was also supplied through a soaked cotton wad placed into the described arena. Every day the food was renewed and the experimental arena was cleaned. The parameters of the immature developmental (i.e. development time and survival) were recorded twice a day (i.e. every 12 hours). The period between two successive molts was defined as the developmental time of a single-stage, while the whole developmental time was defined as the period between the egg hatching and the emergence of the adult stage. A hundred newly hatching larvae were reared for testing each diet regimen.

3.2.4. Effect of diet regimens on adults

Newly emerged adults, developed feeding upon the four tested diets, were sex-determined following the key proposed by McCornack (2007) and weighted on a 10-mg-sensitive microbalance (Mettler-Toledo GmbH Giessen, Germany). The adult body length (i.e., the distance between the labrum and the distal part of the elytra) was measured under a stereomicroscope (Leica M205FA) through a digital measuring tool (Leica Application Suite X, Leica Microsystems, Switzerland Ltd.).

To study the effect of the food on the longevity and the reproductive traits of the coccinellids, adults of *H. axyridis* that received the same dietary regimes at juvenile stage were coupled (i.e. 1 female and 1 male) and reared in a ventilated Petri dish (10 cm in diameter) and fed maintaining the same dietary regimen for the whole lifetime. Also in this arena, the dietary regimen was daily provided *ad libitum*, the experimental arenas were cleaned daily, and the same honey-water solution source was offered to the adults for all the treatments. A Z-fold filter paper was also fixed into the experimental arena as an artificial oviposition substrate. Oviposition and egg-hatching of each female were recorded for 30 days since the first egg-laying event, while the longevity was counted for both sexes. The fecundity (i.e. number of laid eggs) of the females fed with different food regimens was daily recorded from the first egg-laying event. Each fresh egg-batch was isolated in a single Petri dish and daily checked for two weeks to assess the fertility (i.e., the number of hatched eggs).

3.2.5. Data analysis

Dataset was first proved for the normality distribution (Shapiro–Wilk test) and the variance homoscedasticity (Levene's test). The survival response of coccinellid juveniles and adults to different dietary food regimens was analyzed using the Kaplan-Meier procedure followed by Log Rank (Mantel-Cox) test to compare the survival curves among the treatments. The effect of the diet regimens on the adult body weight and the adult body length for both sexes were analyzed performing a General Linear Model (GLM). The reproductive parameters were analyzed conducting a Kaplan–Meier estimate for the preoviposition period and a GLM for the fecundity and the fertility data. Differences among the dietary treatments in the performed GLMs were highlighted by Tukey's HSD test.

Age-specific survival and progeny values of each female were used to construct life tables and to calculate the following demographic growth parameters (Jervis 2012, Biondi et al. 2013). Net reproductive rate:

$$R_o = \sum l_x m_x$$

expresses the growth rate of a population per generation, where lx and mx are respectively the age-specific survival and the age-specific female progeny produced at the individual pivotal age (x). The mean generation time:

$$T = \sum \frac{xl_x m_x}{R_o}$$

is the average interval between births of one generation from the next one. The intrinsic rate of increase

$$r_m = \sum e^{r_m x} l_m m_x$$

that describes the growth potential of a given species, the Finite capacity for increase:

$$\lambda = e^{r_m}$$

that is the rate at which a population will multiply itself per unit of time,

Doubling time:

$$DT = \frac{log_e 2}{r_m}$$

the time, expressed in days, required for a population to double its size at a given r_m. Statistical analysis was carried out on IBM® SPSS® Statistics for Macintosh, Version 23.0.0.0 software (IBM Corp. Released 2015. Armonk, NY: IBM Corp.).

3.3. Results

3.3.1. Effect of diet regimens on juveniles

The food regimens significantly affected the survival of the immature stages (from egg to adult) of the harlequin ladybird (F=5.430; d.f.=3, 400; *P* <0.001). Among the tested food, the highest survivorship was highlighted for coccinellid larvae when they fed upon AG (87.7%), while an opposite trend was recorded for the AD (65.6%) (Table 2). The developmental time was significantly affected by the food regimes as supported by the Kaplan-Meier estimation (Mantel-Cox logrank test, χ^2 = 187.15; d.f.=3, 397; *P* <0.001). The time from egg to adult was shorter when larvae fed EE (~14.07 days) compared to AD treatment (~21.67 days); however, no statistical differences were found between the control group (AG) and the EF dietary regimen which the juvenile development lasted about 15 days.

3.3.2. Effect of diet regimens on adults

The tested foods significantly affected the adult body weight in both sexes (F=79.661; d.f. = 3, 136; *P* <0.001 for females; F=86.309; d. f. = 3, 168; *P* <0.001 for males). Compared to the natural prey (AG), *Harmonia axyridis* adults had the significantly highest weight when cultured upon EE (33.62±0.5 mg) that was almost twice than the weight for coccinellids reared over AD (17.95±0.51 mg). Conversely, no statistical differences were found in the weight between females reared on EE or EF, as well as for the males reared on AG or EF. The adult body length was significantly affected by the different tested food regimens (F= 3.204; d.f. = 3, 70; *P* =0.029 for females; F=10.254 ; d.f. = 3, 70; *P* <0.001 for males). The adult longevity analysis showed significant differences among the tested food regimes for both sexes and, in general, female adults lived longer than males (Table 3). In particular, adult females fed with EE and EF lived significantly longer compared to individuals cultured upon AG. The dietary regimens significantly affected all the studied reproductive traits: preoviposition period (χ^2 = 61.44; d.f.=3, 99; *P* <0.001), number of laid eggs (F=18.42 d.f.=3, 98; *P* <0.001) and fertility (F= 15.05; d.f.=3, 90; *P* <0.001). The Kaplan-Meier estimation revealed a significant delay in the preoviposition period of coccinellids fed with EF and AD that was 2.8 and 2.9 times longer in comparison with beetles reared on the cotton aphid, respectively. Although adult females fed with EE began to oviposit one week later than females reared on *A. gossypii*, they showed the highest fecundity in comparison to the dietary treatments, in which the mean fecundity values ranged from 60.7 to 236.28 eggs per female in 30 days. Although the fertility rate did not vary significantly between adult females fed with EE and the control group (AG) (71.96±3.91% and 67.26±3.37%, respectively), this reproductive parameter was double compared than for coccinellids developed on EF and AD (Table3).

According to the estimation of the demographic growth indexes, the highest value of net reproductive rate (R_o) was obtained for coccinellid reared on moth eggs, followed by aphid prey, moth eggs in combination with brine shrimp cysts and artificial diet (Table 4). The mean generation time was faster for coccinellids fed upon its natural prey and slower over the AD. The higher values of the intrinsic rate of increase (r_m) and the finite rate of increase (λ) were estimated for females fed on natural prey and EE if compared as resulted in EF and AD.

Table 1. Row composition of the factitious tested foods.

Food	**Composition**	**Amount (%)**	**Unitary cost per 0.1 x Kg^{-1} ($)**	**Manufacturer**
Moth eggs **(EE)**	*Ephestia kuehniella* eggs	100	200	Bioplanet Cesena, Italy
Entofood **(EF)**	*Artemia* sp. cysts	83		Koppert B.V. Berkel en Rodenrijs, The Netherlands
	Ephestia kuehniella eggs	17	50	
Artificial diet **(AD)**	Sucrose	24	2	
	Ground beef	16		
	Liver	16		
	Egg white	12		
	Egg	8		
	Wheat germ	8		
	Honey	6		
	Brewer’s yeast	5		
	Condensed milk	3		
	Multivitamin	0.5		
	Sodium Benzoate	0.4		
	Nipagin	0.1		

Table 2. Juvenile (egg-adult) survivorship, developmental time, and adult body size parameters of *Harmonia axyridis* fed on different diets. Means (±SE) followed by the same letter within a column are not significantly different (*P* >0.05; Tukey's post hoc test).

Diet	**Juvenile survival (%)**	**Developmental time (h)**	**Weight (mg)**		**Length (mm)**	
			female	**male**	**female**	**male**
Aphis gossypii **(AG)**	87.70±3.90a	370.96±7.25a	27.77±0.76a	24.62±0.62a	7.85±0.13a	7.15±0.12a
Moth eggs **(EE)**	82.10±3.80ab	337.70±9.49b	33.62±0.50b	28.59±0.45b	8.14±0.25ab	7.79±0.10b
Entofood **(EF)**	78.40±.4.00b	358.85±10.15a	31.75±0.81b	24.42±0.80a	7.63±0.15a	6.83±0.16ac
Artificial diet **(AD)**	65.60±4.10c	520.26± 23.11c	17.95±0.51c	15.87±0.48c	6.69±0.31ac	5.80±0.08c

Table 3. Reproduction parameters and longevity of *Harmonia axyridis* fed with different diet regimens. Means (± SE) followed by the same letter within a column are not significantly different (*P* >0.05; Tukey's post hoc test).

Diet	**Preoviposition period (d)**	**Fecundity in 30 days (# eggs/female)**	**Fertility (%)**	**Longevity (d)**	
				Female	**Male**
Aphis gossypii **(AG)**	13.9±0.44a	236.28±17.55a	67.26±3.37a	51.44±3.19a	55.41±3.97a
Moth eggs **(EE)**	21.34±3.39b	387.77±48.66b	71.96±3.91a	96.31±11.32b	91.14± 9.33b
Entofood **(EF)**	38.55±4.72c	154.89±42.81ac	36.24±6.03b	83.37±9.45b	74.07±9.77c
Artificial diet **(AD)**	40.7±3.54c	60.70±8.88c	37.09±5.75b	51.67±11.90c	50.92±10.27c

Table 4. Estimated demographic growth parameters of *Harmonia axyridis* developed towards four different diet regimens.

Demographic growth parameters	**Diet regimen**			
	Aphis gossypii **(AG)**	Moth eggs **(EE)**	Entofood **(EF)**	Artificial diet **(AD)**
Net reproductive rate (Ro)	94.861	168.460	37.980	4.727
Generation time (T) [day]	39.262	47.034	59.398	67.493
Intrinsic rate of increase (rm)	0.116	0.109	0.061	0.0230
Finite rate of population increase (λ)	1.123	1.115	1.063	1.023
Doubling time (DT) [day]	5.978	6.359	11.32	30.116

3.4. Discussion

Several factors can influence the developmental and reproductive traits of coccinellid beetles but food has undoubtedly a key role on these biological parameters (Hodek et al. 2012). The study of the effect of different food regimes on life table parameters is important not only to understand the predator population dynamic but also to optimize the insect rearing conditions (Morales-Ramos et al. 2013). Our study has revealed that *H. axyridis* can complete its development and to reproduce differently over the tested dietary regimes which included the moth eggs, moth eggs combined with *Artemia* sp. cysts, a liver-based artificial diet and the cotton aphid *A. gossypii* as natural and colony-rearing prey. The estimated population growth indexes of the harlequin ladybird cultured upon different diet differed significantly. These results are particularly relevant regarding the role of food in predator nutrition for assessing effects in terms of population demographics in insect rearing optimization.

In our experiments, when the harlequin ladybird was reared on *E. kuehniella* eggs showed significantly higher survival rate, shorter developmental time and increased body size in terms of weight and length, compared to the alternative tested dietary regimes. Moreover, also the investigated reproductive parameters highlighted that the species reproduces optimally when fed with lepidopteran eggs and this result is fully in accordance with previous studies. Many authors proved that *H. axyridis* developed successfully on *E. kuehniella* eggs as factitious prey (Specty et al. 2003; Berkvens 2008; de Castro-Guedes et al. 2016). Similar highest rearing performances were also reported when *H. axyridi*s was cultured upon the eggs of Angoumois grain moth, *Sitotroga cerealella* (Olivier) (Lepidoptera: Gelechiidae) (Chen et al. 2012; Abdel-Salam and Abdel-Baky 2001). The larval survivorship and development, body size and fecundity of the coccinellid were increased in comparison to the examined dietary regimes because lepidopteran eggs are richer in protein and lipids rather than carbohydrates (Specty et al. 2003).

However, according to Abdel-Salam and Abdel-Baky (2001) *H. axyridis* developed and reproduced optimally when reared on fresh eggs instead of previously-frozen eggs of *S. cerealella* this can be due to the degradation of nutritional elements caused by lower temperature. Therefore, since we used frozen *E. kuehniella* eggs, less efficient rearing performance can be expected in comparison to fresh lepidopteran eggs. However, this aspect needs to be further investigated.

We also reared the harlequin ladybird on commercial food for entomophagous predators which is made by *Artemia* sp. cysts as main ingredient and a low proportion of *E. kuehniella* devitalized eggs. This food is routinely used in protected crops as an alternative food source for mirid predators to sustain their population growth when prey is lacking or scarce (Vandekerkhove et al. 2009). When *H. axiridis* larvae fed upon the mixture of moth eggs and brine shrimp cysts (i.e. EF), the lowest proportion of individuals reached adulthood in comparison with *E. kuehniella*. This effect can be due because of the low water content of this food. Young coccinellid larvae generally use the extra-oral digestion for prey-consumption and this process requires water availability in the food. Therefore, continuous water depletion may have cause mortality due to energy losses for prey consumption (Hodek et al. 2012). Although we provided an extra water source, this may not solely compensate the body water loss caused by the digestion, as previously suggested by De Clercq et al. (2005). The authors found that larvae of *A. bipunctata* reared on hydrated or dry decapsulated cysts of the brine shrimp *A. franciscana* or the lyophilized meat and liver diet regimes did not reach adulthood. According to the study, the low survival rate of immature stage was probably own to the lack of physiologic accessibility to food by coccinellids. The authors reported that *A. franciscana* cysts tended to lump together and as they dry out, the mass hardened and impeded to be process by the predator larvae. Nevertheless, *Artemia* sp. cysts mixed with *E. kuehniella* egg had a better impact in comparison to the artificial diet and the natural prey. We did not observe significant differences in the developmental time of larvae between the cotton aphid and the EF. Therefore, we can suggest the latter as a valid substitute for the natural prey, since it is commercially available because and less labor is needed.

