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INVESTIGATIONS ON ECOLOGY AND BEHAVIOUR OF THE INVASIVE *HALYOMORPHA HALYS* (HETEROPTERA: PENTATOMIDAE) AIMED AT ITS SUISTAINABLE MANAGEMENT IN AGRO-ECOSYSTEMS

Candidato: Bulgarini Giacomo

Relatore: Prof.ssa Lara Maistrello

Coordinatore: Dr. Alessandro Ulrici

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Abstract

Halyomorpha halys (Heteroptera, Pentatomidae), nativa dell'Asia orientale, è una specie molto polifaga che rende frutti e semi non commercializzabili. Essendo un autostoppista su oggetti inanimati, ha un'elevata capacità di dispersione facilitata dal commercio, che permette una rapida espansione globale. Per contrastarla, gli agricoltori hanno aumentato l'uso di insetticidi ad ampio spettro, stravolgendo i piani di difesa integrata, con gravi rischi economici e ambientali. Inoltre, a causa delle aggregazioni di svernanti negli edifici, è anche un fastidio urbano. In Europa, l'Italia è il Paese che ha subito le maggiori perdite economiche, come testimoniano i danni stimati di 600 milioni di € nel 2019. Per ottimizzare la gestione sostenibile negli agroecosistemi, è fondamentale la conoscenza dei tratti ecologici e comportamentali degli organismi invasivi. Questo lavoro è incentrato su *H. halys*, in particolare su: i) ruolo dei predatori generalisti nativi come agenti di biocontrollo, e ii) valutazione dell'uso di oli essenziali per ridurre i problemi causati sia in ambito agricolo che urbano. Considerando il ruolo dei predatori nativi, un primo gruppo di prove ha studiato in laboratorio l'abilità predatoria di diverse specie solitarie sulle uova e le neanidi. I risultati hanno indicato un'accettazione piuttosto bassa di *H. halys* come preda, poiché solo due specie (l'ortottero *Eupholidoptera chabrieri* e il reduvide *Rhynocoris iracundus*) hanno causato l'80% di mortalità su almeno un tipo di preda e la mortalità dovuta agli altri predatori non superava mai il 60%. Una seconda serie di prove ha testato il potenziale di *Lasius niger*, la formica più comune nei pereti. I risultati hanno mostrato predazione sul secondo e il terzo stadio di *H. halys* ma non su uova e ninfe. Un approccio complementare per studiare la predazione da parte di specie autoctone è stato quello di eseguire analisi molecolari del contenuto intestinale di esemplari raccolti in campo. I risultati hanno indicato la presenza di *H. halys* nell'intestino di insetti (Coccinellidae, Forficulidae, Nabidae, Reduvidae, Mogoplistidae, Tettigoniidae) e di aracnidi (Phalangiidae, Anyphaenidae, Araneidae, Philodromidae e Salticidae). Individuando specie in grado di sfruttare *H. halys* come preda, questa parte del lavoro, che è stata eseguita nel nord Italia, fornisce un importante contributo sul controllo biologico conservativo di questo insetto nell'Europa meridionale. Ulteriori prove sono state eseguite in Canada sul ragno *Phiddipus audax*, un predatore generalista molto comune in Nord America, che di solito si trova in numero elevato in campo nelle trappole innescate con feromone di aggregazione di *H. halys* a fine estate. Indagini in campo e test di laboratorio con l'olfattometro hanno permesso di accertare che *P. audax* può utilizzare il feromone di aggregazione come indizio per identificare *H. halys* come preda e hanno identificato una chiara risposta stagionale in quanto i ragni rispondevano ai segnali solo in autunno, suggerendo cambiamenti fisiologici in risposta alle condizioni ambientali. La potenziale utilità degli oli essenziali per ridurre i problemi causati da *H. halys* è stata valutata testando la repellenza di quattro composti a diverse concentrazioni su adulti di tre fasi fisiologiche-comportamentali: uscita da svernamento, attività in estate, entrata in svernamento. Tutti gli oli testati erano repellenti a concentrazioni superiori al 3%. I risultati hanno suggerito che gli oli essenziali di curcuma e chiodo di garofano sono candidati promettenti per ridurre gli attacchi alle colture sensibili in estate, nonché per prevenire l'ingresso di adulti svernanti nelle case.

Abstract

Halyomorpha halys (Heteroptera, Pentatomidae), native to East Asia, is a polyphagous species that causes serious damage on fruits and seeds, rendering the products unmarketable. Being a hitchhiker on inanimate objects, it has a high dispersion capacity facilitated by human activities and trade, which has allowed fast colonization worldwide. To manage the invasion of this pest, farmers have increased the use of broad spectrum insecticides, disrupting IPM programs, with serious risks both for the economy and the environment. Besides, due to the overwintering aggregations inside buildings, *H. halys* is also a dwelling nuisance. In Europe, Italy is the country that has suffered the greatest economic losses from *H. halys*, as evidenced by the estimated € 600 million damage in 2019. In order to optimize management and sustainability in agroecosystems, knowledge of ecological and behavioural traits of invasive pests is crucial. This work is targeted on *H. halys*, focusing in particular i) on the role of native generalist predators as biocontrol agents for this pest, and ii) on verifying if essential oils could be used to reduce the trouble it causes in both agricultural and urban contexts. Considering the role of native predators, the first group of experiments investigated the predatory ability of several solitary generalist predators on the eggs and the first two juvenile instars of *H. halys* in no-choice laboratory trials. Results indicated that the tested species showed a quite low acceptance of *H. halys*, as only two species (the orthopteran *Eupholidoptera chabrieri* and the reduviid *Rhynocoris iracundus*) caused 80% mortality on at least one item and the mortality due the other predators never exceed 60%. A second set of laboratory trials tested the potential of *Lasius niger*, the most common ant in pear orchards. Results showed that it consumed second and third instars of *H. halys* but not the eggs or older nymphs. A complementary approach to investigate predation by native species was to perform molecular analysis of the gut content of specimens collected in the field. Results indicated presence of *H. halys* in the gut of insects belonging to Coccinellidae, Forficulidae, Nabidae, Reduvidae, Mogoplistidae, Tettigoniidae and of arachnids belonging to Phalangiidae, Anyphaenidae, Araneidae, Philodromidae and Salticidae. By identifying species that can exploit *H. halys* as suitable prey, this part of the work in northern Italy, provides an important contribution for conservation biological control of this pest in southern Europe. Further trials were performed in Canada on the spider *Phiddipus audax*, a very common generalist predator in North America, which is usually found in high numbers inside field traps baited with aggregation pheromone of *H. halys* in late summer. Field investigations and laboratory tests with an olfactometer allowed to ascertain that *P. audax* can use the aggregation pheromone as a clue to identify *H. halys* as a potential prey and identified a clear seasonal response as the spiders responded to the chemical cues only in fall, suggesting physiological changes in response to environmental conditions. The potential usefulness of essential oils to reduce troubles caused by *H. halys* was tested in a two-choice apparatus where the repellence of four compounds at different concentrations was evaluated on adults of three different physiological-behavioural phases: exiting overwintering, active during summer, entering overwintering. All tested oils were repellent at concentrations higher than 3%. Results suggested that turmeric and clove essential oils are promising candidates to reduce attacks to susceptible crops in summer, as well as to prevent the entrance of overwintering adults in houses.

CHAPTER 1

GENERAL INTRODUCTION

1. Invasive species

In biology, and according to the Convention on Biological Diversity (CBD), a taxon (animal, plant, fungus or pathogen) is defined as an alien species if it is introduced, established, naturalized and spread in a habitat other than its historical range. Alien species found in a new habitat can become extinct in the new range or maintain very low population levels, or may increase exponentially. If the latter, alien species may thrive in the new environment, increasing in abundance and over long periods of time, becoming invasive alien species. By invasive alien species, or pest, we mean exotic species that alter the ecosystem, compete with or consume native species and alter the fundamental rules of existence of organisms in the area. This can have significant social and economic effects if they were to alter any of the ecosystem services that affect humanity (Vitousek 1990). Among the invasive pathogens that have spread from continent to continent and have caused serious consequences for mankind, causing millions of victims, there are the bubonic plague, the black plague, Spanish flu and Covid-19. But biological invasions do not only affect human health, and the ones that involve biodiversity and ecosystem services often have serious economic consequences. Species have always spread to new ranges and habitats even without human help through long-term dispersal events, but this happened less often, more slowly and in areas not so distant from each other (Simberloff & Rejmanek 2011). Thanks to the industrial revolution there have been technical improvements that have allowed the transportation of increasing amounts and types of materials over great distances, thus facilitating the spread of alien species. Some plants and vertebrates were introduced intentionally (i.e. voluntary release of wild game, biological control agents, plants used to shape the landscape), while the majority of invertebrates, including insects, were introduced accidentally (Pimentel et al. 2005; Jones & Pedro 2008).

This revolution has revealed one of the most elusive classes of invasive species, namely the hitchhikers. Hitchhikers are those organisms that are transported and dispersed involuntarily through the transport of goods and which are therefore destined to increase with globalization (Hulme 2007). Examples include aquatic organisms that are transported with the ballast water of ships (Hulme 2007). Thus, international trade has facilitated the movement of the species and its degree affects the number of alien species (Mooney & Cleland 2001; Westphal et al. 2008). The introduction of alien species has increased since the 20th century and this has caused threats to native species and ecosystems and to human well-being (Simberloff et al. 2013). In New Zealand, the number of native plant species equals the number of invasive species, and in general 20% of plant species are alien in many countries. In the past 500 years, alien species have invaded 3% of the land that is not frozen (Mooney & Cleland 2001).

Not only trade, but also the development of agriculture has facilitated the establishment of alien species. In agriculture, new species are often introduced as they can lead to economic gain, satisfy consumer demand for exotic products, suppress or feed on other species or simply because they are liked by people (Davis & Landis 2011). This occurs from the period of the great explorations, as the introduced and acclimatized species were considered exotic curiosities and also considered as resources, and currently many alien species are introduced for aesthetic and commercial purposes (Simberloff et al. 2013). Furthermore, modern agriculture greatly facilitates the invasion and establishment of alien species due to the increase in monocultures and the continuous disturbances caused by agricultural management techniques that open up biological space for exploitation (Davis & Landis 2011). Furthermore, the agricultural environment offers an availability of resources capable of facilitating invaders with respect to the natives, and the high connectivity of the agricultural environments facilitates the dispersion of alien species (Davis & Landis 2011).

The increase in trade has certainly increased the dispersion of invasive species, however among all the alien species, only 10% manage to establish in the new environment and only a fraction of these (from 1% to 10%) become invasive (Mooney & Cleland 2001; Westphal et al. 2008). One of the elements that affects the success of an invasion is the propagule pressure, which includes both the number of individuals introduced and the number of times the introduction occurs (Meyerson & Mooney 2007; Westphal et al. 2008). Therefore the greater the number of individuals introduced and the greater the number of times the individuals are introduced, the greater the probability that an alien population will be able to establish itself in the new environment. Considering only the hitchhikers coming from the ships, it is very likely that some individuals were already present in the ports and the continuous exchange of goods has done nothing but increase the propagation pressure, also increasing the genetic diversity due to the arrival of new individuals, allowing the new species to establish themselves (Levine & D'Antonio 2003; Lockwood et al. 2005). There is also another mechanism that make alien species invasive in the long period: the lag effect, which is the mechanism that renders species invasive after years of introduction at low levels (Mooney & Cleland 2001).

A further factor that has allowed the increase of invasive species is climate change (Mooney & Hobbs 2000). Invasives are significant components of human-caused environmental changes (Westphal et al. 2008). The abiotic environment is highly altered due to climate change and massive alterations such as the exploitation of the land. With the increase in CO_2 levels, it is expected that invasive plants will respond better than native species, managing to grow more by increasing photosynthetic rates and shifting to early emergence of their leaves (Mooney & Cleland 2001; Farnsworth & Meyerson 2003; Ziska & George 2004). Climate change pushes organisms to seek new habitats with a more compatible climate for them, consequently increasing biological invasions (Mooney & Cleland 2001). Arthropod invasions ranging from agricultural pests to disease carriers (i.e. mosquitoes and ticks) are occurring and are expected to increase, with significant impacts on human well-being (Ogden et al. 2014). Fluctuations in climate and biota have already occurred in the past, but the rate of change has accelerated (Mooney & Cleland 2001).

In addition to external or anthropogenic factors, what allows some species to become invasive are some characteristics of the species themselves, which are shared among invaders, such as: i) short generation time, ii) high fecundity and growth rate (with a population dynamics based on the r strategy), iii) high dispersal capacity, iv) behavioral flexibility, v) phenotypic plasticity and vi) wide variety of hosts (Simberloff 1989; Williamson 1996). All these characteristics allow invasive species to compete with native ones for resources and in some cases to surpass them.

1.1 Ecological impacts

From the ecosystem point of view, invasive species have a great impact. By ecological impacts we mean significant changes in ecological processes, and invasive species are able to cause higher changes compared to natives (Simberloff et al. 2013). They are in fact the second cause of extinction since 1500 A.D., after habitat loss (Wilcove et al. 1998; Mooney & Cleland 2001; Horan & Lupi 2005; Bellard et al. 2016). Among the best documented invasive species there are ants, such as the red ant *Solenopsis invicta* (Buren, 1972). This ant, native to South America, has invaded the southern regions of North America and is a threat to various arthropods also attacking beneficial insects, and is also dangerous to reptiles, birds and mammals (Morrison 2002; Holway et al. 2002). Even herbivorous invertebrate species can cause serious ecological damage, eating native plants leading them to extinction, as it is occurring to the Fraser fir (*Abies fraseri*) in

North America, due to the balsam woolly adelgid *Adelges piceae* Ratzeburg (Hemiptera: Adelgidae) (Small et al. 2005; Kenis et al. 2009). In the USA, 49% of endangered species are due, at least in part, to the effects of alien species (Wilcove et al. 1998). This can be due to the fact that in the invaded areas there are no specific natural antagonists to keep the invasive population uder control and/or due to the fact that invasives can successfully compete with the natives or prey on them (Horan & Lupi 2005). Furthermore, invasive species could be vectors/vehicles of diseases for plants, as well as for humans (Horan & Lupi 2005).

Invasives are therefore able to adapt to the new environment causing direct changes, but they can alter the biological community also indirectly through changes in the genetic structure of the populations, with hybridization and introgressions with native species (Long 2003; Mallet 2005; Mooney & Cleland 2001). Hybridization can lead to decreased fitness and thus extinction. This phenomenon is greater in small isolated populations. Three out of 24 endangered species in the US became extinct because they hybridized with alien species (Mooney & Cleland 2001). The arrival in a new habitat can also lead to a change in behavior, both on the part of the invader and of the natives (Mooney & Cleland 2001). In addition to predation and hybridization, another factor that can lead to extinction of native species is competition, an invasive species can in fact steal resources from the natives if they are more efficient in procuring them (Westphal et al. 2008; Mooney & Cleland 2001; Hulme 2007; Meyerson & Mooney 2007). When the invasives differ substantially from the natives in the use and acquisition of resources, an alteration of the trophic structures of the invaded area occurs, altering the frequency and/or intensity of disturbance. For example the moth *Lymantria dispar* L. has defoliated the oaks in North America, significantly affecting native bird populations (Vitousek 1990; Simberloff et al. 2013; Bell & Whitmore 2000; Gale et al. 2001).

Although there is always a positive correlation between the decline in native species and the expansion of invasive species, the latter are not always the main cause of this phenomenon (Didham et al. 2005). The degree of invasion is also dictated by the properties of the ecosystem (Westphal et al. 2008). A disturbed ecosystem or habitat can facilitate invaders if they are able to take advantage of changes in the ecosystem (Cohen & Carlton 1998; MacDougall & Turkington 2005). Furthermore, regions with endemic species are more susceptible and more easily invaded (Cohen & Carlton 1998; Westphal et al. 2008). Habitats with a great species richness are also more susceptible to biological invasions, as they have greater resource heterogeneity (Westphal et al. 2008).

1.2 Economic damage

Although the ecological damages are great, they are difficult to decipher compared to the economic ones, which are perceived and reported more quickly, in addition to the fact that economic pests attract more scientific attention (Vilà et al. 2010). Some countries are more vulnerable to biological invasions and this may depend on several factors, such as its openness of the economy, its geography, existing regulations and the importance that agriculture, forestry and tourism have in the country (Perring et al. 2002). Invasive species are capable of causing significant damage especially in the agricultural sector, being in fact the major cause of crop losses, with about 500 species of insects and mites that are crop pests (Paini et al. 2016).

Just by estimating the costs caused by 79 invasive species in the U.S.A. between the years 1906 and 1991, the economic impact had reached 97 billion dollars a year (Office of Technological Assessment 1993). Currently, eonomic costs in U.S.A. caused by alien spcies has reached an estimate of 120 billion US dollars a year (Pimentel et al. 2005; Westphal et al. 2008). At least 145 alien species have been introduced into the great lakes since 1830 and a third in just the last 30 years. 10% of these species were found to be invasive and caused significant economic damage.

For example, zebra mussel (*Dreissena polymorpha*) (Pallas, 1771) caused damage for 5 billion in the last 10 years (Horan & Lupi 2005).

In Europe, on the other hand, estimates of costs due to invasive species exceed 20 billion euros per year (Pyšek & Richardson 2010). In the UK, annual damage to crops caused by alien arthropods is estimated as € 2.8 billion, and by adding to the damage inflicted by alien pathogens and vertebrates, it increases to € 3.8 billion per year (Pimentel et al. 2001). In Germany, only three invasive arthropods of stored cereals cost about € 12 million per year (Reinhardt et al. 2003). In Italy, in Lombardy, € 18 million were spent from the region to remove the trees hosting the Asian long-horned beetle *Anoplophora chinensis* (Thomson, 1865), apparently without success (Ciampitti & Cavagna 2014). New Zealand is estimated to have been overrun by 2,200 species of invertebrates, some of which are serious agricultural pests (i.e. the Mediterranean fruit fly and the Argentine ant). These invasions cost about US$ 195 million in crop losses and another US$ 242 million in control costs (Barlow & Goldson 2002). In Australia, invasive weeds caused an impact of 3.9 billion Australian dollars (Sinden et al. 2004).

The management of invasive species involves various costs that are added to the direct damage of the aliens. For this reason, preventing their entry is more effective than managing them after the invasion (Shine & Williams 2000). Early monitoring and diagnosis are useful tools for scientists to collect the information necessary to organize techniques suitable for limiting the invasion and expansion (Hobbs & Humphries 1995). If the prevention is ineffective or late, the next steps are eradication (easier at the beginning of the invasion and with species easy to recognize and eliminate), followed by containment and control, aimed at limiting the further expansion of the pest, or adaptation (Horan & Lupi 2005).

2. The target invasive pest*: Halyomorpha halys*

2.1 Morphology and biological cycle

Halyomorpha halys (Stål, 1855) (Hemipetra, Pentatomidae), commonly called brown marmorated stink bug (BMSB), is native to China, Japan, Taiwan and South Korea (Lee et al. 2013a; Haye et al. 2015). The adult is gray-brown in color and 12 to 17 mm long. It has antennae with white bands, basal and apical on the 4th segment and only basal on the 5th segment, an hemielithral membrane with longitudinal dark spots on the ribs and the connective membrane with a triangular-shaped median yellow spot (Hoebeke & Carter 2003). Adults also have copper-colored spots or bluish punctures on the head and pronotum and whitish calluses present in the discal part of the pronotum and at the base of the scutellum. The odoriferous glands are found on the dorsal surface of the abdomen and the underside of the thorax. These glands are responsible for producing the pungent odor used by the stink bug as a defense mechanism (Malumphy & Eyre 2011). Adult males are usually slightly smaller than females and can be easily identified by the pterygopodium located in the last urite. The females are polyandrous and lay their egg masses, composed on average of 28 eggs, on the abaxial plane of leaves. As soon as they are laid, the eggs are pale-green and turn white just before hatching. From birth to the adult stage, *H. halys* exhibits five nymphal stages. Newly hatched nymphs emerge 3–6 days after egg-laying and remain on eggs during the first instar to acquire endosymbionts left by the mother (Taylor et al. 2014). Table 1 shows some characteristics of the five instar nymphs. Growth from hatching to adult stage requires 32–5 days at 30 ° C with a minimum and maximum development threshold of 14° and 35° C. At 25° C (Rice et al. 2014; Lee 2015).

Instar nymph	Emerging from the egglaying (days)	Measures (mm)	Colour	Morphology
1°	3–6	2.4	Black head; red eyes; reddish-orange abdomen with black spots	
2°	12–14	3.7	Black and white colours	Spines on the pronotum margins; white central band on the tibia
3°	15–16	5.5	Dark brown colorus	Spines on the pronotum margins; white central band on the tibia
4°	22–23	8.5	Dark brown colours	Spines on the pronotum margins; white central band on the tibia; wing pads
5°	29–30	12	Dark brown colors	Spines on the pronotum margins; white central band on the tibia; wing pads

Tab 1. Morphological characteristic of the five instar nymphs of *Halyomorpha halys* (from Rice et al. 2014)

H. halys is a multivoltine species with a number of generations ranging from one to four per year in southern China (Lee et al. 2013a). The average longevity of females and males, where they have only one generation per year (like in Pennsylvania and northern US), is 84 and 119 days, respectively (Medal et al. 2013). Non-reproductive adults overwinter in antropogenic structures and natural hosts, gradually emerging from these sites in March to April and becoming active when daily maximum temperatures exceed 25°C (Nielsen et al. 2008; Nielsen & Hamilton 2009; Haye et al. 2014a). Females are synovigenic and begin to lay eggs at the end of May and then continue throughout the summer (Nielsen & Hamilton 2009; Haye et al. 2014a). The copulation time ranges from a minimum of 8.43 minutes to a maximum of 11 with an average period of 10.15 minutes and the interval between depositions is 4.3 days at 25° C in the laboratory (Medal et al. 2013; Nielsen et al. 2008). The total number of eggs laid per female over the lifespan ranges from 168 to 212 (Medal et al. 2013; Nielsen et al. 2008). Egg mortality in the fields varies from 66% in the second half of August to 100% in the second half of September, when the temperature drops below 15° C (Haye et al. 2014a). Overwintering adults start to move to overwintering sites at the beginning of October (Nielsen & Hamilton 2009).

In the regions where *H. halys* has two generations per year, like in Beijing province in China, Southern Europe, and Mid-Atlantic or Southern US (Bergh et al. 2017; Zhang et al. 1993; Bakken et al.2015), the exit of adults from the overwintering sites starts in late March, when the maximum temperatures exceed 14 °C, with a photoperiod of 13h. Females begin to lay eggs in mid May and after the second week of July the adults of the overwintering generation start to die, but in the same period the adults of the first generation starts to lay eggs. In this period it is possible to observe an overlap of generations, the overwintering one and the first one. The reproductive potential shown in southern Europe (Italy) is high, with on average 285 (R_0 = 24.04) and 215 (R_0 = 5.44) eggs each for the overwintering and first generation females, respetively. The adults of the second generation will not reproduce and are the adults that enter in overwintering from the end of September and in October (Costi et al. 2017).

H. halys has a high dispersal capacity, an adult can fly 2 km in 24h (Lee & Leskey 2015) and up to 26m in height, and from field observations it has emerged that it can fly at about 3m/s along a straight line from take-off upon landing (Rice et al. 2014; Lee et al. 2013b). The longest flights occur in late summer and autumn, when stink bugs begin to gather to shelter in overwintering sites (Wiman et al. 2015; Lee & Leskey 2015). Being wingless, nymphs disperse while walking. It seems that temperature also affects movements, in fact at 25° C or higher temperatures the juvenile instars were able to travel more than 3m in 30 minutes and on average can travel 20m in 5 hours (Lee *et al.* 2014a).

Like other pentatomids, *H. halys* overwinters in the wild in crevices of dead trees and thick-barked trees (Lee et al. 2014b). Male *H. halys* produce a two-component aggregation pheromone (Khrimian et al. 2014; Zhang et al. 2013). This pheromone elicits a response on all stages of growth, and is used when a resource is identified, such as food, partners or overwintering sites. Furthermore, the aggregation pheromone does not push the attracted individuals to identify the exact point of the stimulus source, but they can be arrested several meters earlier (Morrison et al. 2016a).

2.2 Host range

Halyomorpha halys has piercing-sucking mouthparts and feeds by inserting its stylet inside the host plant tissues. It has a preference for reproductive structures, in particular for the unripe berries (Haye et al. 2015). Adults tend to feed on fruits, while nymphs can also feed on leaves and stems (Malumphy & Eyre 2011; Haye et al. 2015). This stink bug also showed the unique ability to feed through the bark, an unusual feature for stink bugs in general (Panizzi 1997; Martinson et al. 2013). *Halyomorpha halys* is a polyphagous species with a host range that includes more than 300 species of plants, both angiosperms and gymnosperms, belonging to more than 45 families, with a predilection for Rosaceae and Fabaceae (Lee et al. 2013a; Dioli et al. 2016). Both adults and juvenile stages of *H. halys* move from wild hosts to crops depending on the phenology of the host plant and their dietary needs (Lee et al*.* 2014a). Plants attacked by *H. halys* include fruit trees (i.e. apple, apricot, cherry, olive, plum, peach, pear, kiwi, persimmon), small fruits (i.e. raspberry, blueberry, blackberry, grapes), hazelnuts, walnuts, vegetables (i.e. okra, asparagus, tomato, sunflower, eggplant, sweet pepper), row crops (i.e. soy, cotton, hops, bean, sorghum, wheat, corn) and spontaneous and ornamental plants (i.e. tree of paradise, buddleia, greater ash, holly) (Pfeiffer et al. 2012; Leskey et al. 2012a; Lee et al. 2013a; Rice et al. 2014; Haye et al. 2014b; Kuhar et al. 2012; Haye et al. 2015; Northeastern IPM Center 2014).

2.3 Distribution

Human activities and trade have allowed this insect to exploit its nature as a hitchhiker on inanimate objects, allowing it to expand its range and also colonize other continents (Maistrello et al. 2018; Rice et al*.* 2014). In the United States, the first confirmed identification of an established population of *H. halys* occurred in 2001 in Allentown (Pennsylvania), however some of those specimens may have originally been collected in 1996 (Hoebeke & Carter 2003; Hamilton 2009). The species gradually expanded to other states, until 2010, which was the large outbreak year of *H. halys* in the eastern US. Up to 2020, *H. halys* is present in 46 states in the United States and 4 provinces of Canada (StopBMSB 2020). Most recently it was detected also in South America, specifically in Chile (Faúndez & Rider 2017).

In Europe the first sighting of a stable population of *H. halys* occurred in 2007 in Zürich, Switzerland (Wermelinger et al. 2008), however photographic evidence testifies to its presence since 2004 (Haye et al. 2014b). In the same year, an individual was captured in Liechtenstein (Arnold 2009). In the following years, between 2007 and 2010, the population of *H. halys* grew exponentially (Mueller et al. 2011), so much so that in 2013 *H. halys* was present in 11 cantons (Haye et al. 2014b). In 2011, many individuals were registered in Greece, while in 2012 it was detected in Germany and France (Milonas & Partsinevelos 2014; Heckman 2012; Callot & Brua 2013; Garrouste et al. 2014). In 2013 *H. halys* adults and nymphs were identified in Hungary (Vétek et al. 2014), in 2015 in Austria, Romania and Serbia (Rabitsch & Friebe 2015; Macavei et al. 2015; Šeat 2015) and in 2016 in Spain, Russia and Bulgaria (Dioli et al. 2016; Mityushev 2016; Simov 2016). Single individuals, but not stable populations, were intercepted on baggage of passengers in transit in Great Britain (Haye et al. 2015; Malumphy 2014). In November 2020*, H. halys* is reported with established populations in 28 countries in Europe and along the Black Sea (Inaturalist 2020).

2.4 Halyomorpha halys in Italy

In Italy the first official record was in 2012 in the province of Modena, Emilia Romagna (Maistrello et al. 2016). However, thanks to the use of a model based on its spatiotemporal dispersion, it was possible to identify 2009 as the possible year of arrival of *H. halys* in the country (Maistrello et al. 2018). In the following years, the pest rapidly spread to the rest of the country, invading the entire territory, including the major islands of the Mediterranean (Sardinia, Sicily and Corsica) (Maistrello et al. 2018). In Italy, *H. halys* has two generations per year, with a partial overlap of the oviposition periods, and contemporary presence of different stages of development throughout late spring and summer. In Italy the reproductive potential of this pest is high, with an average 285 eggs for the overwintering generation and an average 215 eggs for first-generation females (R_0 = 24.04 and 5.44 respectively for the two generations) (Costi et al. 2017). The overwintering period lasts between the end of September to the end of March-mid of April and mating occurs since mid May to the end of August (Costi et al. 2017).

Genetic analyses have shown that there are at least two different populations in Italy, which may have arisen from two different introduction events. One may be due to a normal expansion by the Swiss population, the other one may have originated from a few individuals as a result of passive transport through trade with North America (Cesari et al. 2014). The genetic analyses of samples collected over time have also shown that the Italian populations have the highest biodiversity outside the Asian range, probably due to the multiple introductions that still occur (Cesari *et al.* 2018).

2.5 Damages and economic losses

Direct damage occurs to plants as a consequence of the feeding activity, which can cause scars, malformations, suberifications, discolored areas, necrotic areas, brownish spots and premature fruit drop, in addition to the abortion of flowers and young fruiting bodies (Rice et al. 2014; Malumphy & Eyre 2011). The damage also changes during the ripening of the fruit, an attack by *H. halys* on a young fruit causes malformation or premature drop, but as it matures the reactions of the affected tissues change, leading to suberification, necrotic areas, discolored areas, "cat-faced"or watery rot (deliquescence) of the pulp (Rice et al. 2014). This pest can also cause the "stay green" syndrome, found in particular on the edges of soybean crops, a syndrome that delays senescence in plants (Leskey et al. 2012b). Attacks on dried fruit, such as hazelnut, increase the incidence of empty shells. All of this damage makes agricultural products unmarketable, causing considerable economic damage. In the Midwestern US states, in 2010 *H. halys* caused more than $37 million in damages in apple alone (Leskey et al. 2012a). In Italy, it has became a key pest of fruit orchards since 2015, when the incidence of damaged fruits reached 60% in the plants on the edge of the fields (Maistrello et al. 2017). According to a recent estimate, in 2019 *H. halys* caused 80–90% yield loss to fruit production (pears, apples, peaches, kiwi) in northern Italy, with an economic impact of € 588 million (CSO Italy 2020). *H. halys* in Asia is also a vector of Paulownia phytoplasma (Sun et al. 1999), which alters the development and growth of affected plants, and of yeasts such as *Eremothecium coryli* (Rice et al. 2014).

In addition to the damage caused in agriculture, *H. halys* is also considered a nuisance pest, as it uses human dwellings as overwintering sites (Inkley 2012). Large *H. halys* clusters can block ventilation ducts and outlets, and particles can be aerosolized and distributed throughout the home. Clinical tests have confirmed that the *H. halys* is a significant allergen that could play an important role in allergies in the United States in the future (Mertz et al. 2012). Crop workers complained of a mild allergic reaction to insect chemicals and a case of irritant contact dermatitis caused by *H. halys* was also recorded (Anderson et al. 2012). There is a risk that a similar scenario could also occur in Europe (Haye et al. 2015; Lee 2015).

2.6 Natural antagonists

In its native habitat, several antagonists co-evolved with *H. halys*. *Arma chinensis* (Fallou, 1881) (Hemiptera: Pentatomidae) and *Misumena tricuspidata* (F.) (Araneae: Thomisidae) feed on eggs and adults, *Isyndus obscurus* (Dallas, 1850) (Rhyncota: Reduviidae) feed on adults and nymphs, a species belonging to the genus *Orius* (Anthocoridae) feeds on eggs and *Nyctereutes procyonoides* (Gray, 1834) (Carnivora: Canidae) on adults (Oda et al. 1982; Qiu 2007; Sasaki & Kawabata 1994; Kawada & Kitamura 1992). As regards the parasitoids, egg parasitoids, especially the genus *Trissolcus* (Hymenoptera: Scelionidae) are the most numerous with 6 species, including *T. mitsukurii* (Ashmed), *T. japonicus* (Ashmead, 1904) and *T. flavipes* (Thomson). Other parasitoids of *H. halys* are *Telenomus nigripedius* (Nakagawa) and *Telenomus mitsukurii* (Ashmead) (Hymenoptera: Scelionidae) and species belonging to the genera *Anastatus*, *Bogosia* and *Ooencyrtus* (Arakawa & Namura 2002; Chu et al. 1997; Hou et al. 2009; Kawada & Kitamura 1992; Lim et al. 2007; Qiu 2007; Yang et al. 2009; Zhang et al. 1993). In Japan there are also entomopathogens capable of infecting nymphs and adults and are *Ophiocordyceps nutans* (Pat.) and *Plautia stali* intestine virus (PSIV) (Nakashima et al. 1998; Sasaki et al. 2012).

In the invaded areas there are no specialist antagonists, so to understand which generalist native species could attack *H. halys* several tests were carried out. Many studies have focused on the use of sentinel eggs (both fresh and frozen) exposed in nature to understand by whom they could be attacked. In the USA, both parasitization and predation were low and with damage due to chewing mouthparts greater than that due to piercing-sucking mouthparts (Ogburn et al. 2016; Cornelius et al. 2016; Morrison et al., 2016; Abram et al. 2017; Shanovich et al. 2020). With these studies it was not possible to identify predators, unlike parasitoids, that were recognized after emergence from parasitized eggs. Four parasitoid families have been identified: Scelionidae (*Telenomus*, *Trissolc*us and *Gyron*), Eupelmidae (*Anastatus*), Encyrtidae (*Ooencyrtus*), Pteromalidae (Abram et al. 2017; Morrison et al. 2018; Leskey & Nielsen 2018). These parasitoids are able to attack *H. halys* eggs but are not always able to develop and emerge, thus the *H. halys* eggs act as an evolutionary trap (Abram et al. 2014a). Studies with sentinel eggs have also been carried out in Europe and again both parasitization and predation were low. *Anastatus bifasciatus* (Geoffory, 1785) (Hymenoptera: Eupelmidae) was the predominant species in all studies (Haye et al. 2015b; Costi et al. 2019; Kereselidze et al. 2018; Moraglio et al. 2020). Other species capable of parasitizing eggs were *Trissolcus kozlovi* Rjachovsky, *Trissolcus belenus* (Walker) (Tortorici et al. 2019), *Tr. basalis*, *Telenomus turesis* Walker (Scelionidae) and the hyperparasitoid *Acroclisoides sinicus* (Huang & Liao) (Pteromalidae) (Sabbatini Peverieri et al. 2019; Moraglio et al. 2020).

A few studies performed in USA identified species of generalist predators of eggs and nymphs (Orthoptera, Neuroptera, Dermaptera, Coleoptera, Araneae, Hemiptera, Hymenoptera) and adults (Hemiptera, Araneae) (Abram et al. 2014b; Morrison et al. 2016b; Pote & Nielsen 2017; Poley et al. 2018; Arellano et al. 2019; Biddinger et al. 2017; Morrison et al. 2017). The only sudy performed in Europe identified *Crematogaster scutellaris* (Olivier, 1792) (Hymenoptera: Formicidae) as predator on *H. halys* nymphs (Castracani et al. 2017).

2.7 Monitoring and containment techniques

Monitoring of *H. halys* is performed by active and passive techniques. Active techniques include tree beating, visual sampling and sweep net. These sampling methods allow comparisons of the stink bug densities among plant species and changes in densities in time (Rice et al. 2014). However they are not always effective, as they depend on the type of plant and crop to be monitored. Sweep net is more effective with soybean than in orchards, while tree beating and visual sampling are used principally to quantify *H. halys* activity on ornamentals (Maistrello et al. 2017; Bakken et al. 2015; Nielsen & Hamilton 2009).

Passive techniques are represented by traps, such as the the light traps and phromone traps. Black light traps provide an efficient sampling method for pentatomids, detecting *H. halys* populations at low densities and early-season populations, also they can monitor the spread of an invasive species in a large geographic region (Nielsen et al. 2013). *H. halys* therefore exhibits a phototactic response. In particular it has been observed, both in the laboratory and in the field, that wavelength-restricted black light is more attractive to *H. halys*, followed by white light and then by blue light (Leskey et al. 2015a). However, currently the mostly used worldwide are the traps baited with aggregation pheromones.

Halyomorpha halys aggregation pheromone was identified by Khrimian et al. (2014) and consists of a 3.5:1 mixture of the 2 stereisomers (3*S*, 6*S*, 7*R*, 10*S*) -10,11-epoxy-1-bis-abolen-3-ol (SSRS) and (3*R*, 6*S*, 7*R*, 10*S*) -10,11-epoxy-1-bisabolen-3-ol (RSRS). Although the SSRS isomer is more attractive than RSRS one, it has been observed that their mixtures 1:1.7 (commercial synthetic mixture) and 3.5: 1 (natural mixture) are more effective, in particular the latter is even more attractive to females (Khrimian et al. 2014; Weber et al. 2017). It was also observed that the pheromones do not need to be highly purified to be attractive for *H. halys* and that other stereoisomers of 10,11-epoxy-1-bisabolen-3-ol (murgantiol) do not cause inhibition of the response (Leskey et al. 2015b). This suggests that it is possible to use impure mixtures of the main pheromonal components without compromising biological activity (Weber et al. 2017). The main problem with the use of pheromone traps is due to the fact that this pheromone attracts *H. halys* individuals, but does not push them to seek the source of the pheromone (unlike the use of sexual pheromones, Morrison et al. 2016a), so that many individuals are not caught, remaining near the trap and causing damage in its proximity (Sargent et al. 2014; Aldrich et al. 2009).

AIM OF THE THESIS

Since its introduction on continents other than its original one, *H. halys* has caused extensive damage to crops. The solutions implemented so far do not show complete effectiveness against this pest. The invasion of *H. halys* has led farmers to increase the use of pesticides, in particular broad-spectrum insecticides such as organophosphates, which however affect also non-target, useful insects such as pollinators, predators and parasitoids. This type of management disrupted the most innovative IPM strategies and is causing both economic and environmental damage (Leskey et al. 2012a; Maistrello et al. 2017).

In order to increase the sustainability of management for this invasive pest, alternative containment techniques have been developed, that include prevention by means of exclusion netting (Candian et al. 2020) as well as the behaviourally-based strategies IPM-CPR (Blaauw et al. 2015), trap-crop (Mathews et al. 2017) and “attract and kill” (Morrison et al. 2019).

In order to sustainably reduce the population of this pest, several studies have focused on searching native antagonists, but most of these have been conducted in the United States. In Europe there is a large gap in understanding the effect of native predators on *H. halys*; in fact, only one species of ant has been tested in the laboratory and the exposure of sentinel egg masses does not allow the identification of the full suite of predators as it is limited to only one of the life stages of the pest (Castracani et al. 2017; Costi et al. 2019).

Therefore, there are no data regarding the predatory community of this pest in southern Europe and their identification may be useful in the development of conservation biocontrol projects. Behavioural studies, both of the potential predators and of pest, can also be used, namely by studying both the attraction of certain predators to the pheromones of *H. halys*, and by verifying the repellent effect that some substances can have on the pest. The behavioral response of natural enemies may change over the season as well as their sensitivity to kairomones from *H.halys*, so this too must be taken when developing integrated pest management programs.

This thesis is focused on the sustainable management of *H. halys* and is divided into two main sections. The first one is focused on generalist predators, where, in order to better understand the role of native generalist predators of the invaded areas, the predatory ability of southern European generalist predators was studied in the laboratory on the eggs and juvenile stages of *H. halys,* both with solitary (Chapter 2) and social (Chapter 3) species. Through the analysis of real-time PCR products using the gut content of generalist predators collected in nature to see if the DNA of *H. halys* was present (Chapter 4). Finally the behaviour of a North American jumping spider, *Phidippus audax*, was studied in Canada, focusing in particular on its attraction to the aggregation pheromone of *H. halys*, to understand if it is actually attracted to it and at what time of the year (Chapter 5).

The second section is dedicated to the identification of some repellent substances to be potentially used in behavioural manipulation strategies (i.e. push-pull strategy). In particular, four essential oils at different concentrations were tested on three different physiological phases of *H. halys* to verify repellence and understand in which period of the year the stink bugs are more sensitive (Chapter 6).

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CHAPTER 2

Predatory ability of generalist predators on eggs, young nymphs and adults of the invasive *Halyomorpha halys* in southern Europe

Giacomo Bulgarini[1,], Zaid Badra[2], Stefano Leonardi[3], Lara Maistrello[1]

[1]Dipartimento Scienze della Vita, Università di Modena e Reggio Emilia, Via G. Amendola 2, 41122, Reggio Emilia, Italy
[2]Department of Mountain Environment and Agriculture, Free University of Bolzano, Piazza Università 5, 39100, Bolzano, Italy
[3]Dipartimento di Scienze Chimiche, della Vita e della Soostenibilità Ambientale, Parco Area delle Scienze 11/a, 43124, Parma, Italy

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Abstract

Halyomorpha halys (Stål, 1855) is an invasive pest causing serious damage to agricultural crops in Europe and United States. Very little is known about *H. halys* predators in Europe. This survey evaluated the potential of generalist predators/omnivorous species by means of predation bioassays in tri-dimensional arenas, where the predator had to locate *H. halys* prey-items on the leaves of a bean plant. Eleven species of different taxa were tested and the prey-items consisted in fresh eggmasses, 1st and 2nd instar nymphs. One species was also tested against adults. Some predators were species commercially available as biocontrol agents against plant pests, other predators were wild, captured in habitats shared with *H. halys*. All tested specimens were starved 24 h before starting the experiment. The survivorship of control prey-items in predator-excluding cages was compared to that of predator treatment groups to determine the effect of predator presence. According to the results, the generalist species showed a quite low acceptance of *H. halys* prey-items, since only two species caused 80% mortality on at least one item (*Eupholidoptera chabrieri* and *Rhynocoris iracundus*) and mortality due the other species never exceed 60%. Among commercially available species only *Adalia bipunctata* adults and *Chrysoperla carnea* larvae were effective, predating the eggs and 1st instar nymphs, respectively. Among the field collected specimens, the orthopteran *E. chabrieri* and the predatory hemipterans *R. iracundus, Nagusta goedelii* and *Himacerus mirmicoides* showed efficacy against 1st instar nymphs, *E. chabrieri* and *R. iracundus* showed efficacy against 2nd instar nymphs, whereas only *E. chabrieri* and *N. goedelii* predated the eggs. *R. iracundus* was also tested on the adults and successfully predated them. By identifying some of the species that can exploit *H. halys* as a suitable prey in southern Europe, the present investigation provides an important contribution for conservation biological control of this pest.

KEYWORDS: Brown Marmorated Stink Bug; conservation biocontrol; predatory hemipterans; predation bioassay

1. Introduction

Halyomorpha halys (Stål, 1855) (Heteroptera, Pentatomidae), is an invasive insect native to eastern Asia, specifically China, Japan, Taiwan and South Korea (Leet et al. 2013; Haye et al. 2015a). Being a hitchhiker on inanimate objects, it has a high dispersion capacity facilitated by human activities and trade (Maistrello et al. 2018), that allows a fast colonization worldwide (Rice et al. 2014). It has been present in North America since 1996 (Hoebeke & Carter 2003; Hamilton

2009) and in April 2020 *H. halys* was reported in 46 states in the United States and in 4 provinces of Canada (Stopbmsb 2020). Most recently it was detected also in South America, specifically in Chile (Faúndez & Rider 2017). In the European continent, after the initial detection in Switzerland in 2004 (Haye et al. 2015a) the spread into the different countries was extremely fast. As of April 2020, *H. halys* is reported with established populations in 28 countries in Europe and along the Black Sea (Inaturalist 2020).