A similar trend in the juvenile survival rate was already reported for *H. axyridis* reared on the Mediterranean moth eggs and *Acyrthosiphon pisum* as natural prey (Specty et al. 2003). By contrast, *H. axyridis* was able to reproduce successfully over *Cinara atlantica* (Wilson) (Hemiptera: Aphididae), *Brevicoryne brassicae* (L.) (Hemiptera: Aphididae), and frozen eggs of *E. kuehniella* (Zeller) (de Castro-Guedes et al. 2016). By contrast, Tsaganou et al. (2004) evaluated the influence of three aphid species, including *A. gossypii*, on the development of the harlequin ladybird. Like our findings, *H. axyridis* successfully developed on *A. gossypii*, but larvae did not reach adulthood when fed upon *Megoura viciae* Buckton (Hemiptera: Aphidoidea). The unsuccessful rearing of coccinellids on aphid prey can be caused by the deficient in nutritive quality value of this prey. Besides, aphids can be an unsuitable prey because of the inclusion of plant-derived allelochemicals which may negatively affect the physiology of coccinellid beetles (Hodek et al. 2012).

Among the tested food, the artificial diet was not fully suitable for the larval development of the harlequin ladybird, because only 75% of larval stages reached adulthood. Similarly, the larval development time was longer compared to the tested food, with an average of 21 days. As a consequence, this result means that the artificial diet we tested was not nutritionally adequate. We choose this artificial diet because Haramboure et al. (2016) proved that this food had optimal nutritional properties to improve the mass-rearing of the predatory lacewing *Chrysoperla externa* (Neuroptera: Chrysopidae) despite the low-cost compared to aphid natural prey and/or alternative factitious food. According to Riddick (2009), one of the animal protein-based artificial diets (where the chicken was the major constituent) was able in maintaining oviposition rates of *H. axyridis* at the same level to those of cohorts cultured over factitious or natural prey. Previous studies confirmed that liver-based artificial diets decreased survival rates, lengthened larval development time and reduce the adult body size of the harlequin ladybird (Sighinolfi et al. 2008; Chen et al. 2012). Nevertheless, Ali et al. (2016) demonstrated that an artificial diet can improve the fitness of the species by reducing the development time and by increasing the emergence rates. In our case, the failure of artificial diet in comparison with natural prey and factitious food can be due to the lack of nutritional elements such as amino and fatty acids (Sighinolfi et al. 2008). For this reason, the biochemical analysis of the artificial diet is required for the assessment of quality parameters to enhance its composition. Moreover, because symbionts may influence predator ability to absorb specific molecules from the diet, the use of preservatives that do not alter symbionts should be taken in account (Riddick 2009). Besides that, although the artificial diet was expensive in terms of both labor and ingredients, it is cheaper if compared to the costly EE and the rearing of natural prey. Hence, we suggest using the artificial diet for adult maintenance in case of a shortage of natural prey.

We can also suggest to rear young instar on artificial diet and then supplied coccinellid adults with *E. kuehniella* eggs since it has been proved that females fed with artificial diet during their larval life showed a significant fitness recovery when fed with moth eggs over their adulthood (Sighinolfi et al. 2008). Many polyphagous coccinellids have a ‘mixed’ feeding habit which allows the species to select the most favorable balance of important nutrients from plant and animal sources (Hodek et al. 2012). Similarly, the harlequin ladybird can utilize a wider range of prey species, compensating thus suboptimal diet of factitious prey by supplemental feeding on plant products, such as flower pollen (Berkvens et al. 2008), having thus a considerable plasticity to different food sources and compensate accordingly.

Harmonia axyridis showed a good adaptability to various foods at a biological level by completing successfully their lifecycle on different food, as described for other entomophagous insect predators (De Clercq et al. 2005; Haramboure et al. 2016). Nevertheless, the adaptation to suboptimal food may negatively affect the biological traits of the harlequin ladybeetle. As a consequence, chemical food analysis associated with the biochemical analyses of the coccinellid should be taken into account to prove the efficiency of the food conversion (Specty et al. 2003).

The estimation of demographic parameters is important for the evaluation of the effect of multiple external factors (e.g. food, pesticides) on the biological performances of natural enemies (Jervis 2012; Biondi et al. 2013; Benelli et al. 2017). In our study, the demographic parameters calculated for the harlequin ladybird changed according to the different tested food regimes. The net reproductive rate (Ro) had the highest value when the coccinellid fed on EE; however, this index dramatically decreased when females were cultured upon the AD. We obtained a net reproductive rate lower (168.46) than the index calculated by Abdel-Salam & Abdel-Baky (2001) on *H. axyridis* fed with frozen eggs of *S. cerealella* (234.96). By contrast, our estimated intrinsic rate of increase (rm) was similar to the findings of de Castro-Guedes et al. (2016) who reared *H. axyridis* on *E. kuheniella* eggs. The same authors found also similar reproductive indicators when the species fed upon two aphid prey.

To fully understand the value of our investigated diets for rearing *H. axyridis*, further experiments should be addressed towards the effect of the diet regimens on the predator fitness in communal rearing systems and over various generations. For example, the culture of the harlequin ladybird upon *Ephestia* eggs over many generations may negatively affect the searching activity for prey and the recognition capacity of chemical cues by larval stages (Morales-Ramos et al. 2013). We also agree with the importance of maintaining a standard experimental approach in the evaluation of different diet regimens, because differences about experimental conditions (e.g. temperature, prey condition,) may result difficult to correlate the scientific outputs between other investigations (Raak-van den Berg et al. 2017).

Nowadays, one of the main challenges in coccinellids production is a cost-effective technique because many of the current rearing methods rely on the rearing of a tri-trophic system (host plant, herbivore and predator) which is costly and labor-intensive. Overall, we propose the combined use of *E. kuehniella* eggs with *Artemia* sp. cysts for the laboratory culture of *H. axyridis,* as it allows the constant rearing production and it is promptly commercially available, as it requires minimal cleaning labor, comparing the natural prey and it is definitely cheaper than the costly routinely used lepidopteran eggs.

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CHAPTER IV.

Can low concentrations of systemic insecticides affect the voracity of the harlequin ladybird?

Abstract

Systemic neurotoxic insecticides are widely used to control aphid pests worldwide and their potential non-target effects on aphid predators are often unknown. Behavioural response linked to biological control services is crucial when assessing the compatibility of chemicals with biocontrol organisms. This is particularly relevant for insecticides at low and sublethal concentrations. We studied the acute toxicity and the sublethal effect on the voracity of the generalist predator *Harmonia axyridis* (Coleoptera: Coccinellidae) of three systemic insecticides routinely used against aphids. The tested insecticide concentrations where the Lethal Concentration 50% $(LC)_{50}$, the LC_{20} and the LC_1 estimated for the target pest *Aphis gossypii* (Hemiptera: Aphididae). The survival and the voracity differed among the tested chemicals and concentrations, but only thiamethoxam at LC_{50} caused the highest mortality. In most of the pesticide-concentration combinations, the predators showed a density independent functional response, while an inverse density dependence of the prey consumption rate was observed for coccinellids exposed to sulfoxaflor and thiamethoxam at their lowest concentration. The estimated parameters, i.e. the attack rate and the prey handling time, were affected at higher concentrations by both imidacloprid and sulfoxaflor. Our findings stress the importance of carefully evaluating the potential behavioral side effects of insecticides on natural enemies in the risk assessment, even at very low concentrations, of insecticides used in sustainable pest control programmes.

Keywords

Ecotoxicology, cotton aphid, sulfoxaflor, neonicotinoid, biological control, IPM.

4.1. Introduction

The control of agricultural pests often relies on chemical pesticides, but the overuse of these substances cause numerous undesired side effects (Köhler & Triebskorn 2013). The major consequences regard resistance phenomena for key pests, outbreaks of secondary pests, increases in production costs, human health risks and disruption of the ecosystem functioning and services (Wilson & Tisdell 2001; Alavanja et al. 2004, Whalon et al. 2008; Lu et al. 2012). In the Integrated Pest Management (IPM) context, the harmonization of different control tools for reducing pest population to tolerable levels is the main goal and the compatibility of the chemical tool towards the preferred biological means is crucial to reach this purpose (Radcliffe et al. 2009). On this basis, the risk assessment of potential pesticide side effects on beneficial arthropods has become essential in developing successful IPM strategies. The evaluation of non-target effects of pesticides on beneficial organisms generally includes both lethal and sublethal effects (Desneux et al. 2007). The former investigates the acute toxicity and it is used as first-step for the pesticide-selectivity assessment, giving thus immediate feedback on the direct mortality caused by the pesticide (Youn et al. 2003). The latter takes into account the physiological and behavioural impairments due to the non-lethal dose/concentration/rate of the chemical to the non-target species, which may lead to the reduction of the ecological services the organism provides.

During the last decade, a growing research literature body has been addressed towards the study of pesticide sublethal effects (Desneux et al., 2007, Guedes et. 2016). Taking into account that in the field natural enemies can be exposed to pesticides directly and/or indirectly through the contact over spray foliar residues when foraging on the crop and/or the contaminated diet when feeding on food (e.g. pollen, prey), different bioassays mimicking a realistic field-scenario of exposure for the risk assessment evaluation on non-target arthropods have been developed (Biondi et al. 2012a, 2012b, 2013, Tan et al. 2012; Abbes et al. 2015; Passos et al. 2018, Calvo-Agudo et al. 2019, Taning et al. 2019). For the risk assessment, insecticides are generally evaluated to non-target organisms at the dose/concentration/rate suggested and/or applied in the field to control the target pest (i.e., according to the label indications). However, new research on plant-systemic insecticides is moving towards the effect of pesticide at low dose/concentration/rate on natural enemies, because this condition frequently occurs in the field after pesticide application (Guedes et al. 2016).

Systemic insecticides are largely used for the control of economically important crop sap-feeding pests (e.g. aphids, whiteflies, mealybugs) because of their property to translocate throughout plant tissues, regardless the application method and the route of entry. Moreover, because sap-feeding pests can also transmit plant viruses, systemic insecticides contribute to controlling insect vectors of plant diseases (Jeschke et al. 2011, Simon-Delso et al. 2015; Wang et al. 2017; Mohammed et al. 2018). Insecticides with systemic properties are mostly represented by neonicotinoids with an over $4.6 billion estimated market value (Sparks & Nauen 2015). Neonicotinoids generally act as nicotinic acetylcholine receptors agonists (nAChRs) in the insect central nervous system and are classified in different-generation groups depending on variation in the molecular structure (Simon-Delso et al. 2015). Also the newly developed sulfoxamine insecticide class, commercialized as sulfoxaflor, although it differs from neonicotinoids (Sparks et al. 2013), could be considered a four-generation neonicotinoid because of the similar mode of action (Cutler et al. 2013, Simon-Delso et al. 2015). The success and the widespread use of neonicotinoids over the last three decades was essentially due to the lower binding efficiency to vertebrate compared to invertebrate receptors, which make these compounds selective for fish and mammals, the high persistence and water solubility associated with the systemic nature that allow versatile applications (e.g., seed treatment and spray) (Simon-Delso et al. 2015, Sparks & Nauen 2015). Nevertheless, the overuse of systemic insecticides has raised concerns over the environmental risks because of the ecological services they may disrupt (Chagnon et al. 2015). A plethora of studies collectively recognized the negative impacts of neonicotinoids on natural enemies and in particular on pollinators (Cloyd and Bethke 2011, Blacquiere et al., 2012). For these reasons, the outdoor application of three neonicotinoid insecticides (i.e., imidacloprid, clothianidin and thiamethoxam) has been recently banned in Europe, but their use in greenhouse crops is still authorized (Official Journal of the European Union, 2018).

Coccinellid predators play a key role in controlling several pests across multiple agricultural crops and the multicolored Asian lady beetle, *Harmonia axyridis* Pallas (Coleoptera: Coccinellidae), widely contributes in suppressing sap-sucking pests for its widespread presence in both natural and agricultural landscapes (Hodek et al. 2012). Even though the harlequin ladybird has been considered a noxious invasive organism owing to the several undesired effects it may cause (Roy et al. 2016), its positive role as biocontrol agent remains undiscussed (Seko et al. 2014; Riddick 2017). To the best of our knowledge, few studies have investigated the effect of non-lethal concentrations of neonicotinoid insecticides on the behaviour, and in particular on the functional of coccinellid predators (He et al. 2012; Martinou & Stavrinides 2015; Yao et al. 2015; Wang et al. 2018). Such an attribute is a fundamental biological trait for the estimation of prey depletion rates (i.e., a good proxy for biological control services) and prey population dynamic predictions (Jervis 2012).

Within this framework, we investigated the impact of low concentrations of systemic insecticides routinely applied for the control of the cotton aphid *Aphis gossypii* Glover (Homoptera: Aphididae) on the widespread predator *H. axyridis*. For this, we assessed the acute toxicity of Lethal Concentrations (LC) of three neurotoxic aphicides previously estimated in a companion study (see chapter II) for the target pest by exposing the coccinellid on sprayed plants infested by *A. gossypii*. The functional response of the survived exposed predators was estimated by offering them several densities of a substitute prey for evaluating the sublethal effect of these insecticides on the predation capacity of the harlequin ladybird. This work may also provide the basis for future guidelines in optimizing IPM programs involving the protection of natural enemies and the use of systemic insecticides for the control of sap-sucking pests such as the cotton aphid.

4.2. Materials and methods

4.2.1. Biological materials

Laboratory colonies of *H. axyrids* and *A. gossypii* were established by collecting several specimens from a field survey during the spring season on citrus orchards and zucchini crops, respectively, in Sicily (Italy) in 2015. Individuals of the harlequin ladybird were reared on cotton aphid-infested zucchini plants, which were weekly offered within a fine mesh net plastic cage with a honey solution source. The cotton aphid culture was maintained inside ventilated plastic cages (50 x 50 x 50 cm) by providing weekly zucchini seedlings (cv "Bianca di Trieste") grown on 2L pots in greenhouse conditions following the best routine agricultural practices and avoiding any pesticide application. Insect rearing and the following experiments were kept at standard environmental conditions on a climatic cabinet (Refrigerated incubator model IRE-475, Raypa® R. Espinar, s.l. Spain) held at 25 ± 1°C, 65 ± 5% RH and a 16L:8D photoperiod.