In Italy, the first official sighting occurred in 2012 in the province of Modena, Emilia Romagna (Maistrello et al. 2016). A crowdsourcing survey indicated a very fast spread all over the country and the surrounding main islands (Sicily, Sardinia, Corsica), and a model on its spatiotemporal dynamics tracked the possible date of entry back to 2009 (Maistrello et al. 2018). The genetic analysis of the samples collected overtime shows that the Italian populations of *H. halys* have the highest biodiversity outside of its native Asia, as a result of multiple invasions that are still in progress (Cesari et al. 2018). In Italy, *H. halys* completes two generations per year with a partial overlap of the oviposition periods and contemporary presence of different development instars during summer. The reproductive potential shown in Italy is high, with on average 285 and 215 eggs each for the overwintering and first-generation females respectively (R0 = 24.04 and 5.44 respectively for the two generations) (Costi et al. 2017).

Halyomorpha halys is a polyphagous species with more than 300 host plants, including fruit trees, crops, vegetables, ornamental and spontaneous plants (Rice et al. 2014; Haye et al. 2015a). Damage occurs mainly to fruits and seeds as a consequence of the feeding activity with the piercing-sucking mouthparts, that cause scarring, malformations, suberifications, discolored areas and/or necrotic areas and brownish spots that render agricultural products unmarketable (Rice et al. 2014), resulting in considerable economic losses. In the Midwest USA states in 2010, *H. halys* caused damages of over $ 37 million for the apple trade (Leskey et al. 2012). In northern Italy, it became a key pest of fruit orchards just a few years after its discovery (Maistrello et al. 2017) and reliable estimates for 2019 report an overall economic impact of € 588 million on northern Italy fruit production (pear, peach, apple, kiwi), with up to 80-100% yield losses in the orchards (CSO Italy 2020). Another feature that makes this pest difficult to manage is its high mobility, typical of all instars. Adults fly on average 2 km per day, but can also reach 116 km per day (Wiman et al. 2015; Lee & Leskey 2015), while the nymphs, from the second stage onwards, can walk over 20 m per day among the different plants (Lee et al. 2014).

Managing *H. halys* poses a big issue in the invaded regions, but no ultimate solution to efficiently manage this pest has been yet been found. Currently, the management of this invasive pest relies on increased applications of broad spectrum-insecticides, thus disrupting previous IPM programs with further negative impact on the economy and the environment (Leskey et al. 2012; Maistrello et al. 2017).

Considering that in invaded areas there are no specific *H. halys* antagonists, field surveys were carried out, both in cultivated and unmanaged areas, exposing either fresh or frozen *H. halys* eggmasses, to verify the potential of generalist antagonists. The eggs were both preyed and parasitized, usually in low percentage for both categories, and as regards to predators, the damages due to chewing mouthparts were on average greater than those due to a pierce-sucking apparatus (Ogburn et al. 2016; Cornelius et al. 2016; Abram et al. 2017; Shanovich et al. 2020). According to Biddinger *et al.* (2017) the wasp *Bicyrtes quadrifasciatus* (Say, 1824) (Hymenoptera: Crabonidae) uses the nymphs of *H. halys* to feed and rear their larvae. In the study by Morrison et al. (2017) spiders were considered, in particular those present in potential overwintering sites of *H. halys* and by studying what remained in the spider webs it emerged that Agelenidae, Pholcidae and Theridiidae can feed on *H. halys*.

In the laboratory, potential predators have been tested on eggs by means of no choice tests. Tettigoniidae, Gryllidae, including *Acheta domesticus* (Linneaus, 1758), Acrididae, like *Melanoplus femurrubrum* (De Geer, 1773), Forficulidae, *Chrysoperla carnea* larvae (Stephens, 1836) (Neuroptera: Crysopidae), Coccinellidae and Salticidae, like *Phidippus audax* (Hentz, 1845) are able to prey on the eggs of *H. halys* or reduce the percentage of hatching (Abram et al. 2014; Morrison et al. 2016; Pote & Nielsen 2017; Poley et al. 2018). In the study of Pote & Nielsen (2017), the predators were also tested on the first two juvenile instars of *H. halys* showing how predatory Hemiptera such as nabids and reduviids can prey on the first instar nymphs and how nabids and *Podisus maculiventris* (Say, 1832) manage to prey on the second instar nymphs. Arellano et al. (2019) tested *Euthyrhynchus floridanus* (Linnaeus, 1767) (Hemiptera: Pentatomidae) on all developmental instars including the adult stage, showing some potential for biological control.

Most of the studies focused on the potential of antagonists in North America, but very little is known about the predatory communities in the European continent. A three year field survey carried out in 2014-2016 using fresh sentinel egg masses in Emilia Romagna, (Italy) showed that impact of predation on eggs never exceeded 6% with similar percentages of damages due to chewing mouthparts and pierce-sucking mouthparts, however this type of survey did not allow identification of the predators (Costi et al. 2019). A laboratory study showed that the arboreal ant species *Crematogaster scutellaris* (Olivier, 1792) (Hymenoptera, Formicidae) cannot prey fresh eggs of *H. halys* but is very efficient in predating all the nymphal instars (Castracani et al. 2017).

The aim of this study is to evaluate the predatory ability of solitary generalist insect predators commonly found in the Italian agroecosystems on the eggs and on the first two juvenile instars of *H. halys*. In this work, we tested both predatory species collected in the field, sharing the same habitat of *H. halys*, and species of predators already used for the biocontrol of other agricultural pests, such as aphids and mealybugs. Among the field collected predators, almost all native to Europe, we included also the invasive *Harmonia axyridis* (Pallas, 1773) (Coleoptera: Coccinellidae), native to Asia and detected in Italy since the 2000s, which is considered an important predator of aphids in many crops (Roy et al. 2016).

2. Materials and methods

2.1. Halyomorpha halys rearing

Halyomorpha halys was reared in climatic chambers at 26 °C, 60% relative humidity, L16:D8 photoperiod inside clear mesh cages (30x30x30cm, approximately 40 individuals/cage) with organic tomatoes, carrots, green bean pods and raw peanuts as food. A bottle cap with a water-soaked cotton swab was used as water supply. Separate cages were used for adults and nymphs. A bean plant (*Phaseolus vulgaris*) with at least two developed leaves and pieces of papers were placed in the adult cage for egg-laying. Food, water and paper were replaced twice per week. Eggs were collected daily and transferred to petri dishes with a bean pod and wet cotton. When the nymphs reached the second instar, they were transferred to the nymphs' cage.

2.2. Predators: a) Biocontrol agents available on the market

Artificially reared insect predators were obtained from a company specialized in production of biocontrol agents for plant pests (Bioplanet s.c.a., Cesena, Italy). The tested species and instars are reported in Tab. 1. Predators were delivered in plastic containers with dispersing material provided by the company. In the laboratory, they were kept in optimal conditions and fed on *Ephestia kuehniella* (Zeller, 1879) (Lepidoptera: Pyralidae) eggs, also supplied by the same company.

2.3. Predators: b) Wild biocontrol agents, field collected

Wild insect predators were collected between April and August 2018 during specific sweep-net and tree-beating sessions (from 7 to 10 am) in four urban parks with mixed trees and shrubs in Reggio Emilia (northern Italy), where *H. halys* was abundant. Field-collected species are reported in Table 1, together with information on the collection method and sites. Predators were individually collected in 50 ml Falcon tubes and kept in a refrigerated bag. Once in the laboratory, the specimens were transferred to plastic cylindrical containers (diameter 11cm, height 6cm with a 5cm diameter anti-aphid net on the lid to allow airflow) kept at room temperature (23 ± 1 C°) and provided with a mixed diet that included 2nd instar larvae of *Hermetia illucens* (Linneaus, 1758) (Diptera: Stratiomyidae), 2nd instar larvae of *Tenebrio molitor* (Linneus, 1758) (Coleoptera: Tenebrionidae), field-collected aphids and *E. kuehniella* eggs. For the omnivorous species *F. auricularia* and *E. chabrieri,* diet included also carrots, green beans and peanuts. A bottle cap with a water-soaked cotton swab was used as water supply. Field-collected specimens were kept in these conditions for 1-3 days before the beginning of the predation test, in order to have at least four individuals of the same species ready. Preliminary trials showed that after 4 days under these conditions, the survival of the field-collected specimens of all the tested species was higher than 90%.

2.4. Predation test

Experimental arenas consisted of transparent cylindrical plastic boxes (20 cm height, 10.5 cm diameter) with a 5 cm diameter anti-aphid net on the lid to allow airflow and one bean plant bearing at least two well-developed leaves, which was placed in the center of the box. Four *H. halys* prey items were considered: a) one freshly laid (<24 hours) egg mass with at least 21 eggs; b) one hatched egg mass with at least 18 first instar nymphs (1-2 days old); c) five second instar nymphs (1-2 days old); d) two adults (1 male and 1 female), only when *R. iracundus* was tested as predator. All prey items were collected from the laboratory rearing; only the egg masses laid on paper were used for the trials. One prey item was randomly placed on one leaf of the plant: in the case of unhatched/hatched egg masses the paper support was clipped to the underside of the leaf. Each tested predator was randomly released, dropping it on the leaf of the plant from above after a 24 hours starvation period. The box was closed with the lid and placed inside in a climatic chamber 26 °C, 60% relative humidity, L16: D8 photoperiod. After 48h, the box was opened, and the status of the prey items was carefully checked under a stereomicroscope for signs of predation according to the predator's mouthparts, recording if they were damaged or dead. The unhatched egg masses were kept inside the climatic chamber at the previously described conditions for five days inside 7 cm diameter petri dishes to check for emergence of nymphs. In case of eggs showing signs of predation, they were considered damaged if after 5 days the nymphs emerged, and dead if no nymphs emerged. In the case of nymphs and adults the ones that showed missing body parts or did not move after stimulation with a brush were considered damaged and dead respectively. The status of the predator at the end of the trial was also recorded (alive/dead).

For each considered predatory species/instar, a minimum of eight replicates was performed for each type of prey item. For each replicate of each combination predator-prey item, a control replicate was carried out in the same time with the same procedure, but without the presence of the predator. Each predator was tested only once. Trials were staggered due to predator availability.

2.5. Statistical analysis

Generalized linear model (glm) with a binomial error structure (logit link function) was used to compare the number of dead prey items and survivors between the treatment (simultaneous presence of predator and prey item) and the respective control (only prey items). For egg mortality, a comparison was made between the number of hatched eggs in treatment and control.

Four separate analyses were run on the different stages of *H. halys*: eggs, first instar nymphs, second instar nymphs and adults. In each analysis a single glm with two factors (treatment and predator type) and their interaction was used. The only exception was the one on *H. halys* adults where only one predator (*R. iracundus*) was tested.

All statistical analyses were performed using R version 3.6.3 (R core Team 2019). To assess the general significance of treatment, predatory type and their interaction, an analysis of deviance of the fitted glm model with Wald statistics χ^2 was performed. To find which predator type produces a significant statistical difference in mortality between the two treatments, a multiple comparison approach was used (Bretz et al. 2011), i.e. the ghlt function of the mulcomp package with *ad hoc* set of contrasts. This procedure is conducted on the scale of the linear predictor (logit) and, assuming asymptotically normally distributed parameter estimates, produces *z-values* and *p-values* associated with this assumption (Hothorn et al. 2008).

In three cases a so-called "complete separation" was found in the data (zero survival or zero deaths in some treatment-predator type combinations). This is a well-known problem in glm analyses because it causes a substantial failure in the maximum likelihood procedure to estimate glm parameters (Lesaffre & Albert 1989). A suitable glm R package (brglm2) was used to deal with this problem.

3. Results

3.1. Predation bioassay

As a preliminary result, a high survival of all predators was recorded in the experiments. Survival was often between 90% and 100% and just in the case of 2nd instar larvae of *C. carnea* the minimum predator survival was 86%.

For each type of prey, there were cases of complete separation. In the case of eggs, no eggs survived in the presence of *E. chabrieri*. In the case of N1 no deaths were recorded in the control of *N. goedelii* and for N2 no deaths were recorded in the presence of *H. axyridis.*

For each type of prey there is a significant general difference between the control and the presence of the predator (eggs:χ^2 = 12.34; df = 1; $p < 0.001$. N1:χ^2 = 78.42; df = 1; $p < 0.001$. N2:χ^2 = 27.18; df = 1; $p < 0.001$). A significant general variability emerges (Fig. 1, Fig. 2, Fig. 3) also among predators for all types of prey (eggs:χ^2 = 926.90; df = 10; $p < 0.001$. N1:χ^2 = 358.19; df = 10; $p < 0.001$; N2:χ^2 = 73.40; df = 10; $p < 0.001$), and this variation affects both control and predator treatments. This underlying natural variability is captured only because the control treatment (absence of predator) was repeated for every predator treatment. More importantly, for the aim of this experiment, several significant differences on prey mortality were detected for the interactions (combinations) of predator and treatment in every prey type analysis (eggs:χ^2 = 70.07; df = 10; $p < 0.001$. N1:χ^2 = 96.06; df = 10; $p = 0.001$. N2:χ^2 = 57.26; df = 10; $p < 0.001$), indicating that for the same type of prey, predators showed remarkable differences in efficacy (Fig. 1, Fig. 2, Fig. 3).

The number of hatched eggs was significantly reduced in presence of *A. bipunctata* (prey mortality treatment = 0.20; prey mortality control = 0.05; z value = 5.22; $p < 0.001$), *E. chabrieri* (prey mortality treatment = 1.00; prey mortality control = 0.09; z value = 8.66; $p < 0.001$) and *N. goedelii* (prey mortality treatment = 0.16; prey mortality control = 0.08; z value = 3.35; $p = 0.009$) (Fig.1). When *F. auricularia* was tested, 4% of the eggs showed attempts of attack with mandibles; nevertheless, this type of damage did not affect eggs hatching.

The survivorship of *H. halys* 1st instar nymphs was significantly reduced in presence of the 2nd instar larvae of *C. carnea* (prey mortality treatment = 0.20; prey mortality control = 0.08; z value = 4.73; $p < 0.001$), and the adults of *E. chabrieri* (prey mortality treatment = 0.71; prey mortality control = 0.20; z value = 10.25; $p < 0.001$), *H. mirmicoides* (prey mortality treatment = 0.21; prey mortality control = 0.16; z value = 4.41; p = 0.001), *R. iracundus* (prey mortality treatment = 0.16; prey mortality control = 0.07; z value = 3.04; p = 0.03) and *N. goedelii* (prey mortality treatment = 0.13; prey mortality control = 0.00; z value = 3.02; p = 0.02) (Fig. 2). The survivorship of *H. halys* 2nd instar nymphs was significantly reduced in presence of adults of *E. chabrieri* (prey mortality treatment = 0.90; prey mortality control = 0.24; z value = 5.76; $p < 0.001$) and *R. iracundus* (prey mortality treatment = 0.80; prey mortality control = 0.14; z value = 5.58; $p < 0.001$) (Fig. 3). *R. iracundus*, the only predator tested on *H. halys* adults, significantly reduced their survivorhip (prey mortality treatment = 0.44 ± 0.22; prey mortality control = 0.00 ± 0.00; z value = 2.08; p = 0.03).

4. Discussion

The present study is the first to screen in Europe the predatory potential of generalist insect predators on the invasive *H. halys*, considering both species commonly reared for biocontrol purpose of plant pests and species commonly found in the field, sharing the same habitat of the invasive pest. According to our results, although six out of ten predators significantly reduced the survival of at least one of the life stages of *H. halys* provided, only two species caused a mortality higher than 80% (*E. chabrieri* and *R. iracundus*) and in the other cases mortality of the prey items never exceeded 60%. Besides, four of the tested species showed no attemps of predation despite starving for 72h in total (24h food deprivation before the trial and 48h of the assay). The low acceptance of the life stages of *H. halys* as prey that occurred in the laboratory during the forced feeding bioassays suggests that in field conditions, where the predators have access to alternative prey items, these species are likely to have a quite low impact on the overall mortality of the invasive stinkbug. Although the present study is not exhaustive, as it did not include other species of arthropod generalists potentially effective in predating *H. halys* (i.e. Coleoptera Carabidae, other species of Orthoptera, spiders), it appears that results obtained with species typical of the European context are substantially similar with the ones obtained in North America (Morrison et al. 2016; Pote & Nielsen 2017).

Considering the candidates with chewing mouthparts, among the tested Coccinellidae only *A. bipunctata* showed significant predation, but only on the eggs of *H. halys*. The ability to consume *H. halys* eggs was observed in a laboratory study in a tridimensional arena only in adults of *Coccinella septempunctata* (Linnaeus, 1758) (Pote & Nielsen 2017). In petri dish studies also *Coleomegilla maculata* (De Geer) adults (Morrison et al. 2016; Abram et al. 2014), *C. maculata* second instar larvae (Abram et al. 2014), and *H. axyridis* adults (Morrison et al. 2016) occasionally fed on the eggs. All the other tested coccinellids, that included also *C. montrouzieri,* were totally ineffective (Morrison et al. 2016; Pote & Nielsen 2017; Poley et al. 2018). In the laboratory study by Pote & Nielsen (2017) no predatory ability was observed on young *H. halys* nymphs by the adults of *C. septempunctata*, *C. maculata* and *H. axyridis*. Coccinellids are essential biocontrol agents of soft-bodied arthropods, especially aphids (Rutledge et al. 2004), but apparently only very few of them seem to have a role in predating eggs and nymphs of this invasive pentatomid, likely only under specific circumstances.

The dermapteran *F. auricularia* was not effective in predating the first and second instar nymphs, but it damaged the eggs, although without affecting their survival. According to Poley et al. (2018), *F. auricularia* did not show significant predation on *H. halys* eggs in laboratory tests (only one specimen over 10 tested was able to predate two eggs after 5 days), whereas in the field Forficulidae species appear to be the most efficient predators of *H. halys* sentinel egg masses (Poley 2017). Plant protection personnel inspecting fruit crops in northern Italy report similar observations (Maistrello, personal communication). According to Morrison et al. (2016), *H. halys* eggs were attacked by specimens of the family Forficulidae, showing incomplete chewing, however the species were not identified in their study. Forficulidae have been recognized as important biocontrol agents of insect pests in fruit orchards (Suckling et al. 2006) and it is therefore likely that some Dermaptera, which are typically omnivorous species, can exploit *H. halys* eggs as food source, but probably *F. auricularia* is not the most efficient predator for this prey item.

The tettigonid *E. chabrieri* proved to be an excellent predator for all the prey items taken into consideration. In general, Orthoptera have always shown good predatory efficacy on eggs (Morrison et al. 2016; Pote & Nielsen 2017; Poley et al. 2018). In our trials *E. chabrieri* successfully predated the young nymphs, probably due to its large size (adults can be 2-3 cm in length). However, being an omnivore, during the predation test it also fed on the bean plant used as support.

The second instar larva of the neuropteran *C. carnea* was able to prey on the first instar nymphs, but not on the eggs. In the study of Abram et al. (2014), *C. carnea* negatively affected the survival of *H. halys* eggs, but in that case, both the egg mass and the predator were positioned inside a petri dish (diameter 10 cm, height 2cm). Our experiments were performed in more natural conditions, with the egg masses placed underneath a leaf, and this might have affected the ability of the predator to detect/attack the prey items.

Among the stylet-sucking predators, the anthocorid *A. nemoralis* did not reduce the survival of any of the prey items provided. Anthocorids are predators of small soft-bodied phytophagous arthropods (mites and insects) and their eggs, and some species are reared as biocontrol agents of plant pests (Lattin 1999). The genus *Orius* is known as an egg predator of *H. halys* in its native range (Lee et al. 2013). Previous studies by Morrison et al. (2016) and Poley et al. (2018) found that *O. insidiosus* can predate *H. halys* eggs, although at quite low rates. Despite having similar size (3 mm), *A. nemoralis* is not as efficient as *Orius sp*., possibly because its mouthparts are too weak to penetrate the *H. halys* egg shell or due to different predatory attitudes and behavior.

The nabid *H. mirmicoides* was effective in predating only the first instar nymphs. These results confirm the previous findings obtained on *Nabis sp*. by Pote & Nielsen (2017) and on unidentified nabids by Morrison et al. (2016), but in our case the identification of the predatory specimens was made at the species level, providing a better insight on the role of these predatory hemipterans.

Considering Reduviidae, *N. goedelii* significantly predated the eggs and the first instar nymphs, while *R. iracundus* proved effective against the first nymph instar and the second instar nymphs and the adults, but not on the eggs. *R. iracundus* predated more males (6 out of 8) than females (3 out of 8), probably because *H. halys* males are generally smaller than females, so perhaps they are easier to be manipulated and successfully predated. The reduviid *Isyndus obscurus* (Dallas) is known to predate nymphs and adults of *H. halys* in native Japan (Lee et al. 2013). In the laboratory, American reduviid species were totally ineffective on eggs of *H. halys* (Morrison et al. 2016; Pote & Nielsen 2017), however *Arilus cristatus* (L.) and *Sinea spinipes* (Herrich-Schaeffer) significantly reduced the survival of first instar nymphs (Pote & Nielsen 2017). Other laboratory trials (Jones 2013) indicate that *A. cristatus* adults were the most efficient predators of *H. halys* adults, and that *A. cristatus* nymphs efficiently predated first, second and third instar nymphs. Our study confirms that predatory Heteroptera like some species of Nabidae and Reduviidae seem to play a significant role in consuming the nymphs and adults of *H. halys*.

The comparisons of performance among the predators that significantly reduced survival on at least one prey item indicated that *E. chabrieri* was the most effective, consuming all the eggs and predating 90% and about 60% of the second and first instar nymphs respectively. Like the majority of the other predators, *R. iracundus* was not able to consume eggs, but it had an efficacy similar to that of *E. chabrieri* in predating second instar nymphs (80%), and successfully predated 43% of the adults. Therefore *E. chabrieri* and *R. iracundus* are the most effective predators of *H. halys* among the species considered in this study, possibly because they were the biggest in size and likely also with the strongest mouthparts.

Interestingly, eggs were the least predated item among the ones offered to the tested predators, as they were consumed only by the tettigonid *E. chabrieri* and to a much lesser extent also by the coccinellid *A. bipunctata* and the reduviid *N. goedelii*. Very little/no consumption was recorded by the predators tested in the already cited studies of Abram et al. (2014), Pote & Nielsen (2017), Morrison et al. (2016) and Castracani et al. (2017). In our experiments, the egg masses were fresh, laid during the previous 24 hours on a paper support, that was clipped underneath a leaf, in order to simulate the natural situation, and no observations were performed during the 48 hours of exposure to the predators. Hypotheses to explain the unsuccessful *H. halys* egg predation include: i) physical-mechanical factors: the mouthparts of the tested predators were either too weak or too small to affect the eggs, due to their size and/or the toughness of the chorion. This is possibly the case of *F. auricularia*, which caused some damage, though not sufficient to prevent hatching; ii) chemical factors: the freshly laid eggs might lack semiochemicals that allow their recognition as suitable prey or possess repellent semiochemicals; iii) behavioral factors: eggs are immobile and are not detected/attacked by those predators that need the mobility of the prey to trigger the predatory behavior, like reduviids (Haridass et al*.* 1988); iv) the position of the egg mass underneath the leaf, that might prevent optimal access, manipulation, grasping/piercing of the eggs by some of the predators; v) a combination of the previous. Further investigations on the physical/chemical egg features and behavioral studies could shed a light on these aspects.

In our experiment, the survivorship of second instar nymphs was usually lower than that of first instar ones and hypotheses to explain this include: i) differences in the production of the defensive compounds typically emitted upon disturbance. Instar-specific compounds have already been demonstrated in other species of Pentatomidae (Borges and Aldrich 1992); ii) behavioral factors related to mobility cues: first instar nymphs have a very reduced mobility as they usually remain on the egg mass to acquire the symbionts necessary for their survival (Taylor et al. 2014). Thus, to some species of predators they might represent less suitable prey items compared to the second instar nymphs, which are very mobile (Lee et al. 2014).

In this work we demonstrated that the commercially available predators are not effective, except for *A. bipunctata*, that slightly decreased the survival of the eggs, and *C. carnea* that slightly decreased the survival of the first instar nymphs. Among the field collected specimens, we showed that only one species proved to be able to predate all the developmental stages of *H. halys*, almost none attack the eggs but some species in the families Tettigonidae, Nabidae and Reduviidae can attack the young nymphs.

H. halys usually lays its eggs under the leaves of shrubs and trees mainly at the top, and a predator capable of preying on both eggs and first instar nymphs and which during our sampling was found mainly on the treetops is *N. goedelii*, a Reduviid known to be a predator of small arthropods inhabiting deciduous trees (Dioli 2013). From the second instar onwards, the nymphs become very mobile and begin walking up and down from plant to plant (Acebes et al. 2017). While on the trees, all nymphal instars can be predated by the arboreal ants *C. scutellaris* (Castracani et al*.* 2017). On their way down, both nymphs and adults can be intercepted by *R. iracundus*, found mainly in shrubs and in the lower parts of the trees (Rieger 1972). While moving on the ground from one plant to the other, the nymphs can be attacked by the nabid *H.*

mirmicoides and the tettigonid *E. chabrieri*, typically found in the grassy areas. Egg masses could be occasionally predated/damaged by some Dermaptera and coccinellids, however they are better exploited by egg parasitoids and the predominant and most successful species in Europe is the generalist *Anastatus bifasciatus* (Geoffroy) (Hymenoptera: Eupelmidae) (Haye et al. 2015b; Costi et al. 2019; Moraglio et al. 2020).

Thus, by identifying some of the species of the community of natural enemies that can exploit *H. halys* as a suitable prey in southern Europe, the present investigation provides an important contribution for conservation biological control of this invasive species. In this view, a significant implementation will be provided by more detailed field and laboratory investigations, such as a study on the gut content of the arthropod predators sharing the habitat with *H. halys* and surveys on the myrmecofauna of the agroecosystems affected by *H. halys.*

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Order	Family	Species	Life stage	Origin	Sampling technique	Sampling sites
Coleoptera	Coccinellidae	*Adalia bipunctata* (Linnaeus, 1758)	adult	Artificially reared		
		Harmonia axyridis (Pallas, 1773)	adult	Field	Tree beating	Middle of the tree
		Cryptolaemus montrouzieri (Mulsant, 1853)	larvae	Artificially reared		
		Cryptolaemus montrouzieri	adult	Artificially reared		
Dermaptera	Forficulidae	*Forficula auricularia* (Linnaeus, 1758)	adult	Field	Tree beating	Top of tree canopy
Orthoptera	Tettigoniidae	*Eupholidoptera chabrieri* (Charpentier, 1825)	adult	Field	Sweep net	Tall grass
Neuroptera	Crysopidae	*Chrysoperla carnea* (Stephens, 1836)	2° instar larvae	Artificially reared		
Hemiptera	Anthocoridae	*Anthocoris nemoralis* (Fabricius, 1794)	adult	Artificially reared		
	Nabidae	*Himacerus mirmicoides* (O. G. Costa, 1834)	adult	Field	Sweep net	Alfalfa, tall grass
	Reduviidae	*Rhynocoris iracundus* (Poda, 1761)	adult	Field	Tree beating and sweep net	Shrubs
		Nagusta goedelii (Kolenati, 1857)	adult	Field	Tree beating	Top of tree canopy

Tab. 1 Details on the tested predatory species, including their origin and, in the case of field-collected ones, the sampling technique and sites.

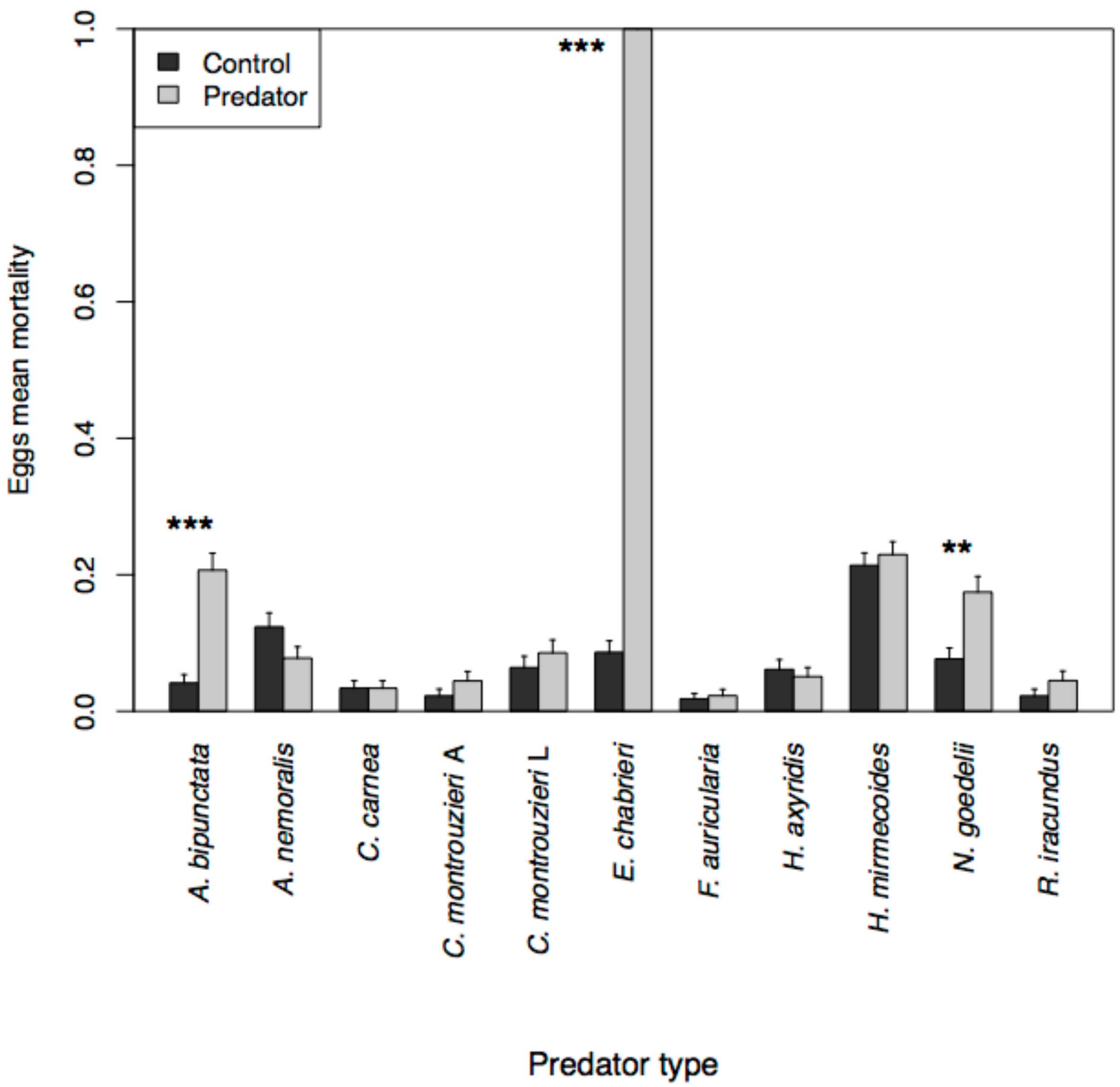

Fig. 1 Comparison of the mean mortality of *Halyomorpha halys* eggsobserved in the presence of the predator and in the relative control after 48 hours. Error bars indicate standard error. Asterisks indicate a significant difference in mortality between control and treatment (predator): *** for $p < 0.001$; ** for $p < 0.01$

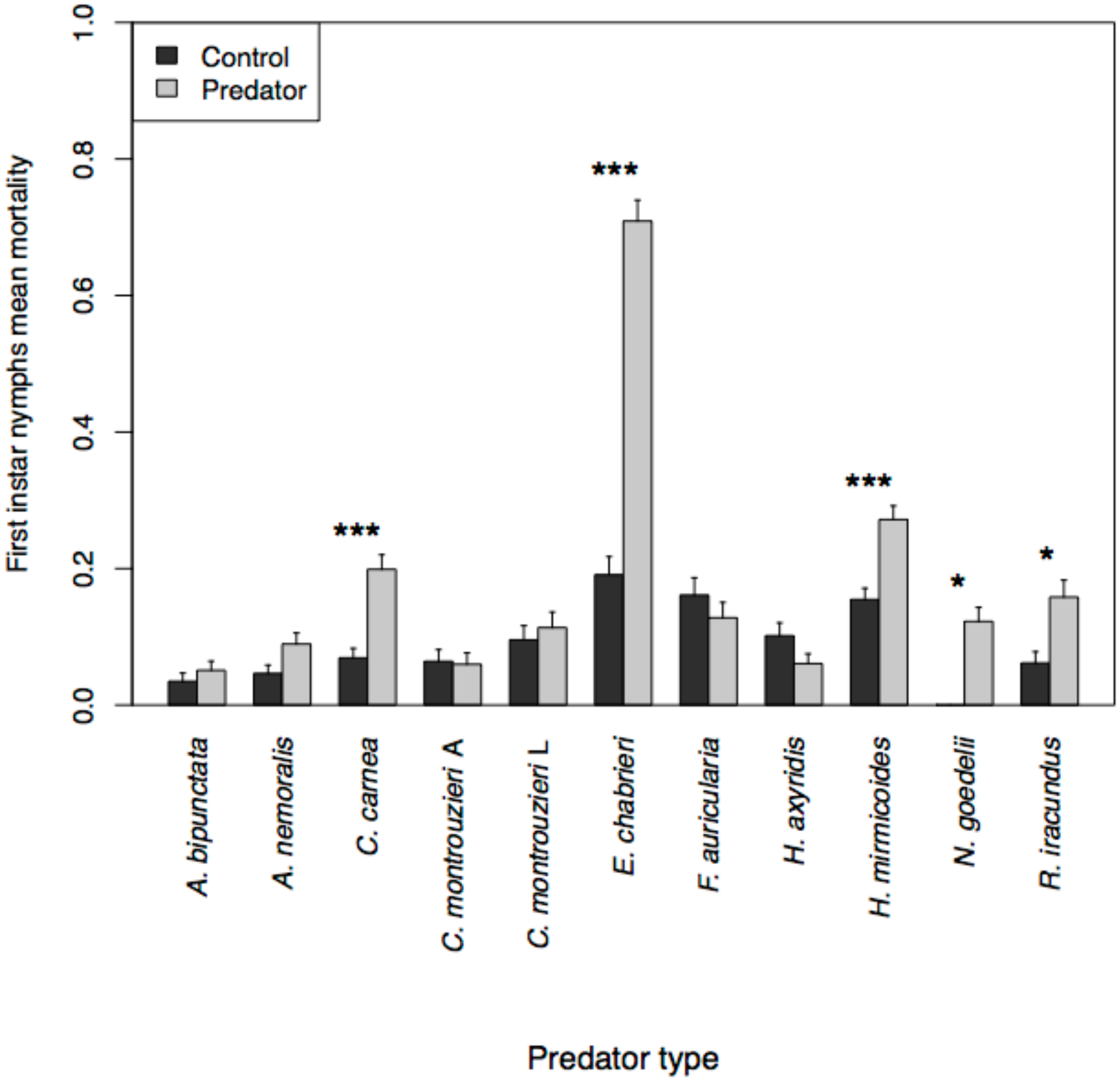

Fig. 2 Comparison of the mean mortality of *Halyomorpha halys* first instar nymphs observed in the presence of the predator and in the relative control after 48 hours. Error bars indicate standard error. Asterisks indicate a significant difference in mortality between control and treatment (predator): *** for $p < 0.001$; * for $p < 0.05$

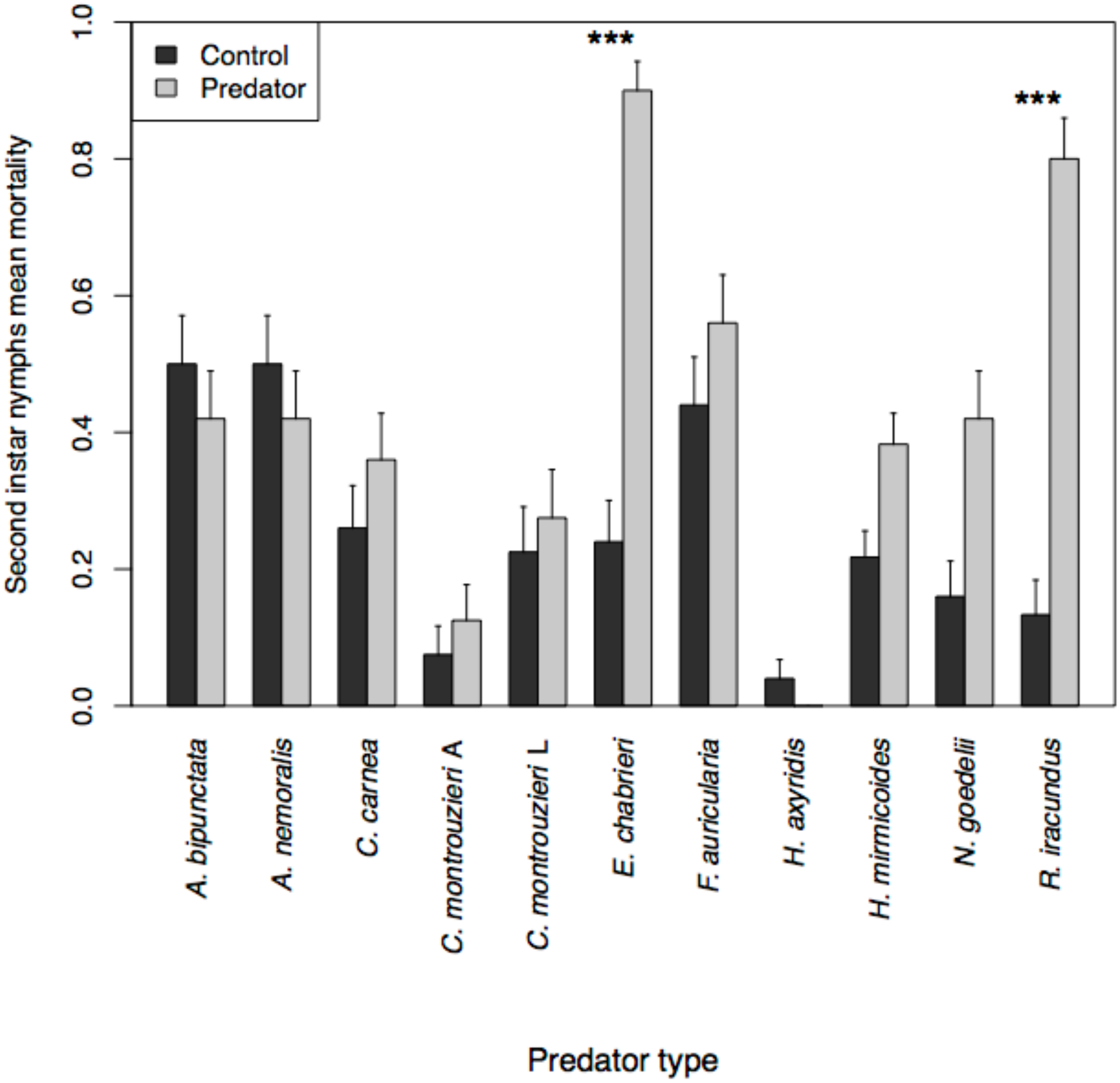

Fig. 3 Comparison of the mean mortality of *Halyomorpha halys* second instar nymphs observed in the presence of the predator and in the relative control after 48 hours. Error bars indicate standard error. Asterisks indicate a significant difference in mortality between control and treatment (predator): *** for $p < 0.001$

CHAPTER 3

Searching for new predators of the invasive *Halyomorpha halys* (Heteroptera, Pentatomidae): the role of the black garden ant *Lasius niger* (Hymenoptera, Formicidae)

Giacomo Bulgarini[1], Cristina Castracani[2], Alessandra Mori[2], Donato A. Grasso[2,*], Lara Maistrello[1,*]

[1] Dipartimento Scienze della Vita, Università di Modena e Reggio Emilia, Via G. Amendola 2, 41122, Reggio Emilia, Italy
[2] Dipartimento di Scienze Chimiche, della Vita e della Sostenibilità Ambientale, Viale delle Scienze 11/a, 43124, Parma, Italy
(*) Co-last Authors

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Abstract

In the recent years *Halyomorpha halys* (Stål, 1855) has became an invasive pest in north America and Europe, where it has caused extensive damage to agriculture, resulting in great economic losses. Evaluating the potential of native predators in the invaded areas, ants might represent good candidates, thanks to their biology, ecology and behavior. In Italy, *H. halys* proved to be the top key pest in pear orchards, where the black garden ant *Lasius niger* (Linnaeus, 1758) (Formicidae, Lasiini) is the most abundant ant species. The aim of this study was to evaluate the predatory ability of *L. niger* on the eggs and on all the juvenile instars of *H. halys* in laboratory conditions. The results indicated that *L. niger* significantly reduces the survival of the second and third nymphal instars by 56% and 58% respectively, but it is not able to reduce the egg hatching and the survival of the first, fourth and fifth nymphal instars. These findings suggest that *L. niger*, a terrestrial and only partly arboreal ant, can only partially contribute to reducing the population of the invasive *H. halys* by preying on the smaller and more mobile nymphal stages. Further investigations, in particular in the field, could help clarifying the effective role of this species as potential biocontrol agents of *H. halys* in fruit orchards in association with other ant species as well as with other predatory insects.

KEYWORDS: Brown Marmorated Stink Bug, conservation biocontrol, ants, predation bioassay, Integrated Pest Management, agroecosystems

1. Introduction

The brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) (Heteroptera, Pentatomidae) is native from China, Japan, Taiwan and South Korea and from the late 90s it has became an invasive pest in Europe and North America (Leskey & Nielsen 2018). At the end of 2020, it was reported in 46 states of the United States and in 4 provinces of Canada (Stopbmsb 2020). Recently, it was detected also in Chile (Faúndez & Rider 2017). In Europe, *H. halys* was recognized for the first time in Switzerland in 2004 (Haye et al. 2015), and it is currently reported with stable populations all over Europe (except Ireland and the Scandinavian peninsula) and in the countries along the Black Sea (Claerebout et al. 2018; Inaturalist 2020). Its incredibly fast spread worldwide is due to the hitchhiking ability of the overwintering adults, which hide inside inanimate objects and packaging of any kind and are carried all over with trade and movement of people (Maistrello et al. 2018).

Halyomorpha halys was officially first detected in Italy in 2012, in the Emilia Romagna region (Maistrello et al. 2016), but a model based on its spatiotemporal dynamics allowed to date back its possible first entry in the country as early as 2009 (Maistrello et al. 2018). Currently, *H. halys* is established in the whole Italian peninsula and Corsica (Maistrello et al. 2018; Maistrello et al. unpublished data). The genetic analysis showed a high biodiversity of the haplotypes, indicating that the Italian populations are the results of multiple invasions from native and invaded countries (Cesari et al. 2018), thus confirming the hitchicking abilities of this species.

A few years since its first discovery in Italy, *H. halys* became a major key pest of fruit crops, especially pear orchards (Maistrello et al. 2017). In 2019, it caused an overall economic impact of € 588 milions on fruit production of Northern Italy (CSO Italy 2020), with up to 80-100% yield losses on pear, peach, apple, and kiwifruit. *H. halys* damages the fruits and seeds by pricking them with its piercing-sucking mouthparts, resulting in malformations, watery rot, suberification and necrotic areas, which render products non-marketable (Rice et al. 2014).