4.2.2. Tested insecticides and predator exposure

We tested three commercial formulations of systemic insecticides having the same mode of action (Nicotinic acetylcholine receptor agonists) and belonging to the chemical groups of neonicotinoids (imidacloprid and thiamethoxam) and sulfoximines (sulfoxaflor). The use of these insecticides is authorized for the control of the cotton aphid on various crops (including zucchini crop) in different countries worldwide and they were prepared following their label guidelines (see Table 1). To evaluate the potential side effects of the three systemic insecticides at low concentrations we used the LC_{50}, LC_{20} LC_1 estimated for *A. gossypii* in a companion study carried out in the same experimental condition of the present study (see chapter II).

4.2.3. Lethal toxicity

The lethal effect of the three concentrations of systemic insecticides to *H. axyridis* was studied by emulating a common field scenario in which the predator is simultaneously exposed to pesticide residues through contact, on both treated-plants and treated-prey, and by the ingestion of contaminated prey (i.e. aphid prey). Newly emerged and unmated females were exposed for three days to pesticide residues of the chosen aphicides at their LC_{50}, LC_{20} and LC_1 on aphid infested zucchini plants.

Through preliminary observations, three zucchini pots infested by mixed cotton aphid colonies (containing approximatively 1000 aphids per pot) were considered optimal to satisfy for three days the feeding uptake of ten harlequin ladybird females (that represented a replicate). Aphid infested zucchini pots were sprayed with the insecticidal solutions until runoff from a distance of 0.5 m through the hand sprayer and were left to dry for 1h in laboratory conditions. Per each replicate, ten females (one to three day old) of the harlequin ladybird were exposed to fresh pesticide residues on treated infested zucchini plants (i.e. three pots) for 3 days inside a ventilated and screened with fine mesh net arena. After the exposure period, predator mortality was assessed. Coccinellids were considered dead when they did not react after being touched with a paintbrush. Two control treatments were also included in this bioassay: an untreated control of infested plants sprayed with water and a blank control of clean and not infested zucchini pots sprayed with water. The first control was meant to evaluate the pesticide-concentration effect on the survival of the predators (i.e. acute toxicity); the latter had the function to include the starvation effect in voracity assessment (see *Functional response*), thus validating a potential antifeedant effect of pesticides on the survived predators. Between 8 and 10 replicates were carried out per each treatment.

<u>*4.2.4. Sublethal effects on functional response*</u>

The functional response to prey density of the adult females survived to the insecticide exposure was estimated for evaluating the effect of systemic insecticides at low concentrations on the voracity of the coccinellids. Each survived female was individualized and tested by offering five prey densities of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) devitalized eggs (Koppert, The Netherlands) inside a ventilated Petri dish (8 cm in diameter) for 24h. The following prey densities were chosen: 200, 400, 600, 800, 1000 eggs / female / 24 hours. The factious food-prey was chosen to standardize the whole experiment, because a potential aphid prey can change numerically over 24h period due to reproduction (personal observation). Moreover, this food source is considered highly suitable for *H. axyridis* development (see Chapter III).

The lepidopteran eggs were counted under the stereomicroscope and then placed in the experimental arena before the release of each adult female. Predators were maintained in a climatic cabinet at the previous controlled environmental conditions for the whole duration of the experiment (i.e. 24 h). The number of non-eaten prey (i.e. residual eggs) was counted under a stereomicroscope to quantify the predator voracity. The number of eaten prey (i.e. preyed eggs) was obtained through the difference between the number of offered prey and the number of non-eaten prey. Between 10 and 15 females (i.e. 10-15 replicates) were tested per each prey density and per each concentration/insecticide combinations and for the two control treatments.

4.2.3. Data analysis

All raw datasets were tested for normality and homogeneity of their variance, using Kolmogorov-Smirnov *D*-test and the Cochran test, respectively. The effect of pesticides, concentrations and their interaction on the mortality of predators after 3 days of exposure to pesticide residues on plants, was analyzed through a Generalized Linear Model (GLM). In the same way, a GLM was performed to study the influence of the insecticides at low concentrations on the behaviour (i.e. voracity) of *H. axyridis* females. The linear model tested the effect of the pesticide, the concentration and the interaction of these two factors on the proportion between the number of eaten prey (N_e) and the number of offered prey (N_o) for 24 hours. For the tested dependent variables (i.e., mortality and N_e), Tukey's honest significant tests for multiple comparisons among the pesticides and the concentrations were carried out ($\alpha=0.05$).

The type of functional response of *H. axyridis* to the prey densities was estimated through the method proposed by Trexler et al. (1988) which best fits this model. As the first step, a logistic regression model between N_e and the proportion (N_e / N_o) was done according to Juliano (2001). Dataset was fit on the polynomial logistic equation showed below:

$$\frac{N_e}{N_o}=\frac{exp(P_0+P_1N_o+P_2N_o^2+P_3N_o^3)}{1+exp(P_0+P_1N_o+P_2N_o^2+P_3N_o^3)}$$

where the intercept (P_0), linear (P_1) and quadratic (P_2) and cubic (P_3) coefficients were estimated through the maximum likelihood method. Because of redundancy, the cubic predictor was not considered (Luna et al. 2007; Savino et al. 2012). The shape of the functional response was thus estimated according to the following expected results: (i) if none of the values among P_1 and P_2 is significant, a Type I functional response is demonstrated; (ii) if P_1 results significantly negative, the functional response is of Type II; while (iii) if P_1 and P_2 are significantly positive and negative respectively, a Type III functional response is proved. After the curve estimation, the *attack rate* (a^i) was calculated through a linear regression applying the Holling's basic assumption:

$$a^i=\frac{N_e}{TN_o}$$

where T is the total exposure time (expressed in day). Thus, the *Handling time* (T_h) was indirectly measured by the disc equation (Holling, 1959):

$$N_e=\frac{a^iN_oT}{1+a^iT_hN_o}$$

A multifactorial GLM was carried out for both the dependent calculated parameters (i.e., a^i and T_h) to highlight the effect of concentration, pesticide and their interaction as fixed factors, followed by Tukey's post hoc test for the multiple comparison of the means. The described statistical analysis was run on IBM® SPSS® Statistics for Macintosh, Version 22.0.0.0 (IBM Corp. Released 2013. Armonk, NY: IBM Corp.).

4.3. Results

4.3.1. Lethal toxicity

The mortality of the females exposed simultaneously to dry residues by contact on sprayed plants and by ingestion of contaminated prey after 3 days was significantly affected by the insecticides ($F_{3,86}$= 3.75; P =0.014), the concentration ($F_{3,86}$= 4.22; P =0.008) and their interaction ($F_{9,86}$=3.68; P =0.009). Only thiamethoxam at LC_{50} caused a significant mortality (≃40%) compared to the other treatments (Fig. 1). The lethal effect was not significantly different from the control for the rest of the treatments.

4.3.2. Sublethal effects on functional response

The GLM analysis revealed a significant effect of the tested factors (i.e. treatment and concentration) on the voracity of *H. axyridis* on *E. kuehniella* eggs after being exposed to pesticide-residues for 3 days ($F_{3,762}$=5.583; P<0.001). The proportion of eaten prey was significantly affected by the concentration ($F_{3,762}$= 3.31; P =0.037) and the interaction between this factor and the pesticide ($F_{9,762}$=8.62; P <0.001). We recorded the lowest proportion of eaten prey for the females previously exposed to LC_{50} of thiamethoxam. Mean values (±SE) of the proportion of eaten prey decreased from 26.36%±11.40 to 9.86%±4.08 in comparison with the untreated control (i.e. aphid-fed coccinellid) in which these values ranged between 59.03%±8.75, and 16.86%±4.56 over the lowest (200) and the highest (1000) offered prey density (Fig. 2). Predator females exposed to LC_{50} of both imidacloprid and sulfoxaflor at the highest prey density consumed more eggs (280.25±60.37 and 343.37±60.24, respectively) when compared to the untreated control (168.58±45.63). Predators that were starved, during the 3 days before the voracity assessment, consumed the highest number of prey at all densities.

According to the logistic regression, *H. axyridis* females mostly showed Type I functional response after being exposed to most of the tested pesticide-concentration combinations and in both the controls (Table 2). However, a Type II functional response best fit for females earlier exposed to sulfoxaflor and thiamethoxam residues at the lowest tested concentration (LC_1) (Table 2).

The interaction between pesticide and concentration affected the parameters estimated for the functional response assessment (i.e. a_i and T_h) (Fig. 3 and 4). Individuals exposed to increased concentrations for both imidacloprid and sulfoxaflor exhibited an increased a_i and a decreased T_h values; this effect was particularly significant between their LC_{50} and the lower concentrations. By contrast, coccinellid females exposed to thiamethoxam residues showed the lowest attack rate (0.16±0.03 d^{-1}) and the highest handling time (0.04±0.01 d) at the highest concentration of the chemical (i.e. LC_{50}), in comparison with the rest of the treatments.

On the other hand, the female group earlier exposed to non-infested plants treated only with water (i.e. control-starved) had the significant highest instantaneous attack rate value (0.73±0.03 d^{-1}) and negligible handling time.

Table 1. Tested systemic neurotoxic insecticides and their Lethal Concentrations 50% (LC_{50}), LC_{20}, LC_1 estimated for the target pest, *Aphis gossypii,* feeding on zucchini plants. LC/FR, ratio between Lethal Concentration and maximum Field Rate reported in the formulation label against the target pest (from Ricupero et al. 2019, see Chapter II).

Active ingredient (a.i.)	Commercial formulation	Chemical class	% a. i.	Label field rate (a.i. ppm)	Estimated Concentration (a.i. ppm)		LC/FR
Imidacloprid	Afidane®200SL	Neonicotinoid	17.7	500	LC_1	0.020	$3.929 \cdot 10^{-5}$
					LC_{20}	0.503	$1.005 \cdot 10^{-3}$
					LC_{50}	3.186	$6.372 \cdot 10^{-3}$
Thiamethoxam	Actara®25WG	Neonicotinoid	25	200	LC_1	0.002	$8.750 \cdot 10^{-6}$
					LC_{20}	0.386	$1.928 \cdot 10^{-3}$
					LC_{50}	1.000	$5.000 \cdot 10^{-3}$
Sulfoxaflor	Transform®WG	Sulfoximine	50	70	LC_1	0.028	$3.929 \cdot 10^{-4}$
					LC_{20}	0.397	$5.671 \cdot 10^{-3}$
					LC_{50}	2.000	$2.857 \cdot 10^{-2}$

Table 2. Estimated parameters for the functional response to different prey densities of *Harmonia axyridis* after being exposed for 3 days to insecticides and their Lethal Concentrations 50% (LC_{50}), LC_{20}, LC_1 estimated for the target pest. Control: predators exposed for 3 days to aphid-infested plants sprayed with water. Control starved: predators exposed for 3 days to plants sprayed with water.

Treatment	**Concentration**	**Estimated coefficients**							**Functional response**
		P_0±SE	***P***	**P_1±SE**	***P***	**P_2±SE**	***P***	**R^2**	
Imidacloprid	LC_1	52.89±11.24	<0.001	-1.02±0.04	0.06	0.67±0	0.23	0.15	I
	LC_{20}	69.78±14.67	<0.001	-0.80±0.06	0.13	0.31±0	0.55	0.25	I
	LC_{50}	78.29±16.25	<0.001	-0.85±0.06	0.13	0.49±0	0.38	0.15	I
Sulfoxaflor	LC_1	59.42±11.20	<0.001	-1.28±0.04	0.02	0.88±0	0.11	0.20	II
	LC_{20}	44.53±13.22	<0.001	-0.19±0.05	0.72	-0.21±0	0.71	0.16	I
	LC_{50}	71.19±15.42	<0.001	-0.70±0.06	0.20	0.39±0	0.47	0.10	I
Thiamethoxam	LC_1	75.88±14.57	<0.001	-1.19±0.05	0.03	0.75±0	0.17	0.22	II
	LC_{20}	70.98±15.42	<0.001	-0.76±0.06	0.17	0.32±0	0.56	0.21	I
	LC_{50}	37.34±15.48	0.02	-0.67±0.06	0.34	0.43±0	0.54	0.07	I
Control	-	72.45±15.07	<0.001	-0.64±0.06	0.21	0.19±0	0.70	0.20	I
Control-Starved	-	109.91±11.42	<0.001	-0.48±0.04	0.27	-0.48±0	0.27	0.49	I

Figure 1. Acute toxicity. Mean percentages (± SE) of mortality of the *Harmonia axyridis* females following the exposure for 3 days to zucchini plants infested by *Aphis gossypii* and sprayed with insecticide at their Lethal Concentrations 50% (LC_{50}), LC_{20}, LC_1 estimated for the target pest. Control: predators exposed for 3 days to aphid-infested plants sprayed with water. Control starved: predators exposed for 3 days to plants sprayed with water. Columns bearing the same letter (upper case letters: within the same concentration regime; lower case letters: within the same tested insecticide) are not significantly different (*P* >0.05; ANOVA with LSD post hoc test for multiple comparisons).

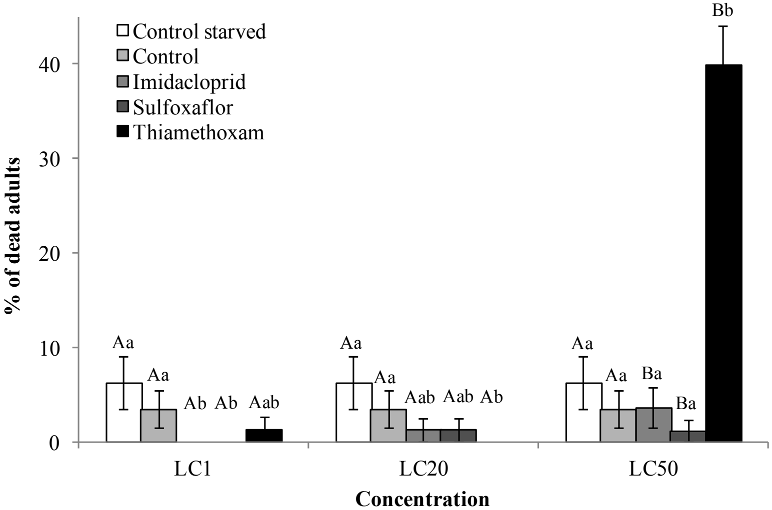


Figure 2. Functional response. Mean proportions (± SE) of *Ephestia kuehniella* eggs consumed by the harlequin ladybird females at different prey densities, after being exposed for 3 days to zucchini plants infested by *Aphis gossypii* and sprayed with insecticide at their Lethal Concentrations 50% (LC_{50}), LC_{20}, LC_1 estimated for the target pest. Control: predators exposed for 3 days to aphid-infested plants sprayed with water. Control starved: predators exposed for 3 days to plants sprayed with water.

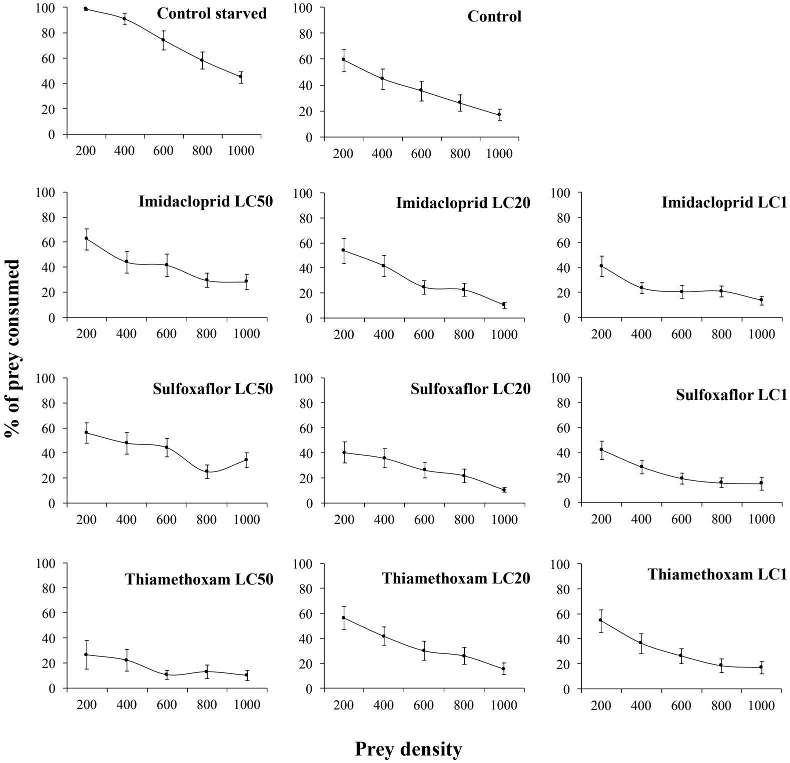


Figure 3. Functional response. Means (± SE) of the attack rate (a_i) expressed as the proportion of eggs eaten by survived females previously exposed for 3 days to zucchini plants infested by *Aphis gossypii* and sprayed with insecticide at their Lethal Concentrations 50% (LC_{50}), LC_{20}, LC_1 estimated for the target pest. Control: predators exposed for 3 days to aphid-infested plants sprayed with water. Control starved: predators exposed for 3 days to plants sprayed with water. Columns bearing the same letter (upper case letters: within the same concentration regime; lower case letters: within the same tested insecticide) are not significantly different (*P* >0.05; ANOVA with LSD post hoc test for multiple comparisons).

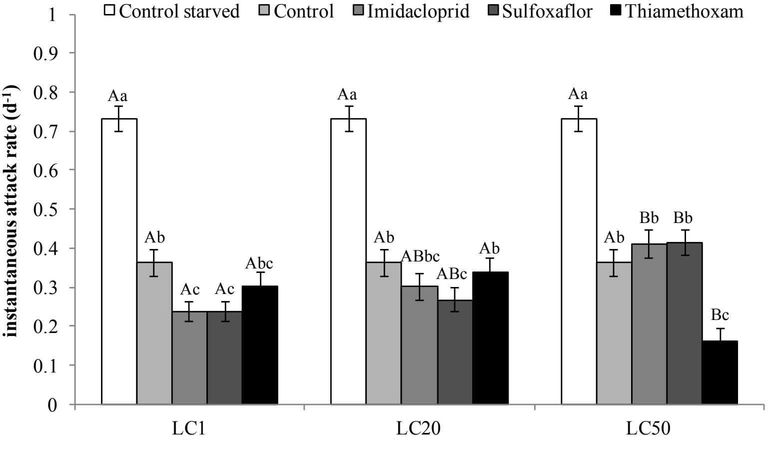
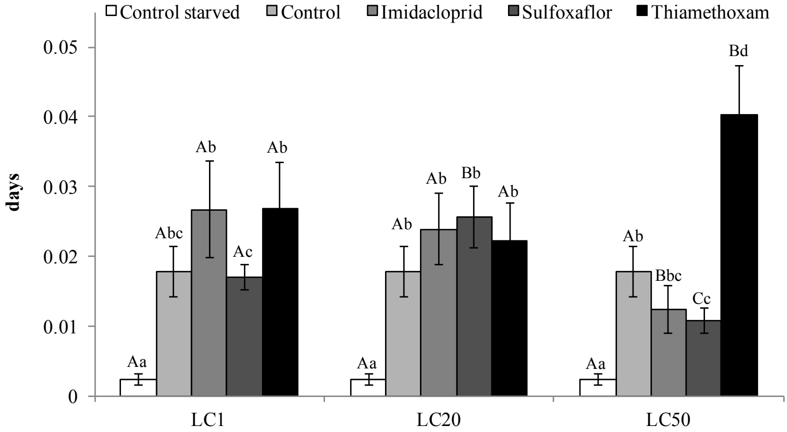


Figure 4. Functional response. Means (± SE) estimated prey handling time (T_h) of survived females previously exposed for 3 days to zucchini plants infested by *Aphis gossypii* and sprayed with insecticide at their Lethal Concentrations 50% (LC_{50}), LC_{20}, LC_1 estimated for the target pest. Control: predators exposed for 3 days to aphid-infested plants sprayed with water. Control starved: predators exposed for 3 days to plants sprayed with water. Columns bearing the same letter (upper case letters: within the same concentration regime; lower case letters: within the same tested insecticide) are not significantly different (*P* >0.05; ANOVA with LSD post hoc test for multiple comparisons).



4.4. Discussion

Pesticide sublethal concentration may negatively impact the survivorship and the behavior of beneficial arthropods (Coats 1991; Guedes et al. 2016). Our study demonstrates that residues on foliage and prey of routinely used systemic insecticides for the control of sap-sucking pests, such as the cotton aphid, can cause acute toxicity and impair the behavior of the generalist predator *H. axyridis,* even at concentrations much lower than the label rates (i.e., 1:200).

In our experiments, only the exposure to thiamethoxam at the highest concentration (LC_{50}) for *A. gossypii* caused high mortality to the predators exposed by contact on treated plants and by ingestion of contaminated prey. Similarly, Barbosa et al. (2018) found that thiamethoxam was highly toxic to *Tenuisvalvae notata* (Mulsant) (Coleoptera: Coccinellidae) when predators were exposed to insecticide dry residues on plants but at the highest field application rate (i.e. 200 g a.i. ha^{-1}). Conversely, larvae of *H. axyridis* feeding upon corn seedlings grown from thiamethoxam treated seeds (at 250 mg a.i./seed) showed neurotoxic symptoms and had the 53% of mortality (Moser & Obrycki 2009). Although the concentration we tested was significantly lower when compared with the field spray application rate, a possible toxic combination of insecticide residual contact and ingestion of pesticide-contaminated prey may have induced a high level in predator mortality. This hypothesis can be supported by the different toxicity caused by thiamethoxam against the harlequin ladybird adults at different exposure routes (Wang et al. 2018).

Conversely, both imidacloprid and sulfoxaflor at all the concentrations and thiamethoxam at the lowest tested concentrations (i.e., LC_{20} and LC_1 calculated for the target pest) caused no significant relevant direct mortality. These results can be explained through the effect of low concentrations and the involved exposure route tested for this bioassay. According to our findings, He et al. (2012) found no lethal effect induced by imidacloprid at 5 ppm to *Serangium japonicum* Chapin (Coleoptera: Coccinellidae), an important predator of the whitefly *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). However, Xiao et al. (2016) found sublethal effects on the reproduction of *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) residually exposed to 10% of LC_5 and LC_5 of imidacloprid. Besides, the progeny of these individuals had a lower demographical growth (i.e., estimated intrinsic rate of increases) compared to the untreated control (transgenerational effects).

Residues of sulfoxaflor at the field recommended rate on glass resulted toxic to *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) (Garzón et al. 2015) and this compound also slightly affected the survival of *Hippodamia convergens* Guerin-Meneville (Coleoptera: Coccinellidae) through the ingestion of contaminated food (Colares et al. 2017). Considering that generalist predators can be exposed to systemic insecticides also by the direct consumption of pollen and extra floral nectar (Cloyd & Bethke 2011), as well as of contaminated honeydew (Calvo-Agudo et al. 2019), different exposure routes can be involved in determining the acute toxicity and they must be taken into account when pesticide lethal effect is assessed (He et al. 2012, Yao et al. 2015, Wang et al. 2018).

Predators earlier exposed to thiamethoxam at LC_{50} consumed less prey showing thus a decreased a_i and an increased T_h. The reduction in predator voracity might be due to a possible intoxication of the survived females exposed to pesticide residues that were not able to recover their predation capacity. This finding appears to be linked with the predator mortality found in the acute toxicity trial for thiamethoxam at the highest tested concentration. Similarly, thiamethoxam negatively affected the voracity of *S. japonicum* to *B. tabaci* eggs when the pesticide was given both by the seed treatment and through the foliar application (Yao et al. 2015). Moreover, several studies support that sublethal doses of systemic insecticides negatively compromised the predation capacity of other generalist predators. For instance, sublethal concentration of thiacloprid significantly reduced the predation rate of *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae) (Martinou et al. 2014) and sublethal exposure to imidacloprid impaired the prey consumption of *H. axyridis* by increasing the T_h (Wang & Shen 2002).

Predators that were in contact and fed upon aphid-infested plants treated with the LC_{50} estimated for the cotton aphid of both imidacloprid and sulfoxaflor were more voracious at the highest offered prey density in comparison with the aphid-infested control. This finding might be explained supposing that the pesticides at the highest concentration exerted an antifeedant effect and starved females increased thus their predation rate. Moreover, this explanation can be corroborated by the higher values of T_h and a_i calculated for the females exposed to plants with no prey (i.e. control starved) in comparison with the values estimated for females that fed on prey-infested plants. The antifeedant effect of sublethal concentrations of pesticide was already reported for imidacloprid and thiamethoxam on Coleoptera, Hemiptera and Hymenoptera (Boina et al. 2009; He et al. 2012, 2013) but to the best of our knowledge, this is the first record for sulfoxaflor towards a coccinellid predator. However, further investigations on the side effect of this newly marketed insecticide on natural enemies should be addressed.

The functional response is defined as the prey consumption rate of a predator in relation to prey density and this biological trait helps in predicting how predators affect prey abundance across trophic levels (Jervis 2012).

We detected for females exposed to the most of pesticide-concentration combinations a density independent functional response (i.e. Type I). The most common functional response reported for coccinellids and more generally for invertebrates is the asymptotic form (i.e., type II) while the linear response in nature is generally linked to sedentary filter feeders (Hodek et al. 2012). However, a density independent functional response may occur when the species encounters the prey at closed proximity and/or it does have a short handling time (Heimpel & Mills 2017). This might be our case because the experimental trial we set up for the functional response assessment included a relatively small and immobile prey (lepidopteran eggs) in a limited arena that had resulted in a reduction of the handling time for predators. Similarly, Koch et. al (2003) found a density independent functional response for adult *H. axyridis* preying on the monarch butterfly *Danaus plexippus* (L.) (Lepidoptera: Nymphalidae) eggs.

Conversely, for female predators exposed to residues of sulfoxaflor and thiamethoxam at the lowest concentration (LC_1) calculated for the target pest, we estimated an inverse density dependent relationship between the predation rate and the prey density. The reduction in food intake and thus the different shape in the functional response might be due to an alteration of the feeding behaviour possibly caused by a neurotoxic effect over the long period that had led to a reduction of the consumed prey (Daniels et al. 2009).

Yao et al. (2015) and He et al. (2012) found no variation in the functional response shape of *S. japonicum* to *B. tabaci* eggs after the exposure to imidacloprid and thiamethoxam, respectively. Wang et al. (2018) reported no significant differences in the functional response patterns of *H. axyridis* to *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) afterword the exposure to sublethal dose (LC_5) of thiamethoxam to different application routes. By contrast, imidacloprid affected the functional response of the predator *Podisus nigrispinus* (Dallas) (Heteroptera: Pentatomidae) and *Tetranychus urticae* Koch (Acari: Tetranychidae) (Poletti et al. 2007, Malaquias et al. 2014), as well as the attack rate (a_i), handling time (T_h) and maximum attack rate (T/T_h) of aphid parasitoid *Lysiphlebus fabarum* (Marshall) (Hymenoptera: Braconidae) exposed to LC_{25} of Imidacloprid (Jam & Saber 2018).

Our study demonstrates that dry residues of systemic insecticides at low concentration on leaves and prey occurring after foliar application may cause direct acute toxicity and induce adverse effects on the predation capacity of *H. axyridis*. Sublethal concentrations of the second-generation neonicotinoid thiamethoxam can exert toxic effect and negatively affect the voracity of *H. axyridis*. Therefore, thiamethoxam applications to control *A. gossypii* are likely not compatible with the presence of *H. axyridis* and its application should be avoided when IPM strategies are set up. Sulfoxaflor and imidacloprid showed a safer toxicological profile in comparison with thiamethoxam because they caused no lethal effect on the coccinellid, therefore, these insecticides may determine an antifeedant effect inhibiting thus the predation activity on the pesticide-treated areas. Besides, our experiment highlighted that sublethal doses of thiamethoxam and sulfoxaflor can also impair the functional response of the harlequin ladybird and thus potentially affecting the ecosystem service provided by the natural enemy.