Currently, its management relies mainly on the use of broad-spectrum insecticides, which however also kill non-target insects, including potential natural enemies, and disrupt the most innovative IPM strategies, causing both economic and ecological damages (Leskey et al. 2012; Maistrello et al. 2017). Among the factors that make management of this pest especially difficult, there are some specific biological, behavioural and ecological traits. These include: i) a wide range of host plants (Rice et al. 2014), such as many fruits, vegetables and row crops, as well as many ornamental and spontaneous shrubs and trees that are usually found on the wooded areas surrounding the crops; ii) the great mobility of both the adults (Wiman et al. 2015; Lee & Leskey 2015), and the nymphs (Lee et al. 2014); iii) the high rate of population growth (Costi et al. 2017); iv) the absence of specific natural antagonists (Abram et al. 2017; Conti et al. 2020).

According to field surveys conducted in the areas of introduction in North America, sentinel egg masses of the invasive stink bug were consumed at quite low percentages, and eggs showed greater damage due to chewing mouthparts rather than to pierce-sucking apparatus (Ogburn et al. 2016; Cornelius et al. 2016; Shanovich et al. 2020). These studies have shown the existence of potential predators, without providing their identification. Laboratory no choice tests with solitary generalist predators showed that some species belonging to the families Tettigoniidae, Gryllidae, Acrididae, Forficulidae, Chrysopidae, Coccinellidae and Salticidae are able to predate *H. halys* eggs (Abram et al. 2014; Morrison et al. 2016; Pote & Nielsen 2017; Poley et al. 2018). Laboratory studies showed that first instar nymphs of *H. halys* are preyed on by species of Nabidae and Reduviidae and that the second instar nymphs are preyed on by Nabidae and by the pentatomid *Podisus maculiventris* (Say, 1832) (Pote & Nielsen 2017). Besides, also the pentaomid *Euthyrhynchus floridanus* (Linnaeus, 1787) showed potential as a predator of all developmental stages, including adults (Arellano et al. 2019).

In Europe, a three-year field survey conducted by Costi et al. (2019) in northern Italy with fresh sentinel eggmasses showed low rates of parasitism (lower than 3%) and predation (2%-5%), but predators could not be identified. In a laboratory study with solitary predators collected in northern Italy, eggs of *H. halys* were predated by *Adalia bipunctata* (Linnaeus, 1758) (Coccinellidae), *Eupholidoptera chabrieri* (Charpentier, 1825) (Tettigoniidae) and *Nagusta goedelii* (Kolenati, 1857) (Reduviidae), the first instar nymphs were predated by *E. chabrieri*, *Chrysoperla carnea* larvae (Stephens, 1836) (Crysopidae), *Himacerus mirmicoides* (O. G. Costa, 1834) (Nabidae), *N. goedelii* and *Rhynocoris iracundus* (Poda, 1761) (Reduviidae) and the second instar nymphs were predated by *E. chabrieri* and *R. iracundus*. The latter species preyed on also the adults of *H. halys* (Bulgarini et al. 2020). From this study the tettigoniid *E. chabrieri* and the reduviid *R. iracundus* appear as the most effective predators, consuming at least three different instars of *H. halys* (Bulgarini et al. 2020).

Ants are globally diverse and abundant, comprise a large fraction of animal biomass in most terrestrial communities, and are key contributors to a range of ecosystem functions (Hölldobler & Wilson 1990; Lach et al. 2010; Gibb et al. 2017). Thanks to their abundance, stable populations and a variety of food habits, ants are involved in the dynamics of multitrophic interactions as well as in soil improvement and the nutrient cycle. Among the different trophic roles, they are also predators of pest species (Cerdá & Dejean 2011). This may represent an important service for those plants hosting ants, for those that are more or less stably visited by ants, or that are surrounded by ant colonies (Campolo et al. 2015; Giannetti et al. 2019; Schifani et al. 2020). Several ant species are robust predators or exhibit very aggressive reactions against other animals including herbivores (Hölldobler & Wilson 1990). In addition, a laboratory study on the European ant *Crematogaster scutellaris* (Olivier, 1792) showed that this ant is able to significantly consume all the nymphal instars of *H. halys*, except for eggs (Castracani et al. 2017).

Predatory ants can be specialists or generalists and the latter are recognized as important for biological control. Generalist ants have several points that make them good candidates for pest control: i) they respond quickly to changes in pest density (Hölldobler & Wilson 1990; Maňák et al. 2013); ii) they can be abundant even when prey is scarce; iii) they are able to store food and continue providing control even if it is not immediately necessary (Hölldobler & Wilson 1990); iv) in case of prey too large to kill, they can drive them away with the use of chemical repellents (Goheen & Palmer 2010; Way & Khoo 1992) or cooperate to subdue the prey; v) have a wide range habitat and feed at different levels of the ecosystem (Hölldobler & Wilson 2009; Castracani et al. 2010; Lucky et al. 2014), and this characteristic makes some defense mechanisms of prey futile, such as jumping away or falling from the plant (Way & Khoo 1992). Furthermore, thanks to the large stable populations and an effective recruiting ability, they can react quickly to the increase in the number of pests. This also leads to the protection of plants at low pest density (Way & Khoo 1992).

Hence, ants have many ideal characteristics that make them a potential tool for biological control of invasive species, and the study by Castracani et al. (2017) showed that *C. scutellaris* can readily recognize all juvenile instars of *H. halys* as prey. On the basis of a previous field survery carried out in pear orchards in Northern Italy, it emerged that *Lasius niger* (Linnaeus, 1758) was among the most abundant ant species both on trees and on the ground (Bulgarini et al. unpublished data; Schifani et al. 2020), and is a species known to be resistant to anthropogenic disturbance such as mowing, fertilization and mechanical stress of the soil (Seifert 2018). *L. niger* is an aggressive and territorial species; it is also a generalist omnivorous that tends to feed on any underground, epigeal or arboreal nutrient source, including other invertebrates, as long as they are available and manageable (Seifert 1992, 2018). The purpose of this study is to evaluate the predatory ability of the autochthonous ant *L. niger*, already present in abundance in the orchards, on the eggs and on all five juvenile instars of the invasive pest *H. halys*.

2.**Materials and methods**

2.1 *Prey rearing*

Adults of *H. halys* were reared in mesh cages (30 x 30 x 30 cm), inside climatic chambers at 26°C, 60% relative umidity and L16:D8 photoperiod. They were fed on organic carrots, tomatoes, green beans and raw peanuts. A bottle cap with water-soaked cotton was used to supply water. Some pieces of paper and a bean plant were inserted in the cages to provide egg-laying substrates. Food and water were changed twice a week and freshly laid eggs were checked daily. Eggs were transfered to petri dishes with a wet piece of cotton and a bean pod. Immediately after their appearance, second instar nymphs were transferred to a new cage (30 x 30 x 30 cm) and reared under the same conditions of the adults.

2.2 *Predator rearing*

During May–September 2020, 23 colony fragments of *L. niger* were collected from different nests located in orchards located in the province of Reggio Emilia (Northern Italy). Ants (from 200 to 500 workers, without queen and/or eggs) were collected together with the nest materials and placed inside plastic containers (29 x 28 x 39.5cm), which were maintained outdoor at natural conditions of temperature, humidity and photoperiod, but sheltered from direct sunlight and precipitation. Ants were fed mainly with sugar cubes and cotton soaked with water. Five dead *Tenebrio molitor* larvae were also supplied to the ants of the numerous nests, when kept in the laboratory for more than a week, as a protein source. The soil was periodically wetted with a sprayer and the food was changed twice a week.

2.3 *Experimental Arenas*

Two arenas were used for the trials. The first one served as ant nest, the other as the testing arena. The nest arena was a rectangular plastic box (10 x 13 x 21 cm) closed with a lid. The testing arena was a cylindrical plastic box (20 cm in diameter, 11 cm height) without lid. The two arenas were connected through a removable transparent plastic tube (2.5 cm in diameter, 7 cm long). In the center of the testing arena, a bean plant (*Phaseolus vulgaris*) with at least two well-developed leaves, was placed. The plant pot (8 cm in diameter) was wrapped in pantyhose to prevent ants from gaining access to the soil. This measure was taken because in the preliminary tests it was observed that the ants tended to dig into the soil of the pot and to stay underground instead of exploring the arena and the plant, thus distracting them from the predation of the prey items. To prevent the ants from escaping, a mixture of vaseline and paraffin was applied to the edges of the testing arena.

2.4 *Experimental Protocol*

On average 100 ants were randomly taken from one of the original nest containers and placed in the rectangular nest arena together with the soil from their own nest. The ants for each trial were used only once. The ants had free access to the circular arena and to the plant. In order to attract the ants on the plant and make it a potential resource for them, a piece of aluminum foil with few drops of honey was wrapped to one of the leaves of the plant. After 24h, the aluminum foil was removed and the ants in the testing arena were moved back to the nest arena, and the access between the two arenas was blocked. After 24h, the potential “prey item” was placed on the leaves of the bean plant. Prey items consisted of one egg mass with at least 21 eggs, or 6 nymphal instars of a given stage (N1, N2, N3, N4, N5) of *H. halys*. Fresh (<24 hours) egg masses laid on the substrate (either paper or bean leaf) were collected from the adult rearing cage and attached to one of the leaves of the bean plant in the testing arena using paper clip. Once the prey item was positioned, the connection between the two arenas was re-established and the ants had free access to the plant. Observations were performed after 1h, 24h and 48h recording the number of dead nymphs and checking if the eggs were removed or damaged (i.e. if the egg shell was pierced/opened). Once the tests were finished, the egg masses were placed in petri dishes inside the thermostatic chamber for five days to count the number of hatched eggs. Ten replicates for each type of prey item were made. For each experiment, a control trial was set up at the same time, consisting of a testing arena with a bean plant not connected with an ant nest arena.

2.5 Statistical analysis

All statistical analyses were performed using R version 3.6.3 (R Core Team 2019). A Generalized linear model (glm) with a binomial error structure (logit link function) was used to compare the prey survival rates (obtained from the ratio between the number of surviving prey and the initial number of prey) between the treatment (presence of ants) and the respective control (absence of ants) at the end of the tests after 48h. For the egg masses, the variable compared was the hatching rate (obtained from the ratio between the number of hatched eggs and the initial number of eggs) and a comparison was made between control (absence of ants) and treatment (presence of ants) with the same analysis. To assess the general significance of treatment (presence and absence of ants), an analysis of deviance of the fitted model with Wald statistics χ2 was performed.

To calculate the effectiveness of predation over time, a one way ANOVA was carried out for each type of prey item (N1, N2, N3, N4, N5), comparing the survival rate of the prey in the three time checks (1h, 24h and 48h) and considering only the survival rates of the treatment (presence of ants). For the egg survival, the number of hatched eggs was used, both in the control and in the treatment, but since hatching necessarily took place after 48h, the eggs were excluded from this analysis. In case of significant differences, Tukey HSD post hoc test was used.

3. Results

3.1 Survival difference between control and treatment

While performing the tests, it was observed that nymphs from the second instar onwards tended to dropping from the plant when threatened by the ants, and that *L. niger* attacked, killed and in some cases dragged into the nest all the types of prey items, with the exception of the eggs. However, this dragging behaviour did not significantly affect the survival of the prey items in all cases compared with the control. The presence of *L. niger* did not affect egg hatching (treatment: prey survival rate (Mean ± SE) = 0.92 ± 0.02; control: = 0.87 ± 0.03; χ^2= 3.05; df = 1; p = 0.08). For juvenile instars, *L. niger* significantly affected the survival of N2 (treatment: = 0.56 ± 0.09; control: = 0.91 ± 0.04; χ^2= 15.47; df = 1; $p < 0.001$) and N3 (treatment: = 0.59 ± 0.05; control: = 0.81 ± 0.07; χ^2= 9.46; df = 1; $p < 0.01$). However, no significant differences between the treatment and control were found for N1 (treatment: = 0.75 ± 0.09; control: = 0.81 ± 0.05; χ^2= 0.74; df = 1; p = 0.38), N4 (treatment: = 0.66 ± 0.04; control: = 0.81 ± 0.07; χ^2= 3.30; df = 1; p = 0.06) and N5 (treatment: = 0.80 ± 0.05; control: = 0.76 ± 0.06; χ^2= 0.55; df = 1; p = 0.45) (Fig. 1).

3.2 Survival difference over time

A significant difference in the survival of life stages at the three time checks was found for each type of prey: N1 ($F_{(2,27)}$ = 3.53; p = 0.04), N2 ($F_{(2,27)}$ = 6.10; p = 0.006), N3 ($F_{(2,27)}$ = 22.30; $p < 0.001$), N4 ($F_{(2,27)}$ = 18.90; $p < 0.001$) and N5 ($F_{(2,27)}$ = 5.85; p = 0.007). Multiple comparisons showed decreasing survival over time for each of the nymphal stages (Figure 2). N3 and N4 survival rates were affected by time, significantly decreasing with greater exposure to predators. N2 survival rate significantly decreased only after 24 hrs, whereas the same effect on N4 survival rate was detected only after 48 hrs. The effect of time on N1 survival rate was less evident but was noticeable when comparing 1hr with 48 hrs.

4. Discussion

Halyomorpha halys is a serious key pest of fruit orchards, and in Italy, pear is the crop where most damage occurs with losses up to 80-100% (CSO Italy 2020). In order to understand the potential of native generalist predators commonly found in agroecosystems as biocontrol agents against this invasive species, this work was performed to test the predatory ability of the ant *L. niger*, a common species in both natural and agroecosystems. After 48 hrs of exposure to different instars of the invasive stink bug, *L. niger* proved to be able to reduce the survival of the second and third instar nymphs by up to 56% and 59% respectively. Very little predatory efficacy was demonstrated against eggs and the other three juvenile instars, despite attempts to attack, kill and drag some individuals into the nest were observed.

In a previous study, Castracani et al. (2017) showed that the European species *C. scutellaris* was able to prey on the juvenile instars of the pest, but left untouched the eggs. In the only study avaible for the USA, field collected ants of unidentified species did not show any predation on *H. halys* eggs in the laboratory (Morrison et al. 2016). However, single individuals were tested, a condition that penalizes the ants, negating the group predation typical of these insects. Thus, all studies available indicate that egg hatching of *H. halys* is never affected by the presence of ants, at least when the ants belong to the three tested species. This is probably due to several factors, including the size and strength of the mouthparts of these ants, which are probably too weak or too little to damage the eggs. Among the potential predators tested, the only ones with a chewing apparatus that were able to consistently damage *H. halys* eggs are mainly tectigonids and carabids, all insects equipped with very strong and large mouthparts (Morrison et al. 2016; Bulgarini et al*.* 2020). Other hypotheses to explain the non-predation of the eggs can be related to: i) the possibility that *H. halys* eggs uses semiochemicals, such as cuticular hydrocarbons or other volatiles, or the lack of any of them to interfere on the ant predatory ability (Schatz & Hossaert-McKey 2010; Cerdá & Dejean 2011); ii) the natural position of the egg mass (maintained during the experiments), typically underside of the leaf, that might prevent a correct and easier detection, and/or an efficient manipulation and ingestion by the predators. A combination of two or more of these hypotheses is also possible. Further studies on both the physical and chemical features of *H. halys* eggs are needed to clarify the reasons for the non-predation by ants and other predator species.

In previous studies, some insects proved to efficiently prey on the first instar nymphs, including tettigoniids, nabids, reduviids and the ant *C. scutellaris* (Pote & Nielsen 2017; Castracani et al*.* 2017; Bulgarini et al*.* 2020). Likely, this occurred because this is the smallest instar with the softest body, and therefore it is much easier to handle. In our study, however, the survival of the first instar was not affected by *L. niger*. These nymphs are the least mobile instar, since in nature they tend to remain aggregated on the egg mass under the leaves to acquire the endosymbionts left by the mother on the eggs (Taylor et al*.* 2014). In our experiments, the first instar nymphs were placed on the plant without the egg masses, however during the test they still tended to remain aggregated under the leaf. This behaviour probably reduced the possibility of these nymphs being detected and/or efficiently handled and thus preyed upon by *L. niger.*

The second and third instars are more mobile than the first and the probability of being located by the ants is greater. However, from the second instar onwards, *H. halys* individuals tends to drop from the leaves when threatened, as observed also during these experiments. This behaviour is a defense mechanism that can help escaping from single predators. However, in the case of ants it is different, due to their ability to feed at different levels of the ecosystem, and their ability to intercept the dropping prey (Way & Khoo 1992). The fourth and fifth instar also use the same defense mechanism, but their survival was not significantly affected by the presence of ants. This is probably due to their larger size, which therefore requires a greater number of individuals to kill them, and also to their greater mobility, which favours a faster escape from the predators.

The results related to mortality over time show that during the first hour of exposure, *L. niger* does not practically affect any of the nymphs tested. The mortality of the different instars increases over time, reaching the highest value after 48h, when the ants have likely fully explored the arena and have also begun to patrol the plant, increasing the probability of interaction between prey and predator. The effectiveness of *L. niger* therefore increases over time, particularly for the second and third instars. The difference between the second instar, whose survival drops drastically after 24h, and the third instar, whose survival drops more gradually over time, is probably due to the difference in size, which makes the second instar easier and faster to kill compared to the third one, which is also faster and more skilled to escape from predators. Experiments with a longer duration could help to clarify these aspects.

These results are partially in line with findings on the arboreal ant *C. scutellaris*, which failed to affect the eggs, but, unlike *L. niger,* successfully preyed on all juvenile instars (Castracani et al. 2017). This difference may be due to physical and behavioural adaptations of the two species linked to their different ecology. In particular, the head size and shape might reflect a different ability to bite: *L. niger*'s head is on average smaller (average between length and width = 977 ± 52 µm for *L. niger*, 1133 ± 105 µm for *C. scutellaris*) and more elongated (1.056 ± 0.02 µm for *L. niger* vs 0.881 ± 0.02 µm for *C. scutellaris*) (Seifert 2018). In ants, the larger shape of the head is generally a symptom of a greater development of the mandibular muscles, and *C. scutellaris* is an arboreal species nesting inside trees, thus biologically linked to the demanding activity of excavating wood. The different morphology and physical ability might explain observed results. Another possibility is that *L. niger* is less prone to search and attack potential prey on plants and/or that it is less aggressive than *C. scutellaris*, which is considered a highly competitive species in the Mediterranean region (Cammell et al. 1996; Way et al. 1997; Santini et al. 2007; Ottonetti et al. 2008). In any case, *L. niger* is a strongly territorial species that tends to attack and drive away intruders from its territory, not necessarily to prey on them (Seifert 2018). This interference and disturbance may have some important effects also in agroecosystems, possibly contributing to reduce crop damages in the case of phytophagous pests.

A possible concern on the use of ants as a tool in biological control regards the eventual disservices caused by the trophobiotic relationships that several species (including *L. niger*) can engage with sup-sucking pests such as aphids and mealybugs (Hölldobler & Wilson 1990). However, it is worth noting that, although this can have some costs for the plant, it is possible that the net cost-benefit balance is definitely beneficial (Rosumek et al. 2009). The ants, in order to protect their source of honeydew or because of their predatory and territorial habit, could provide services to the plant, such as warding off other insects or pathogens that could be much more noxious to the plant (e.g. Giannetti et al. 2019; Schifani et al. 2020, and references therein).

In conclusion, our experiments showed that *L. niger* is able to attack and kill at least some juvenile stages of *H. halys,* representing a potential limiting agent for this pest and thus offering a beneficial role. However, under similar conditions *C. scutellaris* (another ant common in agroecosystems) showed better results (Castracani et al. 2017). This points out the need to consider the diversity in the behaviour and ecological roles of the different ant species as natural pest limiters in agroecosystems (Hölldobler & Wilson 1990; Maňák et al. 2013; Schifani et al. 2020; Way & Khoo 1992). Ant diversity and ecology, different feeding habits, behavioural ecology and impact in the dynamics of species assemblages and community structure, as well specific climate and environmental factors, suggest to consider both single species and community effect on herbivores in agroecosystems to better plan programs of conservation biological control and IPM involving ants (Castracani et al. 2020; Arnan et al. 2018; Gibb et al. 2017). *L. niger* probably has a greater effect on individuals fallen on the ground, possibly disturbed by other ants or other predators. Moreover, *L. niger* foragers are able to form clear associations between odor cues and food locations to orient themselves toward food sources (Czaczkes et al. 2014). This implies that ants may learn to patrol a certain area where food odor is perceived, and this could be potentially used to manipulate ant behavior helping them to encounter food items. This suggests that an association of different species of predators would possibly lead to greater results in reducing pest population. Hence, a better understanding of the specific roles of ants as biocontrol agents of pests in agroecosystems, as well as of the interactions among different ant species, is crucial to provide further elements to feed Integrated Pest Management strategies, where different approaches compensate each other to implement better pest control.

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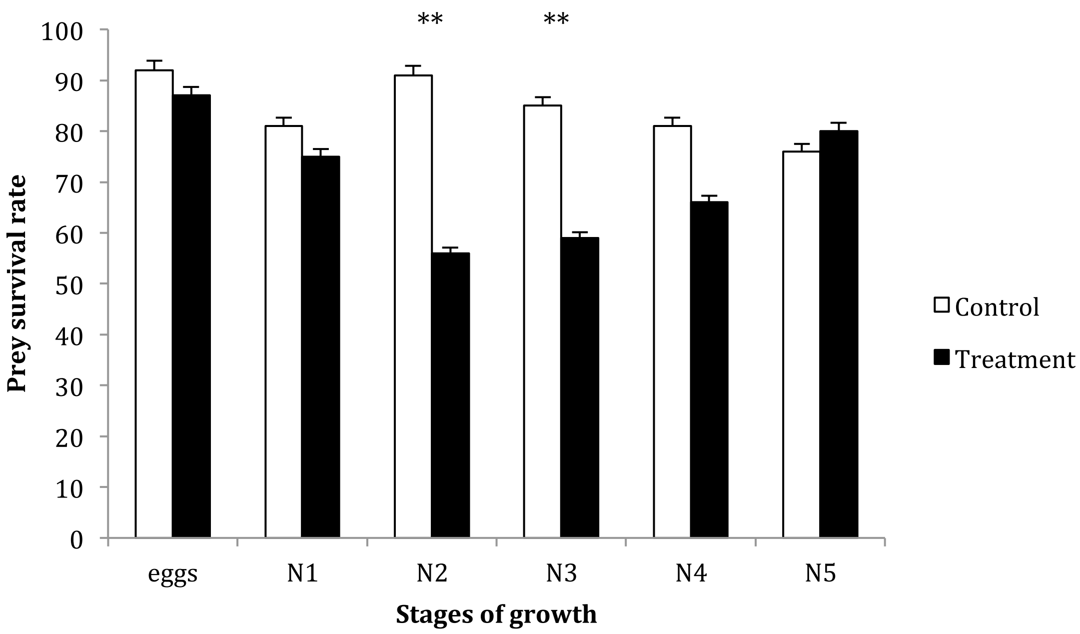

Fig 1. Percentage survival (+SE) of eggs and juvenile instars of *Halyomorpha halys* after 48h in treatment (presence of ants) and control (absence of ants). Asterisks indicate a significant difference between control and treatment (Generalized linear model (glm) with a binomial error structure (logit link function)): ** for $0.01 < p < 0.001$

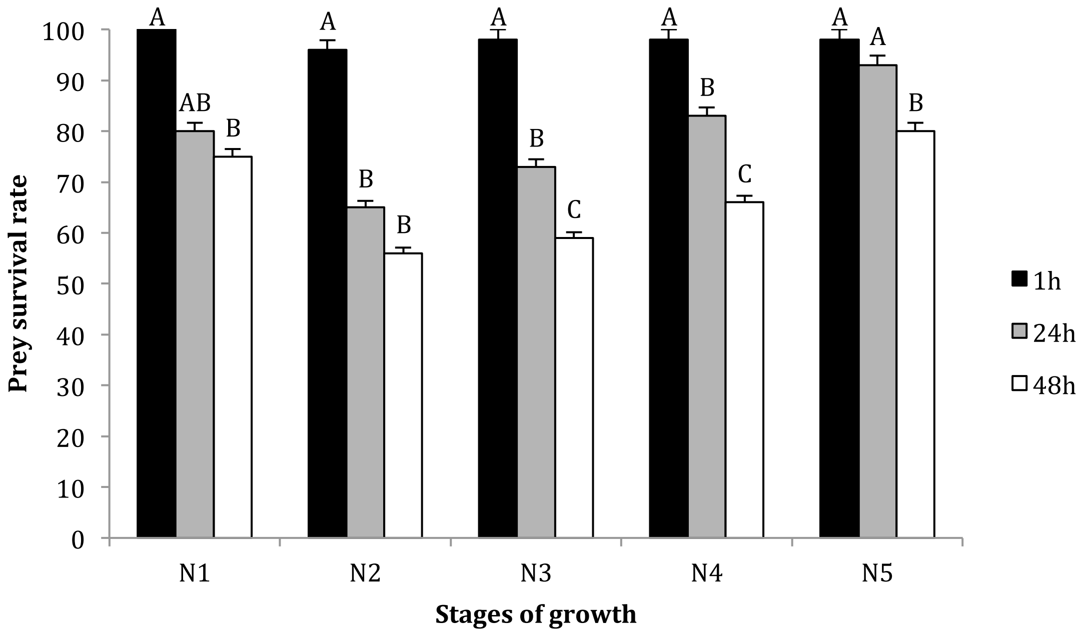

Fig 2. Percentage survival (+SE) of *Halyomorpha halys* juvenile stages over time. A one way ANOVA was carried out for each type of prey item (N1, N2, N3, N4, N5), comparing the survival rate of the prey item between the three time checks (1h, 24h, 48h). Columns bearing different letters indicate a significant difference between the time checks for the same prey item (Tukey's HSD, P< 0.05)

CHAPTER 4

Identification of native predators of the invasive *Halyomorpha halys* through the molecular gut-content analysis

Giacomo Bulgarini[1], Lucia Piemontese[2], Roberto Guidetti[2], Michele Cesari[2], Emanuele di Bella[1], Lara Maistrello[1]

[1] Dipartimento Scienze della Vita, Università di Modena e Reggio Emilia, Via G. Amendola 2, 41122, Reggio Emilia, Italy

[2] Dipartimento Scienze della Vita, Università di Modena e Reggio Emilia, via G. Campi 213/d, 41125 Modena, Italy

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Abstract

Halyomorpha halys (Stål, 1855) is an invasive agricultural pest in North America and Europe. Knowledge of its natural enemy community in the introduced areas is an important step to develop a sustainable management program. This work focused on the molecular identification of the predator species native to northern Italy to determine which feed on *H. halys*. A real-time PCR workflow was applied to detect traces of *H. halys* DNA from the gut content of potential predators collected in the field. Generalist predators belonging to arthropods were collected in four urban parks in Reggio Emilia (northern Italy) using sweep netting and tree beating techniques. Of the 190 analysed samples, 46 were positive for *H. halys* DNA and belonged to 10 insect taxa and at least six arachnid taxa. *Halyomorpha halys* was detected in the gut of several insect predators, namely the Dermaptera *Forficula auricularia*, the Coleoptera *Harmonia axyridis*, *Hippodamia variegata* and *Oenopia conglobata*, the Hemiptera *Nagusta goedelii* and *Himacerus mirmicoides* and the Orthoptera *Arachnocephalus vestitus*, *Phaneroptera falcata*, *Tylopsis liliifolia* and *Yersinella raymondi*, as well as in some arachnids, that include the Opiliones *Mitopus morio* and *Opilio canestrini* and the Araneae belonging to the genera *Anyphaena*, *Araneus*, *Philodromus* and *Calositticus*. The gut content analysis allowed to broaden the range of native generalist predators capable of exploiting the invasive *H. halys* as prey. The integration of this technique with laboratory bioassays and field observations allows to better understand how the invaded ecosystem is responding to the introduction of a new species, providing essential elements for integrated pest management programs.

KEYWORDS**:** Brown Marmorated Stink Bug, real-time PCR, molecular detection, spiders, generalist predators, invasive species, pest management

1. Introduction

Halyomorpha halys (Stål, 1855) (Heteroptera, Pentatomidae) is a serious pest of agricultural crops native to East Asia (Lee et al. 2013). It has a high invasive capacity facilitated by human activities and trade (Maistrello et al. 2018), which has led to a rapid colonization of other continents, namely North America, where it is present in 46 states of the USA and 4 provinces of Canada (Stopbmsb 2020), South America (Chile; Faúndez & Rider 2017), and Europe, where it is reported in 28 countries and around the Black Sea (Inaturalist 2020). In Italy, *H. halys* was first detected in 2012 in northern regions (Maistrello et al. 2016), although it was likely introduced as early as 2009 (Maistrello et al. 2018). Established populations are currently found in all Italian regions (Maistrello et al. 2018; Inaturalist 2020) and are the outcome of multiple invasions from native and already invaded countries (Cesari et al. 2015; Cesari et al. 2017). Due to the very high polyphagy (Rice et al. 2014), high mobility of all instars (Lee et al. 2014; Wiman et al. 2015; Lee & Leskey 2015), and high reproductive potential (Costi et al. 2017) *H. halys* has rapidly become the most important key pest in fruit orchards (Maistrello et al. 2017) and hazelnut grows (Bosco et al. 2018) and proved to be extremely difficult to manage. Damage is caused by the feeding on the fruits and seeds, which render products unmarketable. Losses in fruit production in northern Italy in 2019 were estimated as € 588 millions (CSO Italy 2020). To try to face *H. halys* field invasions, farmers have increased the use of broad-spectrum insecticides, seriously disrupting the previous integrated pest management (IPM) strategies and this is causing negative economic and environmental impacts in the invaded countries (Leskey et al. 2012; Maistrello et al. 2017).

When dealing with an invasive pest, knowledge of the natural enemy community in the introduced regions is an important step to develop a sustainable management program. Among the possible approaches to obtain information on potential natural enemies of the invasive species, one is to perform field surveys collecting naturally laid eggs or using sentinel eggs (fresh or frozen) of the target species, which are exposed in the field for some time, and are then analysed in the laboratory to check for parasitization and/or signs of predation. This approach has been used both in the US (Ogburn et al. 2016; Cornelius et al. 2016; Abram et al. 2017; Shanovich et al. 2020) and Europe (Costi et al. 2019; Haye et al. 2015a; Moraglio et al. 2020) and proved to be very useful to obtain information on parasitoids that use *H. halys* eggs as hosts. However, regarding predators, this approach is not useful to identify the species because it can only provide clues about the type of mouthparts used to pierce or chew on the eggs (Morrison et al. 2016), unless video cameras are associated with the exposed egg masses. Besides, this method can provide information only on biocontrol agents that use eggs as substrate/food items and excludes all the ones that use all the other development instars.

Another approach is to perform laboratory bioassays where the potential biocontrol agents (which, in the case of predators, undergo a period of starvation before the trial) are exposed to eggs or instars of the target species and then it is verify the outcome, in terms of predation/parasitization, after an appropriate period of time. This approach was used to identify some species of generalist predators that can effectively feed upon specific instars of *H. halys*. For example, in the USA, eggs were eaten by some Orthoptera (Tettigoniidae, Gryllidae, Acrididae), Neuroptera (Chrysopidae), Dermaptera (Forficulidae), Coleoptera (Coccinellidae, Carabidae) and Araneae (Salticidae) (Abram et al. 2014; Morrison et al. 2016; Pote & Nielsen 2017; Poley et al. 2018). Young nymphs (N1-N2) were consumed by Hemiptera (Nabidae, Reduviidae, Pentatomidae) (Arellano et al. 2019; Pote & Nielsen 2017), older nymphs (N3-N5) were consumed by Hemiptera (Pentatomidae), Hymenoptera (Crabonidae) and Araneae (Oxyopidae) (Arellano et al. 2019; Biddinger et al. 2017; Athey et al. 2017), and adults were consumed by Hemiptera (Pentatomidae) and Aranea (Agelenidae, Pholcidae, Theridiidae) (Arellano et al. 2019; Morrison et al. 2017). Fewer studies have been performed in Europe, namely in Italy, and the outcome is that eggs were consumed by Coleoptera (Coccinellidae), Orthoptera (Tettigoniidae) and Hemiptera (Reduviidae) (Bulgarini et al. 2020), young nymphs were consumed by Orthoptera (Tettigoniidae), Neuroptera (Chrysopidae), Hemiptera (Nabidae, Reduviidae) (Bulgarini et al. 2020) and by the ants *Crematogaster scutellaris* (Olivier, 1792) (Castracani et al. 2017) and *Lasius niger* (Linnaeus, 1758) (Hymenoptera: Formicidae) (Bulgarini et al. unpublished), older nymphs were consumed by *C. scutellaris* (Castracani et al. 2017), and adults were predated by *Rhynocoris iracundus* (Poda, 1761) (Hemiptera: Reduvidae) (Bulgarini et al. 2020).

An innovative approach to identify predatory species rely on the molecular analysis of their gut content to detect traces of DNA of the prey of interest through different techniques (PCR, real-time PCR, NGS) (Symondson 2002; Greenstone et al. 2010; Dhami et al. 2016; Unruh et al. 2016;

Casey et al. 2019; Siegenthaler et al. 2019). Such an approach is considered more effective than field observations, especially for species of small size, with concealed and/or nocturnal habits or for predation events occuring in places difficult to access (i.e. canopy, dens) or in case of fluid feeder predators like spiders or hemipterans. This rapid and specific identification of predators is crucial in case of invasive species. The molecular analysis can be applied to different matrixes (i.e. gut content, excrementa) and is employed by many laboratories to implement effective pest management programs. With this approach, it was possible to identify 13 different predators of stink bug pests of soybean and cotton (Tillman et al. 2015), the predators of the vineyard pest *Homalodisca vitripennis* (Germar, 1821) (Rhynchota: Cicadellidae) (Fournier et al. 2008), and the predators of the orchard pest *Conotrachelus nenuphar* (Herbst, 1797) (Coleoptera: Curculionidae) (Schmidt et al. 2016). It was also possible to confirm *Orius insidiosus* (Say, 1832) (Hemiptera: Anthocoridae) as predator for the maize pest *Helicoverpa zea* (Boddie, 1850) (Lepidoptera: Noctuidae) (Peterson et al. 2018).

Among molecular techniques for molecular gut content analysis, real-time PCR allows the rapid and specific detection and amplification of a gene or a portion of it from the undigested DNA of a target species. Thus, many protocols rely on dyes that are combined to species-specific primers or probes designed to start replication only in case of complete match with the target DNA, and the results are immediately displayed on the real-time PCR machine. A similar approach was used to detect the vertebrate predators of *H. halys*, by searching the DNA of the target species in their excrements. Through real-time PCR, Valentin et al. (2016) identified the bat *Eptesicus fuscus* (Beauvois, 1796) (Chiroptera: Vespertilionidae) as predator of *H. hal*ys. The purpose of this study was to identify species, native to northern Italy, able to feed on *H. halys*, by means of molecular analysis of the gut content of potential arthropod predators that occupy the same habitat of the invasive pest.

2. Material and methods

2.1 Predator sampling

Potential predators were field-sampled during summer in 2017 and 2018 in four Reggio Emilia urban parks (Mauriziano (44.683747, 10.674409), Rodano (44.677606, 10.664297), Fucini (44.673567, 10.615283), Baragalla (44.666831, 10.601160)), using tree beating and sweep netting techniques. Each selected park was at least 500 square meters, close (30-50 m) to a water source and close to an agro-ecosystem.

In each urban park, 20 trees known to be host plant of *H. halys*, belonging to the genera *Fraxinus*, *Acer*, *Prunus*, *Morus*, *Cornus*, *Corylus*, *Quercus* and *Robinia*, were selected (Haye et al. 2015b; Rice et al. 2014). A tree-beating session consisted of three strong beats in three points at a height between 1 and 3 m, using a stick and a white tray to collect all the dislodged arthropods. The sweep netting session consisted of 10 strokes in five points with tall grass, along a 10 m walk. Sampling was performed in each park every 15 days between 8 and 11 am from the beginning of May to the beginning of October. For each sampled tree, the total number of *H. halys* individuals (adults and nymphs) was recorded. *Halyomorpha halys* was never found during the sweep net sessions. The sampling took place in the same parks and on the same trees in both years, with a total of 22 sampling days in both years.

Captured specimens were individually collected in 50-ml tubes, which were adequately labelled and placed inside a thermally insulated bag, before being transferred in the freezer with absolute ethanol at -20°C. The generalist predators captured during sampling were accurately identified using specific taxonomical keys to the level of genera and possibly also species. All the sampled arthropods were recognized as non-endangered and unprotected species.

2.2 Arthropod preparation and dissection

For the gut content analysis only predators found on trees where *H. halys* was detected on the same sampling session were selected. Prior to dissection, each sample was placed in a 1.5 ml tube and washed in a solution containing 0.001 Triton X-100 by investing the tube for one minute. For individuals too large to enter inside the 1.5 ml tube the legs and/or wings were removed with a flame sterilized cutter. The sample was then transferred to a second 1.5 ml tube and washed with ultra-distilled water for one minute. This process was implemented to remove impurities from the samples and reduce the risk of DNA contamination. After that, each predator was positioned supine and blocked with the use of entomological needles sterilized with alcohol and flame. With the use of microsurgical scissors and entomological tweezers, also sterilized with alcohol and flame, the abdomen of the predator was opened, and the gut was removed and transferred to a 1.5 ml tube and placed in ice. Since spiders and opilionids carry out extraoral pre-digestion and the digestion of food takes place in different parts of the body (midgut diverticula extend throughout the prosoma and legs), the separation of gut content is more difficult (Macías-Hernández et al. 2018). For this reason, the aracnid individuals were used as whole.

2.3 DNA extraction and genetic analysis

Two positive and two negative controls were used for these analyses. Positive controls were represented by i) genomic DNA (gDNA) extracted from the head of *H. ha*lys raised in the laboratory and ii) gDNA extracted from the gut content of a laboratory-reared *Eupholidoptera chabrieri* (Charpentier, 1825) (Orthoptera: Tettigoniidae) fed with adults of *H. halys* and killed by freezing it at -20°C 30 min after eating. The negative controls were represented by the gDNA extracted from the legs and heads of *Nezara viridula* (Linnaeus, 1758) (Hemiptera: Pentatomidae) and *Rhaphigaster nebulosa* (Poda, 1761) (Hemiptera: Pentatomidae), two very common stink bugs in Italy. The used pentatomids were collected in Modena (Italy) and stored in 100% ethanol at -20°C before dissection.

Total DNA extraction was performed on predators and controls using the DNeasy Blood & Tissue kit (Qiagen Sciences, Germantown, MD, USA), following the protocol “Total DNA from Animal Tissue” (Dneasy Blodd & Tissue Handbook, July 2006). The extracted gDNA was re-suspended in 200 µl of Qiagen buffer ATE and then measured with a NanoDrop$^{®}$ spectrophotometer (Life Technologies) to measure the amount of detectable DNA. The negative and positive controls underwent the same extraction protocol.

A very sensitive real-time PCR assay (BMITS1 protocol) was used to detect traces of *H. halys* DNA in the extracted DNAs. The assay was described in Valentin et al. (2016) and was specific to the conserved region of the rDNA internal transcribed spacer 1 (ITS1) of *H. halys*. Three replicates were performed for each sample in 20 µl reactions using 500 nM of each primer (BMITS1F: 5’-CGA GGC CGC CGA TGA-3’; BMITS1R: 5’- CCC ACG AGC CGA GTG ATC-3’), 1× TaqMan^{TM} Fast Advanced Master Mix with Uracil-N glycosylase (UNG), 250 nM of the TaqMan^{TM} fluorescent probe (BMITS1TM 5’- CAG GCA ATG AAG CAC A-3’) with a dye label (VIC) on the 5’ end and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3’ end, and 2 µl of undiluted gDNA from the samples. The optimised real-time PCR protocol consists of an initial step of 2 min at 50°C to activate the UNG, a denaturation phase of 20 s at 95°C followed by 40 denaturation cycles of 10 s at 95°C, an annealing at 67 C for 20 s, 30 s at 72°C for extension and a final extension of 7 min at 72°C.

All reactions were carried out using a BioRad CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc.) and results were analysed using CFX Maestro^{TM} Software version 1.1 (Bio-Rad). The final relative fluorescence units (RFUs) of each positive control and of unknown content wells were compared by the software to the RFUs of the negative control wells. A positive was identified when its RFU value was greater than the mean RFU values of the negative controls plus the default cut-off value.

2.4 Statistical analysis

A Generalized linear model (glm) with a binomial error structure (logit link function) was performed to compare the proportion of positive samples between the two years (2017, 2018) and to compare the proportion of positive samples considering the month of capture, the tree on which they were captured and their interaction as variables. To assess the general significance of year, month, tree and their interaction, an analysis of deviance of the fitted model with Wald statistics χ^2 was performed. All statistical analyses were performed using R version 3.6.3 (R Core Team 2019).

3. Results

3.1 Predator identification

Among the 190 predators analysed in total, the gut content was positive for *H. halys* DNA in 46 samples (24%). Table 1 shows the samples analysed with relative abundance and the proportion of conspecifics of each taxon that resulted in a positive out of the total of those collected for that species. Among insects, the species with positive readings for *H. halys* DNA in their guts are the coccinellids *Harmonia axyridis*, *Hippodamia variegata*, *Oenopia conglobata*, the dermapteran *Forficula auricularia*, the nabid *Himacerus mirmicoides*, the orthopterans *Arachnocephalus vestitus*, *Phaneroptera falcata*, *Tylopsis liliifolia*, *Yersinella raymondi* and the reduvid *Nagusta goedelii*. As for the arachnids, those positive to *H. halys* DNA were the opilionids *Mitopus morio* and *Opilio canestrinii*, and the spiders belonging to the genera *Anyphaena*, *Araneus*, *Philodromus* and *Calossitticus.*

3.2 Statistical analyses

The number of positive samples was significantly higher in 2018 (34) than in 2017 (12) ($\chi^2 = 5.56$; df = 1; $p = 0.01$). According to the glm results, no significant differences were detected considering the month of sampling ($\chi^2 = 0.69$; df = 5; $p = 0.98$), the tree species ($\chi^2 = 10.37$; df = 8; p = 0.23) and their interaction ($\chi^2 = 7.71$; df = 15; $p = 0.93$). However, from Figure 1 and Figure 2 it is possible to observe a trend in the number of predators that resulted positive over time, that showed a peak in September in both years. The greatest number of predators that resulted positive was found mainly on *Acer*, *Prunus, Fraxinus* and *Cornus*, particularly in September and July, respectively.

4. Discussion

This study was performed to obtain a broader view of the potential of native biocontrol agents that are able to prey on the invasive pest *H. halys*, compared to the information obtained from laboratory bioassays, where the potential candidates are chosen by the experimentators. The chosen approach was the molecular analysis of the gut content of species collected in the field, sharing the same habitat of *H. halys*.

The results showed that ca. 25% of the predators collected contained *H. halys* DNA in their gut. This is a considerable result, taking into account that *H. halys* is an invasive species, which could have not been recognized as a typical prey by any of the resident predatory species in the invaded areas. Thus, all the sampled specimens are generalist species with a more or less broad range of prey items, which proved to be able to exploit the invasive stinkbug as a source of food. Remarkably, the time elapsed between the actual consumption of the prey and the moment of sampling the predator in the field is unknown. This can affect the result of the analysis, as the predator might have preyed on *H. halys* but the DNA within the gut has possibly been already digested by enzymes, thus becoming undetectable (Symondson 2002; Dhami et al. 2016). In any case, other hypotheses that may justify this apparently low percentage of positive samples include: i) the predator cannot exploit *H. halys* as prey for physical or physiological reasons, i.e. inadequacy of the mouthparts, absence of cues used to detect potential prey, presence of cues that repel/have an feeding deterrent effect, etc.; ii) the species may exploit *H. halys* as a feeding substrate, but the sampled specimens of that species did not prey on *H. halys* that day; iii) the predator can prey on only on a specific instar of *H. halys*, which was absent on the capture date of the sampled specimen, iv) the DNA was too degraded to be detected.