Moreover, because sublethal rates of neonicotinoid insecticides adversely affect the demographic life traits of coccinellid key predators (Rahmani & Bandani 2013; Moscardini et al. 2015; Xiao et al. 2016), further specific long-term studies are necessary to investigate the effect of low insecticides concentrations on several biological parameters (e.g. development time, fertility, population growth) of *H. axyridis* at different stages. We also highlight the importance in estimating the predator biocontrol activity of over the pesticide effect in combination with other stressors (e.g., temperature fluctuations, starvation, oxygen depletion) because field conditions are generally sub-optimal if compared with laboratory standardized conditions.

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CHAPTER V.

Parasitism and phylogeny of *Dinocampus coccinellae* through different hosts and continents

Abstract

Dinocampus coccinellae (Hymenoptera: Braconidae) is a cosmopolitan parasitoid of coccinellid beetles and little is known about its parasitism among areas invaded in different times by the invasive *Harmonia axyridis* (Coleoptera: Coccinellidae). Genetic differences among parasitoid populations from different geographical areas could help to assess its native origin, as well as potential effects on the *H. axyridis* invasion process. Within this framework, we compared parasitism by *D. coccinellae* on alien and native coccinellids in the native range of *H. axyridis* (China) as well as areas where *H. axyridis* invaded over the last 100 years (USA), more recently (Chile) and very recently (Sicily). About 6,000 specimens of *H. axyridis* and native coccinellids were collected in several sites located in the four continents. We also investigated the genetic relationship of *D. coccinellae* specimens sampled over this geographical distribution using mitochondrial COI and 16S rRNA sequence data. The parasitism rates of *D. coccinellae* varied between native and non-native hosts and among the different sampled areas. Parasitoid phylogenetic analyses highlighted variations in parasitoid COI sequences, while no differences in 16S rRNA region were recorded, making it difficult to pose hypotheses regarding the provenance of this species. Further investigations on spatial genetic structure at narrower scales are needed to elucidate *D. coccinellae* phylogeography, and studies on host-parasitoid interaction mechanisms should be conducted for better understanding the parasitoid ecological role in coccinellid population suppression worldwide.

Keywords phylogeography, koinobiont, barcoding, endoparasitoid, biological invasion

5.1. Introduction

The introduction of alien species and their potential to become invasive have raised exponentially in the last decades because of the intense global trade and, as a consequence, their ecological and economic impact has been widely recognized (Ragsdale et al. 2011; Asplen et al. 2015; Biondi et al. 2018). The success or failure of a new biological invasion mainly relies on the species biological life traits, on the competition with native species and on the interaction with natural enemies (Williamson & Griffiths 1996). The latter is a critical factor in the invasion mechanism and the invasion success in terms of distribution and abundance of the invader, since it may be related to the absence or low efficacy of natural control in the new area, as assumed by the Enemy Release Hypothesis (Keane & Crawley 2002). Natural enemy communities may perform initially low percentage of predation before controlling successfully the exotic species, because they require time to adapt their behavioral and physiological traits to the novel host (Hawkins 2005).

Harmonia axyridis Pallas (Coleoptera: Coccinellidae), native to eastern Asia, is considered a biological model for invasive species worldwide (Roy & Wajnberg 2008; Ovchinnikov et al. 2019). The coccinellid was successfully introduced in several countries as biological control agent against sap-sucking insect pests since the beginning of the last century. Over the past 30 years, the coccinellid beetle has spread in several continents, invading North America, South America, Europe, a less extended part of Africa, the western Asia and, recently, the New Zeland (Ceryngier et al. 2018). In the past decades, the species had caused numerous concerns because of the side effects it may cause, in particular the displacement of native coccinellids, the human nuisance for winter aggregations and the secondary damage to crops (Koch

2003). The invasion success of the harlequin ladybird is undoubtedly own to its life history traits (e.g. size, high efficiency as a predator and wide colonization ability), but also to the reduced control of natural enemy populations in the recently colonized areas (Ceryngier et al. 2018).

The Asian ladybeetle is naturally attacked by *Dinocampus coccinellae* (Schrank) (Hymenoptera: Braconidae), a parasitoid of coccinellid beetles belonging to Coccinellini and Chilocorini tribes (Balduf 1926, Maqbool et al. 2018). The parasitoid reproduces by thelytokous parthenogenesis and its development inside the host is mediated by teratocytes, specialized cells formed during the early embryogenesis which play a role in overcoming host defenses and provide nutrition to the larva (Firley et al. 2007). The parasitoid is also well known to manipulate the host behavior through the transmission of a symbiotic RNA virus that forces the coccinellid to guard the cocoon until the emergence of the adult (Dheilly et al. 2015). *Dinocampus coccinellae* is presumably native within the Holarctic realm (Balduf 1926) with a cosmopolitan distribution, since the species has been reported in different world countries (Firlej et al. 2005; Koyama & Majerus 2007; Berkvens 2010; Dindo et al. 2016). However, the origin of the parasitoid remains unknown and no population genetic information is currently available.

Field data suggest that *H. axyridis* is a marginal host for *D. coccinellae* in the new colonization areas and, these evidences have been widely used as support the enemy release hypothesis to explain the harlequin ladybird invasion success (Berkvens et al. 2010; Ceryngier et al. 2018). The weakness of *D. coccinellae* in the control of *H. axyridis* populations has been explained by the scarce ability of the parasitoid to overcome host immune defenses and to discriminate between hosts depending on their suitability (Firlej et al. 2012).

Parasitism rates of *D. coccinellae* reported for several ladybird species in field and laboratory indicate that the species prefers to attack larger ladybird species because they may provide higher energetic resources for its development in comparison to smaller hosts (Ceryngier et al. 2018). However, laboratory evidences indicated no host preferences when *D. coccinellae* parasitizes *H. axyridis* when compared to other coccinellid beetles of similar size (Dindo et al. 2016). Hence, *H. axyridis* may serve as an egg-sink, depleting thus the parasitoid reproduction (Hoogendoorn & Heimpel 2002). Nevertheless, it is uncertain whether this is the effect of enemy release or of generally low susceptibility of *H. axyridis* to *D. coccinellae*, since the ladybird beetle seems a marginal host also in its native range (Koyama & Majerus 2007) but no additional information is currently available within this area. By contrast, a recent meta-analysis revealed that Czech field populations of *H. axyridis* show higher emergence rates of *D. coccinellae* compared to invasive populations sampled across the world, suggesting an ongoing adaptation of Central European *D. coccinellae* populations to *H. axyridis* (Knapp et al. 2019). These recent findings stress thus the need to carry out investigations on the relationships on the parasitism between the invasive species and its native natural enemy within the environments colonized over different times. Population genetic structure reflects historical and contemporaneous levels of gene flow among populations, this being the result from past or current dispersal patterns, density fluctuations, and mating events (Freeland 2011). The genetic structure of parasitoid is intrinsically linked with its hosts and has important consequences in evolutionary processes, since variations in host numbers and/or geographic range can drive associated changes in parasitoid populations (Althoff & Thompson 1999). For this reason, information regarding parasitoid population genetic structure may help in understanding the interaction between host-parasitoid associations, and in the specific case of *D. coccinellae*-*H. axyridis*, this may contribute in determining factors involved in the coccinellid invasion process worldwide.

Within this context, we compared the parasitism by *D. coccinellae* on alien and native coccinellids in the native range of *H. axyridis* (China) as well as areas where *H. axyridis* invaded over the last 100 years (USA), more recently (Chile) and very recently (Sicily). We also assessed the phylogenetic relationship of *D. coccinellae* populations among this geographical range and their hosts by using generic mitochondrial markers in order to discover the genetic structure at larger scale of this widespread parasitoid and the possible linkage to its hosts.

5.2. Material & methods

5.2.1. Field sampling

In order to assess differences in the parasitism by *D. coccinellae* on exotic and indigenous coccinellid adults in *H. axyridis*-native and invaded areas over time, we conducted field surveys during the spring season in four world locations according the harlequin ladybird invasion timing process (Table 1). *Harmonia axyridis* was sampled in all the chosen areas, while a native species per each site was chosen as control. The indigenous species were: *Propylea japonica* (Thunberg) in China, *Coleomegilla maculata* (De Geer) in Minnesota (USA), *Eriopis chilensis* (Hofmann) in Chile, and *Coccinella septempunctata* L. in Italy (Sicily). Populations of adult coccinellids were weekly sampled for one month during the spring/summer seasons between 2017 and 2018. The sampling was carried out by visual inspection of plants following a Z-trajectory between the rows of the selected plots, having an extension of about 0.50 ha^{-1} for each site. The borders of the cultivated plots were excluded from the sampling, to avoid the influence of external factors, such as for example pesticide applications in the neighboring fields. Two fields were sampled per each chosen area. For each sampled site, adult beetles were picked up by hand and/or by swiping net, stored in ventilated plastic Falcon plastic tubes (50 mL), and were transported to the laboratory in a refrigerated box.

5.2.2. Parasitism assessment

The collected adults of coccinellid beetles were counted and identified morphologically at the species level according the taxonomic identification keys (Hodek et al. 2012). Per each date and collection site, half of the samples were isolated in groups of 8-10 individuals inside ventilated Petri dishes (10 cm in diameter) and reared in laboratory conditions to assess the successful parasitism rate (i.e., parasitoid cocoon emergence) (Hoogendoorn & Heimpel 2002). These samples were maintained at constant environmental conditions (24±1°C, 60±10% RH, 12L:12D of photoperiod) and fed *ad libitum* with frozen eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) provided by Koppert (The Netherlands) and a honey-based jelly. The latter was prepared by stirring distilled water, organic pure honey and agar powder in a volumetric ratio of 1:1:0.1. Petri dishes were cleaned and food was renewed on a daily basis. The ladybird beetles were monitored twice a day for 30 days, because the juvenile parasitoid takes ca. 20 days to develop (Hoogendoorn & Heimpel 2002). Parasitism rate of *D. coccinellae* (i.e., proportion of ladybird individuals that produced a *D. coccinellae* cocoon over the sampled batch) was recorded for each sampled ladybird population.

Parasitized coccinellids were thus isolated in Petri dish (6 cm) up to the emergence of *D. coccinellae* adults that were killed in 95% ethanol and stored at -20°C for DNA barcoding. Emerged adult wasps were also identified through a morphological taxonomic key (Balduf 1926). The rest of collected coccinellids (i.e., half of samples that were not reared) were preserved in 95% ethanol and dissected to understand whether differences exist in the immune response (e.g., encapsulation and/or teratocytes) between native and exotic coccinellid beetles (Firlej et al. 2012). The dissections were performed in a silicone Petri dish for dissection by covering the samples with a drop of Ringer's solution (Jervis et al. 2007). For each dissected sample, the presence of (i) teratocytes, (ii) encapsulated bodies and (iii) the number and the age of parasitoid immature stages were recorded under the stereomicroscope (Leica EZ4 D Microscope 1.2, Leica Microsystems, Switzerland, Ltd). A Generalized Linear Model was conducted to assess the influence of the species and the country on the proportion of total parasitized coccinellid species (i.e., sum of parasitism rates by rearing and by dissection). We used paired t test to highlight differences in the parasitism rates obtained by rearing and by dissection among the species. Statistical analysis was carried out on IBM® SPSS® Statistics for Macintosh, Version 23.0.0.0 (IBM Corp. Released 2015. Armonk, NY: IBM Corp.).

5.2.3. Phylogeny

DNA sequences were generated for two mitochondrial genes: Cytochrome c Oxidase Subunit I (COI) and 16s rRNA (Table 2). These genes have been used in previous studies of insect phylogeography including parasitoids of the family Braconidae at various levels (Kambhampati et al. 2000;

Muirhead et al. 2006; Jourdie et al. 2010; Stigenberg et al. 2015). We choose two individuals per each country and per each host, according to the samplings. Because *H. axyridis* specimens sampled in Sicily were not parasitized (see Results), we included in our analysis wasps emerged from *H. axyridis* collected in Northern Italy, provided by Dindo et al. (2016). Moreover, we added further *D. coccinellae* specimens collected in Canada and in California (USA) to extend the phylogeographic network. Sequence, collection site and host information is available in Table 3.

5.2.3.1. DNA extraction, amplification and sequencing

Each sample was transferred from ethanol to a new clean tube and washed with 2.5% laboratory grade bleach solution in a 40' end-over-end rotation to remove any possible contaminating DNA (Greenstone et al. 2012). The samples were re-washed 4 times with sterile distilled water and rinsed on a paper towel. Parasitoid DNA was extract through the Quiagen® DNeasy® Blood & Tissue Kit (Qiagen, GmbH, Hilden, Germany), according the adapted following protocol. Each parasitoid was placed in a 1.7 mL DNA-free micro-centrifuge tube with three stainless sterilized bead mills (Ø 2mm) and ground for 3' through the Mixer Mill MM 400 (Retsch, Haan, Germany) with 50 µL of AL buffer at 30 Hz. Consequentially, the samples were vortexed with 150 µL of AL buffer and 20µL of Proteinase-K, then incubated at 37°C overnight. An amount of 100 µL of pure ethanol for molecular use was added to the lysed sample; therefore, the mixture was pipetted into a DNeasy Mini spin column and centrifuged for 1' at 8,000 rpm to allow the DNA binding. The DNA extract was thus eluted in 100 µL of Buffer AE with a centrifuge of 1' at 8,000 rpm after two sequential washing cycles with 500 µL of Buffer AW1 for 1'at 8,000 rpm and 500 µL of Buffer

AW2 for 3’ at 14,000 rpm, respectively. Extracted genomic DNA concentration was measured by BioTek™ Epoch™ Microplate Spectrophotometer. Each DNA extraction batch included a negative control (i.e., without sample) which was added when PCRs were performed, to exclude any possible contamination.

5.2.3.2. Molecular markers

DNA corresponding to COI region of 651 bp was PCR amplified using the following primers: LCO1490 and HCO2198 (Folmer et al. 1994). The reaction was performed in 25µL total volume containing: 1 x High Fidelity PCR Buffer (10X), 2.0 mM of $MgSO_4$ (50 mM), 0.2 mM of dNTP Mix (10 mM), 0.2 µM of forward primer (10 µM), 0.2 µM of reverse primer (10 µM), 1µ of DNA template,1 U Platinum® Taq DNA Polymerase High Fidelity (5 U/µL) (Invitrogen™ by Life Technologies™ Platinum® Taq DNA Polymerase High Fidelity, no. 11304-011) and autoclaved distilled water. The PCR conditions were: an initial denaturation at 94°C for 2', 35 cycles of denaturation at 94°C for 15’’, annealing at 50°C for 30’’, elongation at 68°C for 60".

The DNA corresponding to COI region of 831 bp was PCR amplified using the following primers: C1-J-2183 and TL2-N-3014 (Simon et al. 1994). The reaction was performed in 25µL total volume containing: 1 x High Fidelity PCR Buffer (10X), 2.0 mM of MgSO4 (50 mM), 0.2 mM of dNTP Mix (10 mM), 0.2 µM of forward primer (10 µM), 0.2 µM of reverse primer (10 µM), 1µ of DNA template,1 U Platinum® Taq DNA Polymerase High Fidelity (5 U/µL) (Invitrogen™ by Life Technologies™ Platinum® Taq DNA Polymerase High Fidelity, no. 11304-011) and and UltraPure DNase/RNase-Free distilled water. The PCR conditions included an initial denaturation at 94°C for 2', 35 cycles of denaturation at 94°C for 15'', annealing at 56°C for 30'', elongation step at 68°C for 60".

DNA corresponding to mythocondrial 16s rRNA region of 370 bp was PCR amplified using the following primers: 16SWb and 16S outer (Muirhead et al. 2006). The reaction was performed in 25µL total volume containing: 1 x High Fidelity PCR Buffer (10X), 2.0 mM of MgSO4 (50 mM), 0.2 mM of dNTP Mix (10 mM), 0.2 µM of forward primer (10 µM), 0.2 µM of reverse primer (10 µM), 1µ of DNA template,1 U Platinum® Taq DNA Polymerase High Fidelity (5 U/µL) (Invitrogen™ by Life Technologies™ Platinum® Taq DNA Polymerase High Fidelity, no. 11304-011) and autoclaved distilled water. The PCR conditions were: an initial denaturation at 94°C for 2', 35 cycles of denaturation at 94°C for 15'', annealing at 56°C for 60'', elongation at 68°C for 60". All PCR assays were carried out in Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

5.2.3.3. PCR products

PCR-amplified products were combined with loading buffer (Invitrogen BlueJuiceTM Gel Loading Buffer 5x including GelRedTM Nucleic Acid Gel Stain at 3x) and separated in 1.5 % agarose gel (Buffer TAE 1x) by electrophoresis proceeded at 100 V for 90' at room temperature. Then the products were visualized under UV-light camera. Expected product were identified by using Thermo Scientific™ O'RangeRuler™ 50 bp DNA Ladder. All DNA extractions and PCR reactions were carried out in separated sterile laminar flow cabinets in order to avoid any possible cross-contamination. Before each extraction and reaction batch, the cabinets were cleaned with 10% bleach solution for 10' and 75% ethanol. DNA-samples were extracted and processed the Institute of Biology of the University of Talca (Chile). PCR products were purified and sequenced on both strands by Macrogen (Seoul, Korea) using the PCR primers. Sequencing was conducted under BigDyeTM terminator cycling conditions and products purified using ethanol precipitation and run using automatic sequencer ABI3730XL.

5.2.3.4. Phylogenetic analysis

The samples included in this analysis were 31 and GenBank accession numbers of the sequences used are provided in Table 2. The terminal ambigous parts of the aligned sequences from each individual was deleted to obtain a 651 bp, 831 bp and 370 bp segment fragment for COI-F, COI-S and 16s rRNA, respectively. Sequences were edited and aligned manually using Unipro UGENE version 1.26.1 (Okonechnikov et al. 2012). The *16S rRNA* alignment regions were highly conserved, thus gaps were easily inferred by eye and confirmed using MUSCLE (Edgar 2004).

The sequences were checked according their chromatograms and corrected if needed. BLAST searches were conducted for all the sequences to check for any possible contamination. Sequences from closely related taxa to *D. coccinellae* were compared to determine which species were more suitable as outgroups. Sequences from GenBank were obtained in Fasta format.

Meteorus sp. (Hymenoptera: Braconidae) (Stigenberg et al. 2015), *Cotesia salebrosa* (Hymenoptera: Braconidae) (Ruohomäki et al. 2013), *M. pulchricornis* (Whitfield 1997) were chosen as outgroups for COI regions and 16s gene, respectively. Phylogenetic reconstructions were conducted for the two mtDNA genes separately by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei 1993). Phylogeny test was performed with 1000 bootstrap replications. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 32 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

5.3. Results

5.3.1. Field samplings

Approximately 6,000 coccinellids were collected in the field surveys conducted among the sampled sites. In China, *H. axyridis* was the most abundant species with 1,066 collected specimens, and *Propylea japonica* similarly followed with 978 specimens. In Minnesota, between July and September 2017, the native *C. maculata* was the most abundant species, followed with a similar number by the invasive *H. axyridis*. In the Maule region in Chile, a total of 1,081 beetles were collected and the Asian ladybeetle showed the highest presence, followed by *Hippodamia variegata* and the native *E. chilensis.* In Sicily, *C. septempunctata* was the most represented coccinellid among numerous species, while *H. axyridis* was marginally present (Table 4).

5.3.1. Coccinellid parasitism rate

The parasitism rate varied among geographical areas and the host species (Fig. 1). The tested model was statistical significant (χ^2=63.06; d.f.=1; *P* <0.001). The statistical analysis revealed a significant effect of the host species on the proportion of parasitized coccinellid (χ^2=42.15; d.f.=4; *P* <0.001). However, no significant effect of the country on the total parasitism rates was highlighted (χ^2=1.99; d.f.=3; *P*

=0.575). In *H. axyridis* native area (i.e., China), the parasitism rate by rearing was negligible (0.17±0.17%) as also confirmed by the 533 dissected samples which revealed no parasitoid immature stages, teratocytes and chitinized bodies. Conversely, the native *P. japonica* showed 8.02±1.95% of parasitism rate, and no significant differences were found between reared and dissected hosts (*t*=1.22; d.f.=22; *P* =0.65), (Fig. 1A). In Minnesota (USA), the mean parasitism rate by *D. coccinellae* obtained by rearing the coccinellids was 7.65±4.60% for the native *C. maculata* and 2.86±1.90% for *H. axyridis*. Similarly, dissections revealed a higher parasitism for the native species (4.60±1.33%) compared to the invasive *H. axyridis* (1.90±0.64%). Nevertheless, differences in the parasitism rate between dissected and reared coccinellids were significant nor for *H. axyridis* (*t*=0.68; d.f.=24; *P* =0.55), neither for *Coleomegilla maculata* (*t*=0.75; d.f.=33; *P* =0.45) (Fig. 1B). In Chile, a similar parasitism rates were recorded both for native and invasive species and between dissected and reared beetles. In particular, the mean parasitism obtained by rearing was 7.39±2.40% and 8.48±2.06% for *E. chilensis* and *H. axyridis*, respectively. Similarly, we observed the 8.60±2.45% and the 9.13±1.90% of mean parasitism rate by dissected samples of *E. chilensis* and *H. axyridis*, respectively (Fig. 1C). The observed data between reared and dissected specimens were not significant for the native species (*t*=0.11; d.f.=21; *P* =0.91), and neither for the invasive species (*t*=1.50; d.f.=26; *P* =0.15). In harlequin ladybird more recently invaded area (i.e. Sicily), we recorded the 9.05±2.59% of parasitism rate for the reared samples of *C. septempunctata,* while the dissected samples showed lower parasitism rates; however, the difference between the two group was not statistical significant (t=1.88; d.f.=30; *P* =0.06). By contrast, the limited number (n=20) of *H. axyridis* adults collected in Sicily presented no parasitism by *D. coccinellae*, nor by rearing neither by dissection (Fig. 1D).

We observed through the dissection of the samples that both native and invasive parasitized coccinellids collected among different *H. axyridis* invaded areas had teratocytes, while we never recorded the presence of encapsulation bodies (Fig. 2). Teratocytes were present in association with very young immature stages, while they were absent with 4th larval stage (data not shown). In most of the cases, parasitoid larvae were found along the distal part of the abdomen and close to the head of the host. The proportion of parasitized specimens that presented teratocytes changed between native and indigenous species and among the *H. axyridis* invaded territories in different times, ranging from 0±0% (for China and Sicily) to 69±12.60% for Chile (Fig. 2).

5.3.2. Phylogeny

Maximum likelihood analysis of COI (Folmer et al. 1994) sequences showed that the individuals can be divided in 7 groups with high bootstrap values (>50%). No clear geographic structure was observed in *D. coccinellae* populations among different geographical areas (Fig. 3). Samples included in our phylogenetic tree clustered in two main groups. The first small group included only two individuals originated from Minnesota and emerged from the invasive and the native hosts (i.e., US MN 27 HA and US MN 39 CM). Contrary, the second wider group included all the individuals sampled in several hosts and among areas invaded in different times by the invasive *H. axyridis*. The latter group reveled two main branches. The minor branch included all the species originated from California, Canada and four from Chile. Interestingly, no specimens collected in Minnesota, Italy and China were present within this clade. The major branch clustered all the sequences of *D. coccinellae* derived from China, Italy and the remaining from Chile and Minnesota. Within this wide clade no specimens from California and Canada were present. Specimens collected in Chile were separated within the two subgroups according to two different sample sites. Conversely, individuals from Minnesota resulted separated not only by collection sites, but also by host species, since they were widely distributed across the branches. Interestingly, parasitoid specimens emerged from *H. axyridis* and *C. maculata* grouped in different clades. The trees obtained from COI (Simon et. al 1994) and 16s rRNA sequenced regions did not show any discriminative information for the phylogeography of *D. coccinellae* (Fig. 4 and Fig. 5).

Table 1. Sites for coccinellid beetle samplings in the different countries according *Harmonia axyridis* invasion timing process.

Area	**Native**	**Old invaded**	**Recently invaded**	**More recently invaded**
Country	China	Minnesota	Chile	Italy (Sicily)
Location	Harbin	Rosemount	Talca	Catania
Crop	corn and soybean	corn and soybean	alfa-alfa and wheat	wheat and/or citrus and/or stone fruits
Species	*Harmonia axyridis*	*Harmonia axyridis*	*Harmonia axyridis*	*Harmonia axyridis*
	Propylea japonica	*Coleomegilla maculata*	*Eriopis chilensis*	*Coccinella septempunctata*
Sampling period	July 2017	July 2017	November 2017	May 2018

Table 2. Primers used for gene sequencing of *Dinocampus coccinellae.*

Gene	**Primer**	**Sequence (5'-3')**	**Length (bp)**	**Amplicon length (bp)**
COI	F: LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	25	651
	R: HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	26	
COI	F: C1-J-2183	CAA CAT TTA TTT TGA TTT TTT GG	23	831
	R: TL2-N-3014	TCC AAT GCA CTA ATC TGC CAT ATT A	25	
16s rRNA	F: 16SWb	CAC CTG TTT ATC AAA AAC AT	20	370
	R: 16S outer	CTT ATT CAA CAT CGA GGT C	19	

Table 3. Origin of *Dinocampus coccinellae* specimens used for gene sequencing and phylogeny reconstruction.

Specimens ID	Country	Geographic coordinates	Host species
CA 1CM	Canada	45°34'58.8"N 73°14'14.6"W	*Coleomegilla maculata*
CA 2CM	Canada	45°34'58.8"N 73°14'14.6"W	*Coleomegilla maculata*
CL 1HV	Chile	35°25'45.6"S 71°32'43.3"W	*Hippodamia variegata*
CL 2HV	Chile		*Hippodamia variegata*
CL 3HA	Chile	35°22'10.2"S 71°35'40.0"W	*Harmonia axyridis*
CL 4HA	Chile	35°22'10.2"S 71°35'40.0"W	*Harmonia axyridis*
CL 13EC	Chile	35°23'46.5"S 71°47'48.9"W	*Eriopis chilensis*
CL 14EC	Chile	35°23'46.5"S 71°47'48.9"W	*Eriopis chilensis*
CL 16EC	Chile	35°16'36.3"S 71°47'14.9"W	*Eriopis chilensis*
CL 36EC	Chile	35°16'36.3"S 71°47'14.9"W	*Eriopis chilensis*
CN 3HA	China	45°44'59.3N 126° 44' 13.5"E	*Harmonia axyridis*
CN 1PJ	China	45°44'59.3N 126° 44' 13.5"E	*Propylea japonica*
CN 2PJ	China	45°44'59.3N 126° 44' 13.5"E	*Propylea japonica*
CN 4PJ	China	45°44'59.3N 126° 44' 13.5"E	*Propylea japonica*
IT 1C7P	Italy	37°37'01.5"N 14°49'39.3"E	*Coccinella septempunctata*
IT 2C7P	Italy	37°37'24.5"N 14°22'13.9"E	*Coccinella septempunctata*
IT 1HA	Italy	44°48′39"N 11°37'84"E	*Harmonia axyridis*
IT 2HA	Italy	44°48′39"N 11°37'84"E	*Harmonia axyridis*
US CA 1HC	U.S.A. California		*Hippodamia convergens*
US CA 2HC	U.S.A. California		*Hippodamia convergens*
US CA 3HC	U.S.A. California		*Hippodamia convergens*
US CA 4HC	U.S.A. California		*Hippodamia convergens*
US MN 1HC	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Hippodamia convergens*
US MN 11C7P	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Coccinella septempunctata*
US MN 24CYM	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Cycloneda munda*
US MN 25HA	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Harmonia axyridis*
US MN 26CM	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Coleomegilla maculata*
US MN 27HA	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Harmonia axyridis*
US MN 39CM	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Coleomegilla maculata*
US MN 46HC	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Hippodamia convergens*
US MN 47CYM	U.S.A. Minnesota	44°59'32.3"N 93°10'26.4"W	*Cycloneda munda*

Table 4. Cumulative number of coccinellid species collected during field surveys across the different geographical areas.