Looking at the orders of predators that resulted positive, it emerged that 100% of the Hemiptera analysed were positive, however the samples were few (three samples). Also, Opiliones showed a high positivity with 50% of the samples positive, but also in this case the analysed samples did not exceed ten (eight samples). The order that presented the highest ratio of positive samples was Orthoptera with 38% (12 out of 31), followed by Coleoptera with 21% of positive samples (six out of 28) and Dermaptera with 20% positive samples (14 out of 70). Last is the order Araneae, in which only 14% of samples was positive (seven out of 31).

Focusing on the identity of the predators, among the analysed coccinellids, the positive ones were *Ha. axyridis* (four samples out of 19), *H. variegata* (one sample out of four) and *O. conglobata* (one sample out of two). Coccinellidae, where both larvae and adults are efficient predators, are notorious biocontrol agents and many species are often used to suppress pest populations, in particular for aphids (Rutledge et al. 2004). Some coccinellids have been tested as potential predators on the eggs and early juvenile stages of *H. halys*. Adults of *C. septempunctata*, *Adalia bipunctata* (Linnaeus, 1758), *Ha. axyridis* and adult and larvae of *Coleomegilla maculata* (De Geer, 1775) have occasionally been shown to feed on eggs, but none of them consumed nymphs (Pote & Nielsen 2017; Morrison et al. 2016; Abram et al. 2014; Poley et al. 2018; Bulgarini et al. 2020). It is thus possible that the positive specimens detected in the analysis had fed on *H. halys* eggs prior to capture.

The dermapteran *F. auricularia* was positive in almost half of the samples (33 out of 70). Some Forficulidae have been recognized as important biocontrol agents (Suckling et al. 2006) and some of them have been recognized as the most effective predators on *H. halys* sentinel eggs in a field survey (Poley 2017). However, in laboratory studies *F. auricularia* showed either a very poor predatory performance on eggs (Poley et al. 2018) or no predation at all, although it can damage them, and it was never able to predate on young nymphs (Bulgarini et al. 2020). Thus, it is likely that the samples of *F. auricularia* positive to *H. halys* DNA fed on the eggs. The differences between field and laboratory situations could possibly be explained by the fact that the eggs used for lab trials were freshly laid (<24 hours). At this stage they might have some physical or semiochemical features that protect them from being eaten by many predators, which are eventually lost over time, allowing a greater number of predators to feed upon them. Another possibility is that the individuals tested in laboratory and those found in the field do not belong to the same species. In fact, recent studies indicate that the taxon *Forficula auricularia* is in reality a complex of cryptic species that conserve the same external morphology but are genetically differentiated, and the only way to correctly identify those species is by means of specific genetic analyses using mtDNA and nuclear sequence data (González-Miguéns et al. 2020).

The nabid *H. mirmicoides* and the reduvid *N. goedelii* were positive (two of two samples and one of one sample, respectively). In general, reduvids and nabids have been shown to be able to prey on the eggs and young nymphs of *H. halys* (Pote & Nielsen 2017; Morrison et al. 2016; Lee et al. 2013; Jones 2013). More specifically, *H. mirmicoides* successfully preyed on the first instar nymph and *N. goedelii* on both the eggs and the first instar nymphs (Bulgarini et al. 2020).

Among the orthopterans, *A. vestitus* (nine samples out of 25), *P. falcata* (one sample out of three), *T. liliifolia* (one sample out of one) and *Y. raymondi* (one sample out of one) resulted positive. In previous investigations, orthopterans always showed a good predation on *H. halys* eggs (Morrison et al. 2016; Pote & Nielsen 2017; Poley et al. 2018; Bulgarini et al. 2020), and *Eupholidoptera chabrieri* (Charpentier, 1825) can also predate on both the first and second instar nymphs of the pest (Bulgarini et al. 2020). Overall, Orthoptera are medium-large sized insects equipped with big and strong chewing mouthparts that can be effective generalist predators even as immatures.

As for the arachnids, the opilionids *M. morio* and *O. canestrinii* were positive (two samples out of four each). No studies have been published on these animals so far, but in general the opilionids are known as predators of a large number of insects, including the Hemiptera nymphs (Phillipson 1960; Acosta & Machado 2007). It is therefore likely that the sampled opilionids fed on the nymphs of *H. halys*. Considering spiders, four genera resulted in positives tests for *H. halys* DNA: *Anyphaena* (one out of 26 samples), *Araneus* (one out of two samples), *Philodromus* (three out of three samples) and *Calossitticus* (two out of two samples). Among the spiders tested in the laboratory, the Salticidae like *Phidippus audax* (Hentz, 1845) are the ones showing greater predation on the eggs (Morrison et al. 2016; Poley et al. 2018) meanwhile the Oxyopidae *Oxyopes salticus* (Hentz, 1845) consumed the nymphs (Athey et al. 2017). From the studies carried out on the spider webs present in potential overwintering sites of the invasive stink bug, it emerged that Agelenidae, Pholcidae and Theridiidae fed on *H. halys* (Morrison et al. 2017). The low number of positive samples among the sampled spiders could be due to their small size. In fact, spiders usually prey on smaller prey than themselves, as the optimal prey size is 50-80% of the spider's body (Nentwig & Wissel 1986). Therefore, it is likely that the positive samples had fed on the smaller juvenile instars, escluding adults and larger nymphs of *H. halys*.

A significant difference emerged between the two years of sampling, as in 2018 the number of samples positive to *H. halys* DNA was almost three times compared to 2017. This difference is probably due to specific climatic conditions, as the summer of 2017 was exceptionally hot and dry in northern Italy, with very little rain, and this may have adversely affected the survival of many arthropods, including *H. halys* as well as the generalist predators. Many farmers as well as personnel of plant protection services claimed that, in fruit orchards, populations of *H. halys* and related damage were considerably lower in 2017 than in other years (Maistrello, pers. comm.). With fewer pests available, predators were less likely to feed on *H. halys* in 2017 than in 2018.

Despite the lack of significant differences, trends were observed in the number of positive samples both in the months of sampling and considering the tree species. The majority of positive specimens was found in July on *Cornus*, and in September especially on *Acer* and *Fraxinus*. The same trend was observed also in the number of *H. halys*. In July, *Cornus* berries begin to ripen, and these shrubs become more attractive for *H. halys* than other species. Similarly, in September *H. halys* are mostly found on *Acer* and *Fraxinus*, because of their very nutritious fruits (the samaras), whereas the fruits on surrounding trees and shrubs are no longer present. Thus, a higher abundance of potential prey seem to be reflected in a higher number of predators preying on it Other hypotheses that can explain why September is the month with the highest number of positive samples are: i) in September most of the predators have reached the adult and/or the latest development instars, therefore they a have bigger size and they are likely more efficient in predation; ii) September is the pre-overwintering period for *H. halys,* when individuals tend to aggregate on the last available host plants before reaching the recovery sites to spend winter, and the predators are probably taking advantage of the favourable situation.

Thanks to the gut content analysis it was possible to increase the knowledge on the number of species that can use *H. halys* as suitable food. However, this analysis is necessarily not exhaustive, since there are limitations specifically related to the sampling techniques. Tree beating sessions were limited to a maximum of three meters above the ground, thus excluding all the predators that occupy the top parts of the trees, where also *H. halys* tends to stay. Furthermore, ant species were excluded from this study, since some worker ants could have captured and killed *H. halys* and brought it to the nest without eating on it. This would result in a negative sample, giving incorrect results, whereas it has been shown that some species can successfully exploit *H. halys* from laboratory tests (Castracani et al. 2017; Bulgarini et al. unpublished). The molecular analysis of the gut content of the generalist predators has proved to be a fast and effective tool to get a better overview on the potential of biocontrol agents of the invasive *H. halys*, allowing to broaden the number of species that can contribute to the control of this pest. However, real-time PCR analysis is qualitative and cannot provide information on the number or size of the prey eaten, on the stage of development of the prey, and whether the predator has fed on the whole prey or only a a part of it (Greenstone et al. 2010). Thus, the integration of this technique with laboratory bioassays and field observations will provide the necessary information to better understand how the invaded ecosystem is responding to the introduction of a new species. In the case of a serious pest, the combination of the different approaches can provide crucial elements for the development of a sustainable management program.

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Order	Family	Genus/Species	Total	Positive
Coleoptera	Coccinellidae	*Coccinella septempunctata* (Linnaeus, 1758)	2	0
		Harmonia axyridis (Pallas, 1773)	19	4
		Hippodamia variegata (Goeze, 1777)	4	1
		Oenopia conglobata (Linnaeus, 1758)	2	1
		Propylea quatuordecimpunctata (Linnaeus, 1758)	1	0
Dermaptera	Forficulidae	*Forficula auricularia* (Linnaeus, 1758)	70	14
Hemiptera	Nabidae	*Himacerus mirmicoides* (Costa, 1834)	2	2
	Reduviidae	*Nagusta goedelii* (Kolenati, 1857)	1	1
Orthoptera	Gryllidae	*Oecanthus pellucens* (Scopoli, 1763)	1	0
	Mogoplistidae	*Arachnocephalus vestitus* (Costa, 1855)	25	9
	Tettigoniidae	*Phaneroptera falcata* (Poda, 1761)	3	1
		Tylopsis liliifolia (Fabricius, 1793)	1	1
		Yersinella raymondi (Yersin, 1860)	1	1
Araneae	Anyphaenidae	*Anyphaena* sp. (Sundevall, 1833)	26	1
	Araneidae	*Araneus* sp. (Clerick, 1757)	2	1
	Dictynidae	*Dictyna* sp. (Sundevall, 1833)	1	0
	Philodromidae	*Philodromus* sp. (Walckenaer, 1826)	3	3
	Pisauridae	*Pisaura* sp. (Simon, 1885)	1	0
	Salticidae	*Calositticus* sp. (Lohmander, 1944)	2	2
		Europhrys gambosa (Simon, 1868)	1	0
		Icius congener (Simon, 1871)	1	0
		Icius hamatus (C.L. Koch, 1846)	1	0
		Phintella castriesiana (Grube, 1861)	1	0
		Phlegra cinereofasciata (Simon, 1868)	1	0
		Talavera aequipes (O.P.-Cambridge, 1871)	1	0
	Tetragnathidae	Tetragnatha sp. (Latreille, 1804)	2	0
	Theridiidae	*Platnickina tincta* (Walckenaer, 1802)	2	0
	Thomisidae	*Pistius truncatus* (Pallas, 1772)	4	0
Opiliones	Phalangiidae	*Mitopus morio* (Fabricius, 1799)	4	2
		Opilio canestrinii (Thorell, 1876)	4	2
TOTAL			**190**	**46**

Tab. 1 Abundance of the analysed samples divided by order, family, genus and species in alphabetic order, where the first order are of insects (Coleoptera-Orthoptera) and the last two are of arachnids (Araneae, Opiliones)

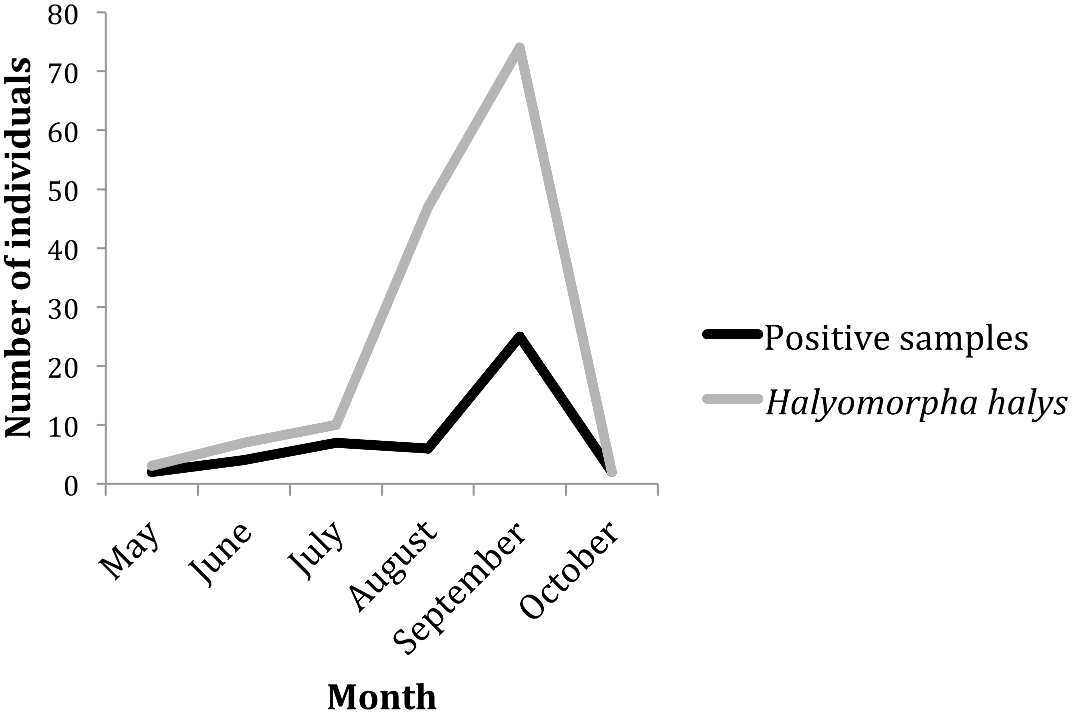

Fig. 1 Trends over time (months) of the abundance of predators that resulted positive for *Halyomorpha halys* DNA and of the number of *H halys* individuals collected during the sampling sessions in 2017 and 2018

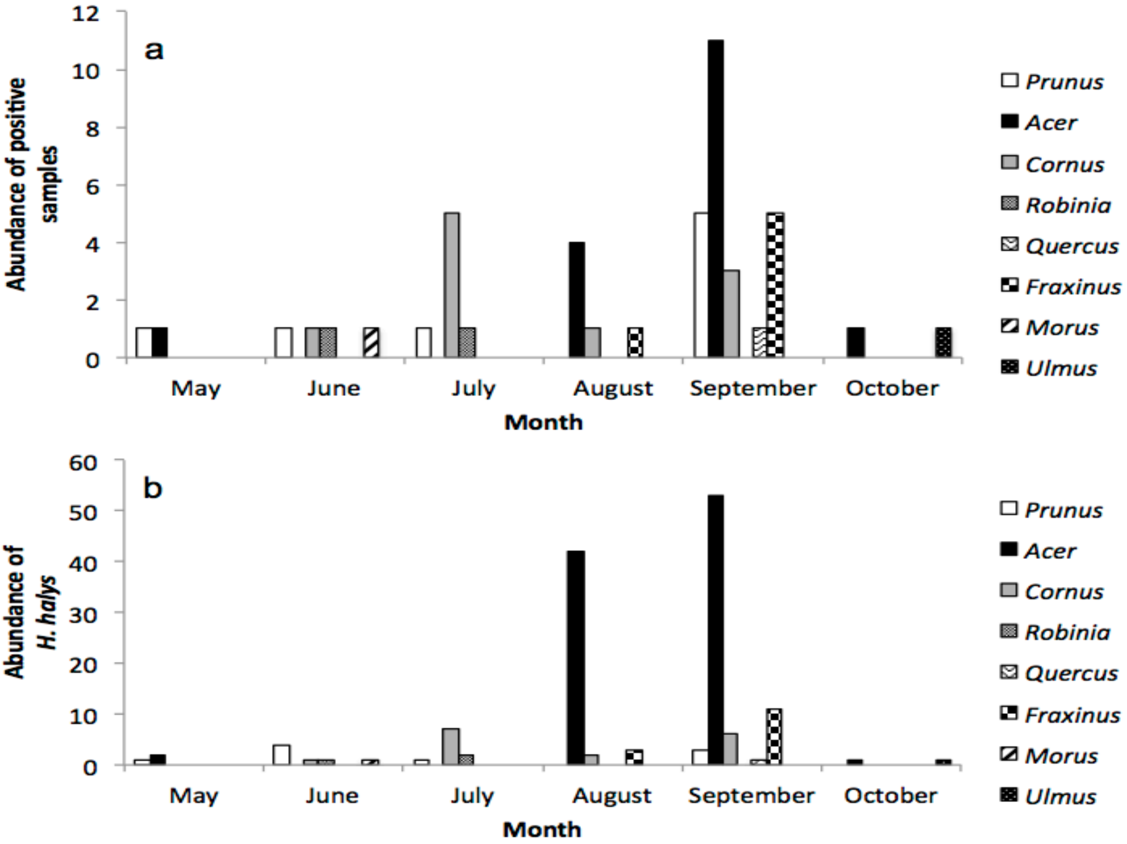

Fig. 2 (a) Number of samples resulted positive to *Halyomorpha halys* DNA over time (months, cumulating data from 2017 and 2018), considering the tree genus (identified at the genus level) on which the predatory specimens were found. (b) Number of individuals of *H. halys* over time (months, cumulating data from 2017 and 2018) on each tree genus

CHAPTER 5

Response of the jumping *spider Phidippus audax* (Araneae: Salticidae*)* to a commercial pheromone lure for *Halyomorpha halys* (Heteroptera: Pentatomidae)

Giacomo Bulgarini[1*], Kyle D. Doward[2*], Lara Maistrello[1] and Jeremy N. McNeil[2]

[1] Dipartimento Scienze della Vita, Università di Modena e Reggio Emilia, Via G. Amendola 2, 41122, Reggio Emilia, Italy
[2] Department of Biology, The University of Western Ontario, London, ON N6A 5B7, Canada
*Joint first authors

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Abstract

Following an observation of the Daring jumping spider, *Phiddipus audax* in traps testing a commercial lure for monitoring the brown marmorated stink bug (BMSB), *Halyomorpha halys,* field and laboratory experiments were undertaken to determine if the predator was responding to the chemical cue. While *P. audax* is present in the agroecosystem throughout the season, in both 2016 and 2019 the vast majority of spiders were captured after the end of August, suggesting a temporal change in responsiveness. This was confirmed in controlled Y tube assays as individuals tested in the summer showed no preferences, while those tested in the fall showed a significant preference for the combo lure over air.In 2016 all adults captured were mature females while in 2019 they were a mix of late-larval stages or adult females, probably due to inter-year temperature differences. Furthermore, in 2016 all individuals were recovered from pheromone baited traps while in 2019 they were found in both control and pheromone baited traps. While this may reflect differential responses by immatures and adults, a more probable cause is a "ghost effect" of contamination as the traps had been used over four seasons and some 2019 controls had pheromone in previous years. A native predator responding to the aggregation pheromone of a recently introduced species seemed somewhat surprising. The lure contains murgantiol, an aggregation pheromone of a native stinkbug, but this alone did not elicit the significant response observed when the complete combo lure was used. Therefore, it will be important to see which stages of *P. audax*, if any, are found associated with overwintering BMSB aggregations and determine any impact this generalist predator might have on the pest populations.

KEYWORDS: Brown marmorated stink bug, aggregation pheromone, daring jumping spider, seasonal responses.

1. Introduction

Halyomorpha halys (Stal) (Heteroptera: Pentatomidae), the brown marmorated stink bug (BMSB), is native to several Asian countries (Lee et al. 2013) but is now an invasive pest in both North America and Europe (Haye et al. 2015a; Leskey & Nielsen 2018; Maistrello et al. 2017; Maistrello et al*.* 2018). In North America the BMSB is now found in four Canadian Provinces and 46 US States (Stopbmsb 2020).

BMSB nymphs and adults exploit more than 300 plant species, including many agricultural crops (Rice et al*.* 2014; Haye et al. 2015a) and as all stages are highly mobile (Wiman et al*.* 2015; Lee & Leskey 2015; Lee et al. 2014) the resulting feeding damage may result in significant economic losses. For example, apple growers in the Midwest US states lost $ 37 million in 2010 (Leskey et al. 2012; Rice et al. 2014), while in 2019 the cost of damage in orchards in northern Italy was € 588 million (CSO Italy 2020). Broad spectrum insecticides have been widely used for control, but as these have had significant detrimental off target effects (Leskey et al. 2012; Maistrello et al. 2017) alternate means of control are desirable.

Native egg parasitoids readily accept BMSB eggs but, as there is a very low incidence of successful larvae development, this introduced species could represent a potential evolutionary trap for native parasitoids (Abram et al. 2014a; Haye et al. 2015b, Konopka et al, 2018, 2019; Costi et al. 2020). Consequently, unless these native species adapt, they will not be effective biological control agents. There are a number of no choice studies reporting native invertebrate predators feeding on BMSB eggs and/or nymphal stages (Abram et al. 2014b; Morrison et al. 2016, 2017; Castracani et al. 2017; Pote & Nielsen 2017; Poley et al. 2018; Bulgarini et al. 2020), but the actual impact of these generalist predators under field conditions remains to be determined.

The BMSB forms overwintering aggregations (Lee 2015) and the possible use of an aggregation pheromone blend, at least as a monitoring tool, has been investigated (Khrimian et al. 2014; Weber et al. 2014; Leskey et al. 2015 a, b). During experiments monitoring local BMSB populations in Ontario, we observed the common Daring jumping spider, *Phidippus audax* (Hentz) (Taylor & Peck 1975), established overwintering tents in the pheromone traps late in the season (August-October). As hemipteran aggregation pheromones can serve as kairomones for natural enemies (Brown et al. 1971; Krupke & Brunner 2003), and other jumping spiders use host odours when foraging (Clark et al. 2000, Jackson et al. 2002), we undertook laboratory and field experiments to examine the spider's temporal response to a commercial BMSB lure.

2. Materials and Methods

2.1 Field trials

The first trial was carried out from June 20 until 20 September 2016 at six agricultural sites in Niagara, Ontario. With two Dead Inn pyramid trap baited with Chemtica P460-Combo stinkbug lure (www.chemtica.com), and two empty ones as controls. The traps were spaced in a line at least 1.5 m apart and 80 cm above the soil. They were emptied and their position rotated every week, at which time the number of *P. audax* was recorded. The lures, which consist of two sachets containing compounds produced by some North American pentatomids and others produced by the *H. halys*, were renewed once every four weeks. The experiment was repeated from June to November in 2019 at three of the previous sites but included an additional component, trap colour. At each site there were two yellow and black traps baited with Combo lure, and two of each colour serving as controls. The number of individuals found in the yellow and black traps were compared to determine if there was any effect of trap colour.