Location	**Species**	**# collected**	**# reared**	**# dissected**
China	*Harmonia axyridis*	1066	533	533
	Propylea japonica	978	489	489
Minnesota	*Coleomegilla maculata*	1142	567	574
	Harmonia axyridis	1113	683	430
Chile	*Eriopis chilensis*	302	156	145
	Harmonia axyridis	400	270	130
Italy (Sicily)	*Coccinella septempunctata*	1202	600	602
	Harmonia axyridis	20	12	8

Figure 1. Mean (±SE) parasitism rates of *Dinocampus coccinellae* by rearing and by dissection of native species and *Harmonia axyridis* over different countries. From left to right, China (A), Minnesota (B), Chile (C) and Italy (Sicily) (D).

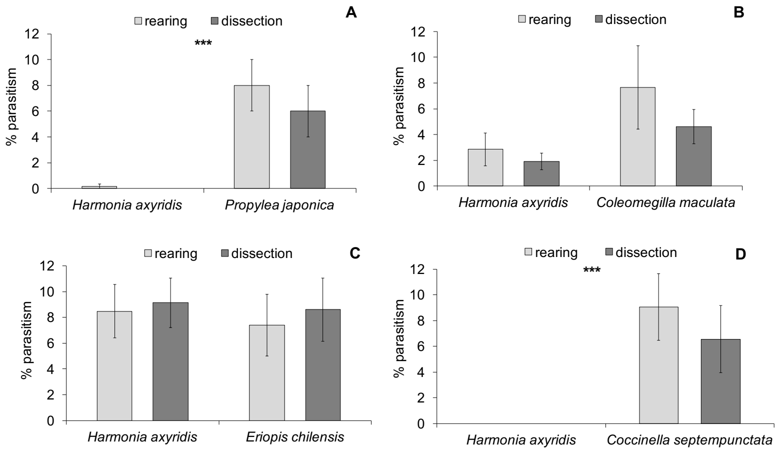


Figure 2. Mean (±SE) percentage of collected coccinellids presenting teratocytes revelead by dissecting native species and *Harmonia axyridis* over different countries. From left to right, China (A), Minnesota (B), Chile (C) and Sicily (D).

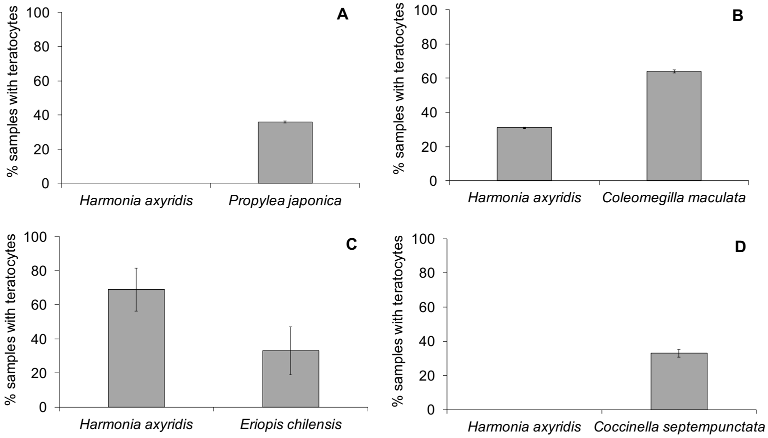


Figure 3. Molecular phylogenetic analysis by Maximum Likelihood method for *Dinocampus coccinellae* (bootstrap=1000). The tree was obtained from the analysis of the mitochondrial marker COI (Folmer et al.1994).

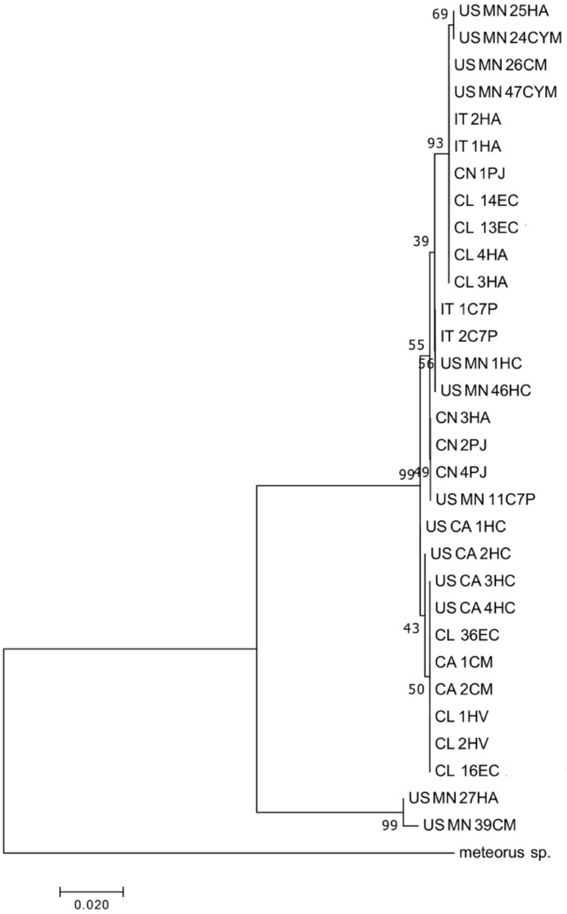


Figure 4. Molecular phylogenetic analysis by Maximum Likelihood method for *Dinocampus coccinellae* (bootstrap=1000). The tree was obtained from the analysis of the mitochondrial marker COI (Simon et al. 1994).

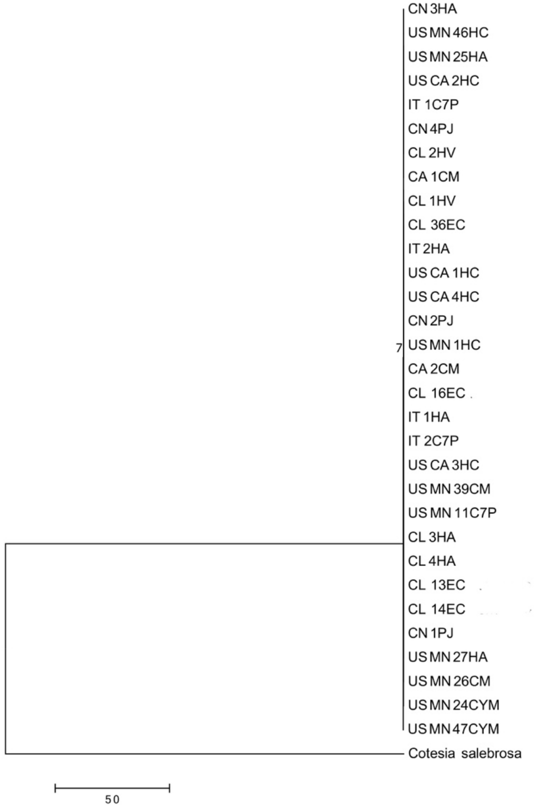
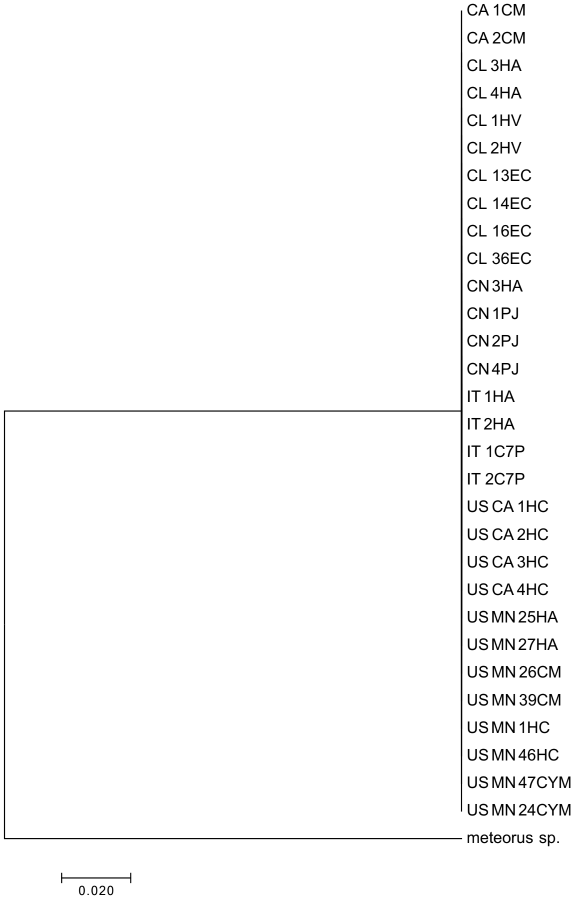


Figure 5. Molecular phylogenetic analysis by Maximum Likelihood method for *Dinocampus coccinellae*. The tree was obtained from the analysis of the mitochondrial marker 16s rRNA.



5.4. Discussion

In our study, the parasitism rate of *D. coccinellae* differed among countries and between the native and the invasive hosts. *Dinocampus coccinellae* parasitism level on non-*H. axyridis* native hosts was similar in all the sampled sites. European and American native coccinellids, as well for *Propylea japonica* in China, were similarly attacked by the parasitoid. By contrast, the parasitism of *D. coccinellae* on *H. axyridis* significantly changed among the sampled sites by recording different values according the invasion timing process of the exotic species.

Harmonia axyridis is a host of *D. coccinellae* with reports both within its native range and in all invaded continents, but parasitism and emergence rates are usually low in comparison with other host species (Hoogendoorn & Heimpel 2002; Koyama & Majerus 2007; Ceryngier et al. 2018). We recorded very low parasitism rates in the native range (China) compared to the rate of parasitism of the native species *Propylea japonica.* Although the harlequin ladybird is native to China across an extended range from the far south, Japan, Korea, to eastern Russia (Brown et al 2011), very little information is present on the parasitism activity of *D. coccinellae* in the native range the coccinellid predator. To the best of our knowledge, Maeta (1969) reported a parasitism rate of 1.2% and 7.5% over a sample size of 164 and 129 coccinellids in two localities in Japan, respectively, at the beginning of 1960. Later, Koyama and Majerus (2007) demonstrated in laboratory conditions that *D. coccinellae* from Japan successfully developed in British *H. axyridis* compared to the British wasps (about 17% of successful parasitism rate vs no successful parasitism). Moreover, parasitoid wasp of Japanese and British origins successfully developed in *H. axyridis* from Japan, suggesting that the noninvasive ecotypes of *H. axyridis* were more susceptible to various strains or geographic populations of *D. coccinellae* than the invasive ecotype. By contrast, the same group reported a higher emergence rate of *D. coccinellae* on *C. septempunctata* compared to *H. axyridis*, in relation to the ovipositional attack.

Varying parasitism rates on the invasive harlequin ladybird compared with similar parasitism rates on native coccinellids by *D. coccinellae* were also recorded in *H. axyridis* non-native countries. Our data are thus partially consistent with the findings of Ceryngier et al. (2018) who found, from the analysis of pooled data, a significantly lower parasitism and emergence rate *H. axyridis* by *D. coccinellae* in its invaded areas compared to the native species. We observed a divergence between the parasitism rates of field collected coccinellids between the *Coleomegilla maculata* and *H. axyridis* in Minnesota. The parasitism level of the native coccinellid beetle was higher than the parasitism rate of the invasive species (8.7% vs 2.9%). *Harmonia axyridis* was introduced in the USA in 1916 and many further release for biological control applications followed; however, the species was reported as permanently established only in the late 80s with a rapid spread across the country during the 90s and 2000 (Brown et al. 2011). Presumably, at the very beginning of its invasion process in the USA, in Oregon between 1993 and 1994, less than 1% over more than 2000 of field collected *H. axyridis* were successfully parasitized by *D. coccinellae.* The low parasitism rate recorded for *H. axyridis* was confirmed in the same country three years later (Ceryngier et al. 2018). By contrast, in Minnesota at the beginning of 2000, the parasitism rate of *H. axyridis* ranged between 23.8% and 8.9% over 63 and 282 collected coccinellids in the field, respectively, followed by very low emergence rate of *D. coccinellae* (Hoogendoorn & Heimpel 2002). According to our findings, in the same work the authors reported higher parasitism levels for *Coleomegilla maculata* which ranged from 14.5% to 18.2% over a sample size of 110 and 435, respectively. Also Firlej et al. (2005) recorded for the first time *H. axyridis* being attacked by *D. coccinellae* in Canada with a parasitism rate of 4.6% over more than 450 field sampled individuals. Conversely, the same authors reported for *Coleomegilla maculata* a parasitism rate of 32.1% in a similar, but slightly higher in size, field collected sample batch. According to previously published works, our findings thus highlight a decrease in the parasitism level of *H. axyridis* by *D. coccinellidae* in Minneota (USA), suggesting a possible host resistance.

Harmonia axyridis was first introduced in Chile in late 90s thorough a flightless strain used in biocontrol programs. However, the first record of species introduction in the wild was in 2003, although no further reports confirmed its permanent establishment until 2010 (Brown et al. 2011). Despite of the results obtained in the other countries, in Chile we found similar parasitism rates between the native *Eriopis chilensis* and the invasive *H. axyridis* both by the rearing and the dissections of the collected coccinellids in field surveys. In 2016, *Eriopis chilensis* showed a parasitism of 29.2% that was almost two times higher than the rate of 10.8% observed for *H. axyridis* (Ceryngier et al. 2018). However, such data should be taken with caution since the number of field samples was very low. Our findings in the Maule region (Chile) suggest a possible parasitoid adaptation to the host whether compared to *H. axyridis* coccinellid beetles collected across the different locations. Some recent sequential data have suggested that some *D. coccinellae* population may become better adapted to the invasive populations of *H. axyridis* (Knapp et al. 2019).

In Italy, *H. axyridis* was deliberately introduced for biological control programs in early 90s, but the species was reported as permanently established in Northern Italy about ten years ago (Burgio et al. 2008). However, this species was recorded for the first time in Sicily much more recently (Menchetti 2016). Within this area, we did not record any parasitism activity of *D. coccinellae* on the invasive ladybird, even though this result might be own to the very low number of collected specimens. On the other hand, the native *C. septempunctata* showed to be parasitized by *D. coccinellae* with similar rates recorded for this species worldwide. In northern Italy, Dindo et al. (2016) found no significant differences in the rates of emergence of *D. coccinellae* by *H. axyridis* and three native ladybird species (i.e., *C. septempunctata, Adalia bipunctata* and *Hippodamia variegata* (Goeze)), although the samples of the two species with low suitability for *D. coccinellae* (*A. bipunctata* and *H. variegata*) were small.

Physiological interactions between *D. coccinellae* and *H. axyridis* or native species have been also assessed. The immune response of *H. axyridis* against the development of *D. coccinellae* immature stages seem to represent the main defensive strategy. Firlej et al. (2007; 2012) demonstrated that *H. axyridis* is able to destroy effectively parasitoid eggs by the encapsulation and increased teratocytes arised in parasitized hosts. Another fascinating hypothesis proposes the resistance of *H. axyridis* to the RNA virus associated to the parasitoid (i.e. *D. coccinellae paralysis* virus, DcPV) (Dheilly et al. 2015; Ceryngier et al. 2018). DcPV was discovered and characterized by demonstrating its presence in the oviduct of parasitoid females and its replication in parasitoid larvae. The virus replication in the host's nervous tissue induces a severe neuropathy and antiviral immune response which is correlated to the paralytic symptoms characterizing the behavior manipulation. After the emergence, the coccinellid recovery to regular behavior is associated to virus clearance. These results provide evidence that changes in ladybeetle behavior is likely the result from DcPV replication in the cerebral ganglia rather than by manipulation by the parasitoid (Dheilly et al. 2015). Hence, investigations on the genetic regions of virus particles that are related to the ability to attack and affect the immune system of the host should be properly investigated. Moreover, since different degrees of parasitoid virulence have been proved when the host is fed with different dietary regimes (Maure et al. 2016), further studies should be addressed towards the effect of ecological and agroecological variables on the parasitoid fitness.

The combination of biology and phylogeography of a parasitoid could provide important information to interpret dispersal patterns, density fluctuations, and mating events correctly (Jourdie et al. 2010). The genetic structure of a parasitoid is also intimately associated with its hosts, because variations in host numbers and/or geographic range can led changes in parasitoid populations and different outcomes at evolutionary levels (Althoff & Thompson 1999). For this reason, molecular genetic data can be helpful in determining origins and routes of spread, estimate the number of founders and identify whether multiple accessions from different geographic areas can contributed to the organism distribution by increasing its genotype diversity (Williamson & Griffiths 1996).

Dinocampus coccinellae specimens included in our phylogenetic analysis showed no clear geographic structure, making difficult to pose hypotheses regarding the provenance of this species. Over the examined sites and hosts, haplotypes from geographically distant sites were sometimes more closely related to haplotypes belonging to the same site. Samples included in our phylogenetic tree clustered in two main groups. Only two parasitoid samples originated from Minnesota and emerged from both the invasive *H. axyridis* and the native *Coleomegilla maculata* clustered in a single small group. The narrow group can represent a population that may have arisen from a single colonization event. However, this result should be considered with caution because the analysis was conducted only on few sequences, since multiple introductions could exist in a single location (Jourdie et al. 2010). Moreover, other specimens derived from Minnesota and different coccinellid hosts were present in other well supported clades, demonstrating thus the paraphyletic origin of these populations. A wider group included all the individuals sampled in several hosts and among areas invaded in different times by the invasive *H. axyridis*. This latter group reveled a subgroup that included all the species originated from California, Canada. Considering that both the Canadian and Californian samples grouped together, we can assume the monophyletic origin of these sampled populations, arguing a possible north-south migration or vice versa. Yet, a more extensive sampling in the involved countries will be necessary to determine the exact geographic origins of this species.

The same main branch clustered a group including all the sequences of *D. coccinellae* derived from China, Italy and the remaining from Chile and Minnesota suggesting thus the paraphyletic origin of the latter two populations. Interestingly, individuals from Minnesota resulted separated also by host species, since they are widely distributed across the branches, suggesting a possible parasitoids specialization toward the invasive host. The high level of mixture at a global scale that we found shows considerable gene flow between populations. For instance, individuals from Minnesota, Chile, Italy and China came out together in a very well supported clade (i.e., sequences were identical in all partitions) despite being geographically so far distant. Similarly, individuals from California (emerged from *Hippodamia convergens*), Chile (emerged from *Eriopis chilensis* and *Hippodamia variegata*) and Canada (emerged from *Coleomegilla maculata*) were identical even though they came from distant places.

The difference between the American individuals which were collected on *H. axyridis, Cycloneda munda, Hippodamia convergens* and *Coleomegilla maculata* and the other individuals from Chile, that emerged from *Hippodamia variegata* and *Eriopis chilensis*, implies that use of different host species may affect evolutionary processes and may have led to genetic differentiation (Hawkins 2005). Interpopulation movements appear thus important even over very large distances. The admixture patterns of population structure found in *D. coccinellae* can be explained by insect human-mediated dispersal such us the contemporaneous trade of commodities and commercial traffic worldwide. The observed genetic structure might also result from the ability of *D. coccinellae* to use a wide host range and this can extend the dispersal patterns and quickly adapt to environmental changes and colonize new environments (Lombaert et al. 2010).

Different possibilities in the phylogenetic relationships of host and parasite species through time could exist: from cospeciation or no speciation, to extinction, host shifting, speciation without a host shift and host speciation without speciation in the parasite (Price 1997). The differences in lineage modes of develop depend on the conditions of the prevailing biological interaction. For instance, geographic patterns have been found in the virulence of the parasitoid *Asobara tabida* (Hymenoptera: Braconidae) and host resistance in populations of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) from the north to the south of Europe (Kraaijeveld & Godfray 1999). As a consequence, parasitoid wasps can present spatial and temporal mosaic variations in their interactions with their hosts for selected traits such as parasitoid virulence and host encapsulation defenses (Althoff & Thompson 1999). For this reason, we need to better understand how the interplay of gene flow and selection determine the distribution of traits observed across populations of interacting species (Bolnick et al. 2011).

The trees obtained from the sequenced16s rRNA region did not show any discriminative information. Hence, the scarce interpretation of genealogical lineage distribution through generic mitochondrial markers can be overcame by the use of specific markers such as microsatellites and AFLPs that provide information on the genetic similarity among populations on the base of allele frequencies (Freeland 2011). Lombaert et al. (2010) suggested that the outbreak of worldwide invasion of *H. axyridis* followed a bridgehead scenario in which an invasive population in eastern North America colonized the European, South American and African continents, with some admixture with a European biocontrol strain. As a result, the widespread invasion can be relied on the success of particular invasive populations rather than native range populations that only serve as colonist sources for the invasion of new territories. For this reason, we can conclude that genetic approach aiming at studying the host resistance patterns towards *D. coccinellae* by tracking phylogenetic host lineages worldwide might reveal new interesting insights within this field.

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General conclusions

In the present thesis work, the efficacy of eight nanoformulated essential oils derived from Mediterranean aromatic plants was evaluated against one of the major agricultural pest, the cotton aphid *A. gossypii*. The tested EO formulations (i.e., anise, fennel, artemisia, garlic, lavender, peppermint, sage and rosemary) caused significant different mortality by topic exposure to the target pest and different residual contact toxicity towards the parasitoid *Aphidius colemani*. Among the EOs, fennel and lavender showed promising for pest control because of the very low estimated lethal Concentration 90% (LC_{90}), compared to the other substances. These nanoemulsions showed also low residual lethal effect towards the non-target species, suggesting to be candidate tools for the sustainable control of the cotton aphid in combination with the parasitoid. However, further experiments in laboratory and field conditions are required to assess the toxicity in a more extended period, including also the behavioral and physiological sublethal effects (Desneux et al. 2007), before their inclusion in IPM programs.

Systemic insecticides are globally used to control agricultural pests and their non-target impact on beneficial arthropods has been often recognized. The evaluation of biocontrol services has a key role for pesticide risk-assessment on natural enemies and this is particularly relevant for insecticides at non-lethal doses. The estimation of baseline toxicity of imidacloprid, thiamethoxam and sulfoxaflor on the polyphagous cotton aphid was conducted and their sublethal effects were assessed on the parasitoid *A. colemani* by residual contact exposure to the LC_{50} LC_{20} and LC_1 calculated for the pest. The LC_{50} calculated for the aphid pest were considerably lower compared to the label concentrations.

Thiamethoxam followed by sulfoxaflor at their LC_{50}s estimated on the pest caused the highest mortality rate on parasitoid compared to the other systemic insecticides at different concentrations, while imidacloprid had the lower impact on the mortality and the fertility of the natural enemy at all the tested concentrations. These findings highlight the importance of case-specific evaluation to optimize pesticide applications in IPM packages, taking into account further ecological services provided by biological control agents.

Harmonia axyridis is a widespread predator and it a biological model to address several questions over different research topics. Although the species is not marketed anymore in Europe, it is commercialized in China for biological control applications (Wang & Shen 2002) and it represents a model species for investigations related to ecotoxicology and biological invasions (Roy et al. 2016; Cabrera et al. 2017). Laboratory colonies of the harlequin ladybird are reared on expensive and labor-intense natural prey, but alternative food regimens are needed to optimize the laboratory rearing though the cost reduction. Within the thesis work, the effect of four different regimes (i.e., moth eggs, moth eggs combined with *Artemia* cysts, a liver-based artificial diet and aphid prey) was tested on the life parameters of the harlequin ladybird at standardized environmental conditions. *Harmonia axyridis* successfully developed on lepidopteran eggs as previously reported in the literature. While the tested artificial liver-based diet did not support very well the development of the coccinellid beetle compared to the other food regimes. By contrast, the commercial mixture of Artemia cysts and *Ephestia kuheniella* eggs determined good performance in the laboratory culture of *H. axyridis*, representing thus a fair compromise between the economic cost and the biological performance of the predator rearing.

The behavioral response of biological control agents is an important trait to evaluate for the compatibility assessment of chemicals used IPM and this is particularly relevant for insecticides at low and sublethal concentrations. The sublethal effect of imidacloprid, thiamethoxam and sulfoxaflor at their LC_{50}, LC_{20} and LC_1 earlier calculated for the target pest *A. gossypii*, was assessed on the functional response of the harlequin ladybird. The survival and the voracity of the coccinellid beetle differed among the three tested aphicides and their concentrations. Thiamethoxam at LC_{50} caused the highest mortality similar to the effect previously recorded for the parasitoid, while the tested systemic pesticides at their low-lethal and sublethal doses were considered safe. After the ingestion of poisoned prey in the most of the pesticide-concentration combinations, the coccinellids showed a density independent functional response, while an inverse density dependence of the prey consumption rate was evidenced for predators exposed to the lowest concentrations of sulfoxaflor and thiamethoxam. Also, the attack rate and the prey handling time were negatively affected by the higher concentrations of imidacloprid and sulfoxaflor. These findings give important information regarding the use of systemic insecticides in protected crops, and stress the importance of evaluating the potential behavioral side effects of insecticides on natural enemies in the risk assessment, even at very low concentrations.

In conclusion, because of the importance of *H. axyridis* as predatory invasive species, the study of potential factors involved in the spreading of the coccinellid worldwide was investigated. In particular, the role of the cosmopolitan parasitoid *D. coccinellae* in the suppression of the invasive populations of the harlequin ladybird was determined in terms of parasitism rate and phylogeography in comparison to native hosts within different world areas, according the *H. axyridis* timing process. The results highlighted that parasitism rates differed among the sampled areas and the hosts. Native non-*H. axyridis* coccinellids were similarly parasitized in all sites, but *H. axyridis* sampled populations showed different parasitism rates, being negligible in the native area (China), low in the old invaded country (Minnesota, USA), and similar to native species in the recently invaded region (Chile). In the more recently invaded site (Sicily, Italy), *H. axyridis* was marginally present with null parasitism activity. These outcomes highlight the need to repeat parasitism investigations over time by extending the sampling site network as well as the study of co-evolutionary factors among parasitoid and host populations. The phylogeography at large scale by using COI marker showed no clear geographic structure in the sampled parasitoid populations in the sampled territories and their hosts, making difficult to identify the origin of the species. Therefore, further studies are needed to determine the spatial genetic structure at anarrow scale and to track the phylogenetic host lineages (Althoff & Thompson 1999).

Concerning the main thesis objectives, new data on the efficacy and the compatibility of different tested commercial formulations and novel compounds on the considered trophic network was acquired. Obtained and processed information on the control of the cotton aphid will allow to establish the formulation's efficacy for pest control and make decisions concerning the use of pesticides in the context defined by IPM and provided by current legislation on plant protection field.

New gained knowledge of side effects of the tested insecticides on beneficial arthropods of applied interest will permit the research community to evaluate the compatibility for the integration in biocontrol programs, supporting farmers and technicians in this field. Research products of this Ph.D. thesis may also contribute to the sustainable use of pesticides throughout the reduction of side effects and integration with other control tools, thus optimizing energetic inputs on agro-ecosystems.

Furthermore, information acquisition on the role and the dynamics of trophic networks throughout the study of simplified biological models will provide useful indications to better understand complex biocenotic relationships which occur in ecological communities. This knowledge can be digested by scientific community to improve acquaintance with applied ecology field, such as biological control strategies in all their aspects. In conclusion, the potential impact of this research product, beyond the merely technical implications, might also contribute to the sustainable protection of the environment.

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