2.2 *Laboratory Bioassays*

In order to carry out bioassays *P. audax* were hand captured at local field sites, taken to the laboratory and reared in individual transparent cylindrical containers (10 cm high, 4.5 cm in diameter) with gauze on the top to allow for air circulation. Those captured in the summer were held at 25° C, 16L:8D, 60% humidity while those captured in the fall were held at 25°C, 10L:14D, 60% RH (2016) or in an insectary under natural conditions (2019). In all cases spiders were fed twice a week with *Drosophila melanogaster* adults until tested.

Bioassays were conducted in Y-tube olfactometer (the common arm was 15 cm and the test arms 25 cm long) with an airflow of 3 L/min (Cole Parmer Acrylic flow meter (max. 5 ml/min)). Air was passed through 225 mL of activated carbon to remove any contaminants before going into the 225 mL beakers with the odour sources that connected with the test arms of the Y tube. As the spiders are very sensitive to movement, the olfactometer was placed inside a 24L x 18W x 18H white LexanTM box to ensure that there were no external stimuli that might influence their behavior.

Spiders were starved for 48h prior to testing, which was carried out from 10:00 to 15:00. Once the spider was introduced into the common arm of the Y tube it was considered to have made a choice if it moved > 3 cm into one of the test arms. If the spider remained in the common arm for 10 min it was classified as "non-responsive". After each trial the system was thoroughly washed with 75% ethanol to remove any excrement or draglines, as these could affect behavior (Clark et al. 2000), and the position of the odour sources switched to avoid any positional bias.

In 2016 the choice of fall adult females was determined when presented with 1) air vs air, ii) pheromone vs air and iii) pheromone vs pheromone. In all cases the pheromone source was the complete Chemtica P460-Combo stinkbug lure. As there was a limited number of females several had to be used twice, but were held for a week between being assayed a second time and were not used in the same choice trial.

In 2019 assays were carried out in both summer (July-mid-August) and fall (September-October), with five different treatments each time: i) air vs air, ii) combo lure vs air, iii) the sachet containing murgantiol vs air, iv) the sachet containing methyl E2,4E,6Z-decatrienoate (MDT) and methyl (2*E*,4*Z*)-decadienoate vs air, and v) the sachet with methyl E2,4E,6Z-decatrienoate (MDT) and methyl (2*E*,4*Z*)-decadienoate vs the sachet with murgantiol. In both time periods spiders were either last instar nymphs (n=20) or adult females (n=33).

2.4 *Statistical analysis*

G tests were used to analyse all data sets using the software R 3.1.1 GUI 1.65 Snow Leopard build (6784) (R Core Team 2019).

3. Results

3.1 *Field trapping*

In both years *P. audax* was observed in the habitat from the start of the field experiments, however, significantly more spiders were captured after mid-August (Figure 1), 62 of 63 in 2016 (G = 77.06; df = 1; p < 0.001) and 48 of 52 in 2019 (G = 43.88; df = 1; p < 0.001).

In 2016 all individuals captured after mid-August were mature females, while in 2019 there were 8 mature females and 40 late instars. In 2016 as all were captured in pheromone baited traps there is a highly significant difference between pheromone and control (G = 85.95; df = 1; p < 0.001). In 2019, of the 48 fall caught individuals significantly more (N=31) were found in pheromone baited traps than in control (N=17) traps (G = 4.14; df = 1; p = 0.04). There were no significant differences in the number captured in yellow or black traps in 2019 (24 in yellow 28 in black G = 0.30: df = 1; p = 0.57)

3.2 *Y tube assays*

Spiders tested in the fall of 2016 made no significant choice in either the air vs air or pheromone vs pheromone assays, however, a significantly higher number of individuals chose pheromone over air (G = 8.37; df = 1; p < 0.01: Figure 2). There were also significantly fewer non responders when at least one of the arms had a pheromone source (G = 9.84; df = 2; p < 0.01: Figure 2).

In 2019 the summer *P. audax* exhibited no preference for the BMSB lure or air in any of the different assays (Figure 3). However, in the fall, while they did not discriminate between air and either one the two sachets that make up the combo lure, they showed a significant preference for the complete blend over air (G = 9.92; df = 1; p < 0.01: Figure 4).

4. Discussion

Phiddipus audax is active in the habitat throughout the summer but is only found in traps baited with the Combo lure at the end of the season (Figure 1). This seasonal change in response is supported by the absence of any choice in Y tube bioassays conducted with summer females (Figure 3) but with significant choice by fall females in both years (Figures 2 and 4). These observations suggest that responsiveness is modulated by changes in abiotic parameters, such as temperature and photoperiod, as seen with the response of true armyworm males to the female sex pheromone (McNeil 1987; Dumont & McNeil 1992).

There were significant inter years differences, both in the developmental stages of *P. audax* captured and the proportions captured pheromone baited and control traps. 2019 was a cooler year, especially in August and September, which could explain the mix of late-stage immatures and adults (all of which can overwinter) in the traps compared to only adults in 2016. In 2016, spiders were only found in the pheromone baited traps while in 2019 there were individuals in control traps. Given many were immatures it is possible that they seek overwintering sites only using visual cues, although trap colour would not be a factor. However, a more likely explanation is that the traps used in 2016 were new, but as they had been used every year thereafter some of the traps used as controls in 2019 could have been used in pheromone treatments in previous years. The resulting contamination could lead to a “ghost effect”, as it has been shown that pheromones can be absorbed and remitted by plants (Wall et al. 1981; Suckling et al. 1996) and humans (Cameron 1983), in the latter case for many years.

The Daring jumping spider is a generalist predator that feeds on native heteropterans, including the brown stink bug, *Euschistus servus* and the tarnish plant bug, *Lygus lineolaris,* (Bailey & Chada 1968; Freed 1984; Young 1989), and has been shown to use chemical cues to locate prey species (Hoefler et al. 2002). The combo lure contains (i) murgantiol, an aggregation pheromone of the native Harlequin stink bug (Zahn et al. 2008), (ii) methyl (2E,4Z)-decadienoate, an aggregation pheromone of *Euschistus* spp (Aldrich et al. 1991) that attracts several other native stink bug species (Leskey & Hogmire 2005), and (iii) methyl (E,E,Z)-2,4,6-decatrienoate which is produced by an Asian stink bug but is attractive also to native stink bug species (Aldrich et al. 2007). The presence of compounds used by native stink bugs could explain *P. audax* presence in the traps but in the Y tube assays only the complete lure resulted in a significant attraction (Figure 4). *Phiddipus audax* readily attacks BMSB nymphs and adults (pers. obs.) so locating aggregations of overwintering BMSB would provide a readily available food source in both fall and spring, although further research is required to determine if *P. audax* responds to the BMSB aggregation pheromone alone, and, if yes, to determine the impact on overwintering pest aggregations.

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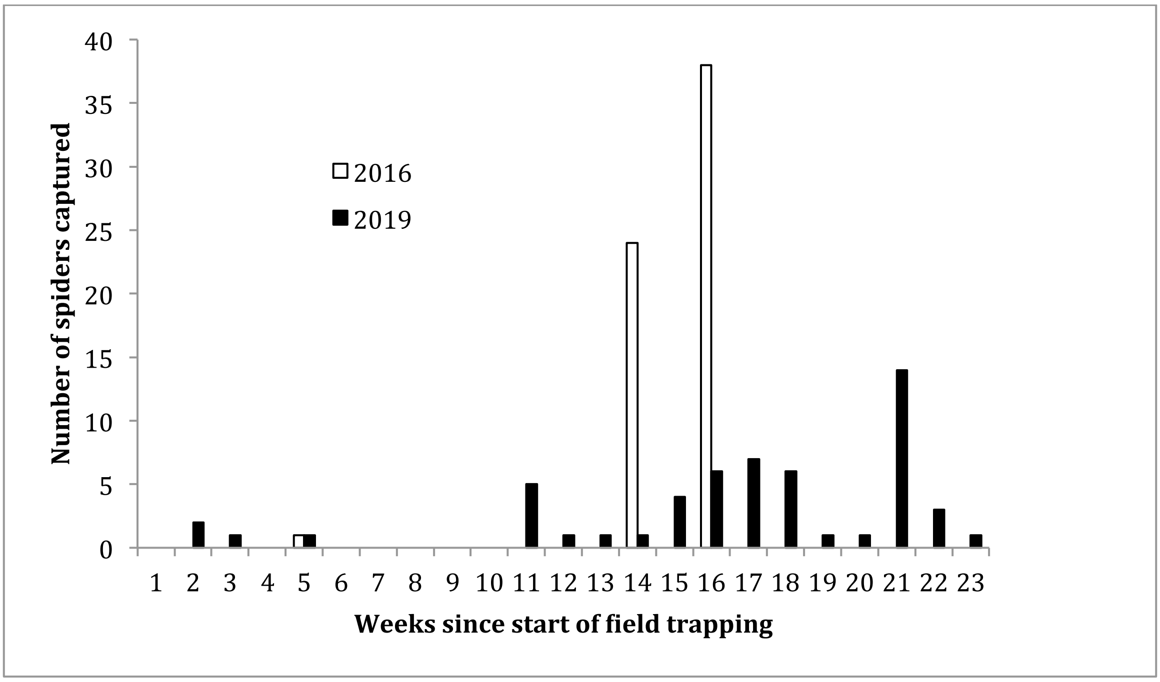

Fig 1. The number of *Phidippus audax* captured each week in traps during a study testing a commercial lure for the brown marmorated stink bug, *Halyomorpha halys*, from 20th June to 19th September 2016 and from 3rd June to 9th November 2019 in the Niagara peninsula, Ontario

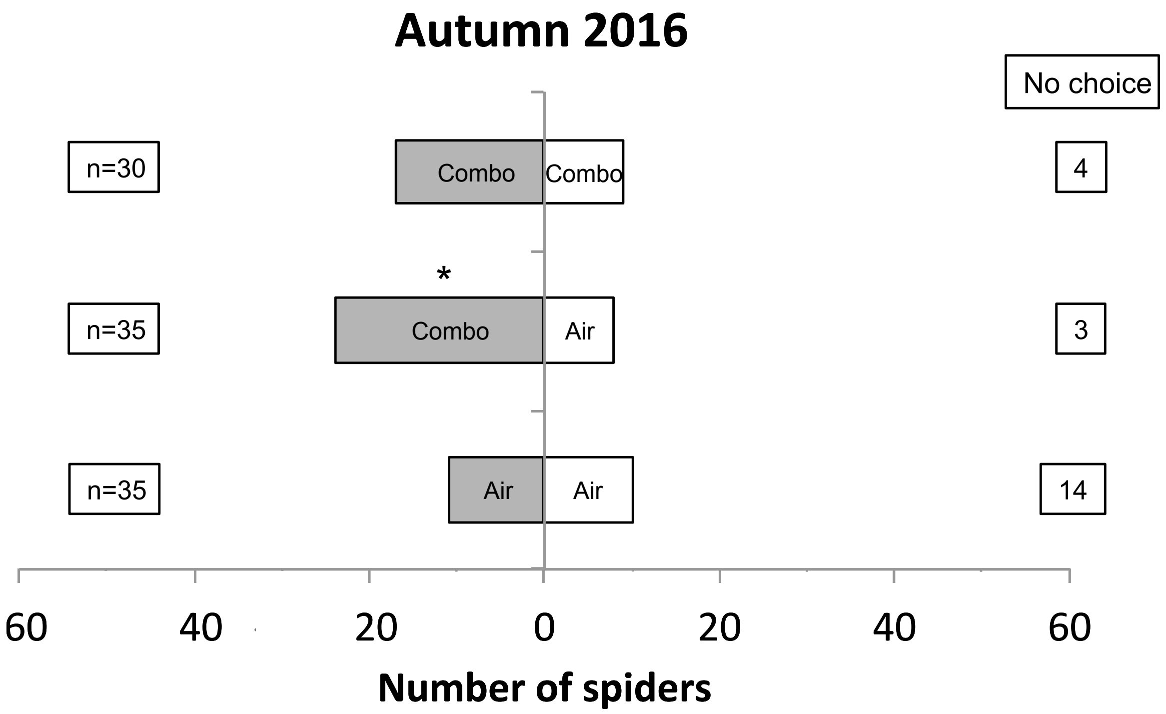

Fig 2. The response of adult female *P. audax* to air or the Chemtica combo lure for the brown marmorated stink bug, *Halyomorpha halys*, in Y tube olfactometer choice assays in autumn of 2016. The asterisk indicates a significant difference of p<0.01

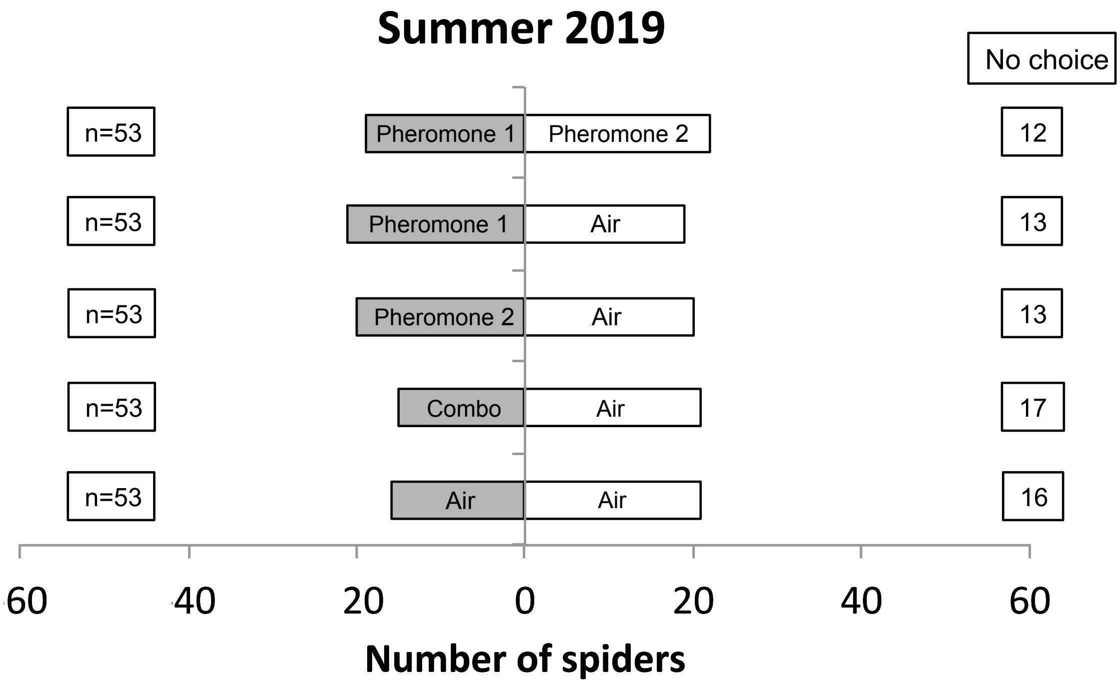

Fig 3. The response of sub adult or adult *P. audax* females to air or different components of the Chemtica combo lure for the brown marmorated stink bug, *Halyomorpha halys*, in Y tube olfactometer choice assays in summer of 2019

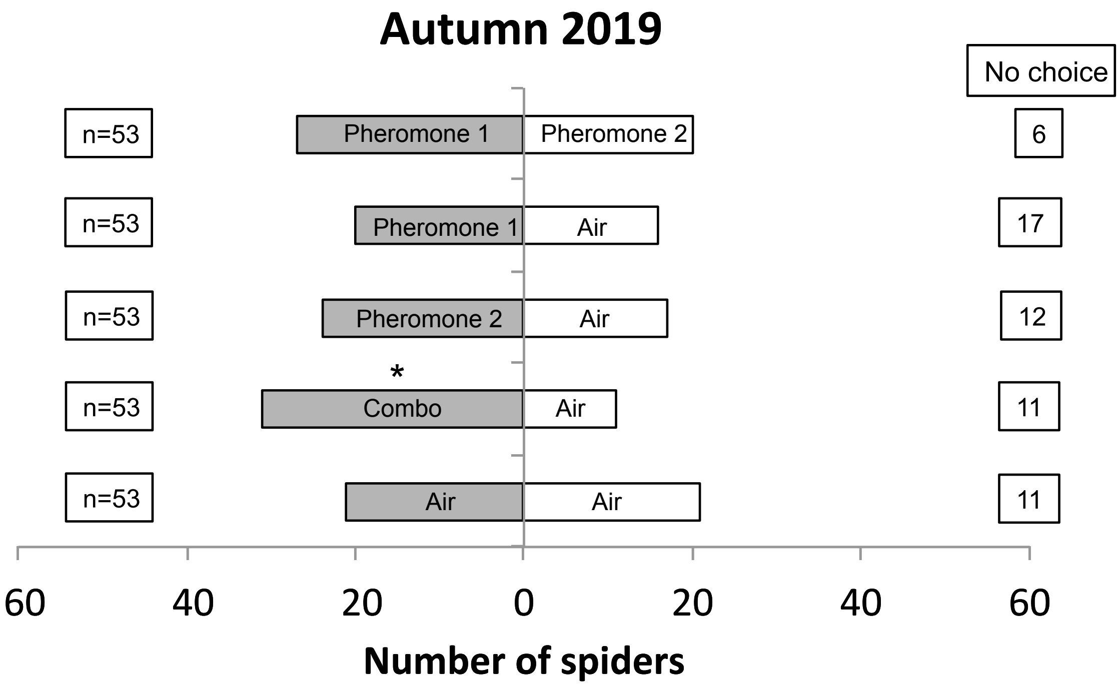

Fig 4. The response of sub adult or adult *P. audax* females to air or different components of the Chemtica combo lure for the brown marmorated stink bug, *Halyomorpha halys*, in Y tube olfactometer choice assays in fall of 2019. The asterisk indicates a significant difference of p<0.01

CHAPTER 6

Repellent activity of essential oils on adults of *Halyomorpha halys* (Heteroptera: Pentatomidae) in different physiological-behavioural phases

Giacomo Bulgarini [1], Sara Bortolini [2] and Lara Maistrello [1]

[1] Department of Life Sciences, Centre BIOGEST-SITEIA, University of Modena and Reggio Emilia, Via G. Amendola 2, 41122, Reggio Emilia, Italy; 235673@studenti.unimore.it; lara.maistrello@unimore.it
[2] Faculty of Science and Technology, Free University of Bolzano, Piazza Università 5, 39100, Bolzano, Italy; sara.bortolini@unibz.it

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Abstract

The brown marmorated stink bug *Halyomorpha halys* is an invasive agricultural pest in North America and Europe, and also a dwelling nuisance in autumn, due to the overwintering adults aggregating inside buildings. The repellent potential of ginger, clove, vetiver and turmeric essential oils (EOs) was tested on *H. halys* adults of three different physiological-behavioural phases: exiting overwintering (EXOV), active during summer (SUMM), entering overwintering (ENOV). In a two-choice apparatus, fresh food was used as an attractant in both sides, together with three males when testing ENOV individuals. A filter paper with different concentrations of the EOs was the treatment side, and a filter paper with ethanol acted as control. The position of the individually tested bugs was recorded after 1, 6, and 24 hours. All tested EOs were repellent at concentrations higher than 3%, independent of sex or length of exposure. Turmeric and clove were the most repellent EOs, whereas ginger and vetiver showed on average a medium to low repellency. Significant differences emerged among the physiological-behavioural phases, with SUMM individuals showing a greater repellency to many of the tested concentrations, and EXOV individuals being overall the least susceptible to these substances. The response to vetiver oil was ambiguous, as at 25% it elicited both a strong repellency in SUMM and a strong attraction in EXOV. Turmeric and clove EOs are promising candidates in integrated pest management strategies to reduce attacks by *H. halys* to susceptible crops especially during summer, as well as to prevent the entrance of overwintering bugs in buildings in autumn.

KEYWORDS**:** Brown marmorated stink bug, ginger (*Zingiber officinale*), vetiver (*Chrysopogon zizanioides*), clove (*Syzygium aromaticum*), turmeric (*Curcuma longa*), integrated pest management

1. Introduction

Halyomorpha halys (Stål, 1855) (Heteroptera: Pentatomidae) is an insect native to China, Japan, South Korea and Taiwan (Lee et al. 2013), that in recent years has become an invasive pest in North America and Europe, due to its hitchhiking on inanimate objects (i.e. containers, vehicles, luggage) combined with human activities and trade (Maistrello et al. 2018). In August 2020, *H. halys* was reported in 46 states of the USA and in four Canadian provinces (Stopbmsb 2020). In 2017 it was also detected in South America, in Chile (Faúndez & Rider 2017). In Europe, the first records occurred in Switzerland in 2004 (Haye et al. 2014) and as of August 2020, *H. halys* reported with established populations in 28 states in Europe and in all the countries surrounding the Black Sea (Inaturalist 2020; Cianferoni et al. 2018).

H. halys has a very wide range of host plants (more than 300) that include fruit trees (i.e. apples, peaches, pears, and kiwi), vegetables (such as tomatoes and peppers), row crops (including wheat, sorghum, maize and soybean), ornamental and non-cultivated plants (Rice et al. 2014). Damage is caused by the piercing-sucking activity resulting in deformities, suberifications, necrotic areas and watery rot, which render the final products non-marketable. In 2010, *H. halys* caused damage of over $ 37 million in U.S.A. apple trade and 100% losses in peach productions in Maryland (Rice et al. 2014; Leskey et al. 2012). In northern Italy, after its first official discovery in 2012 (Maistrello et al. 2016), it became a key pest of fruit orchards from 2015 (Maistrello et al. 2017), and in 2019 it caused up to 80-100% yield losses, with an estimated damage of € 588 million (CSO 2020). Both in Italy and USA, the attempts to counteract *H. halys* outbreaks with chemical control led to a massive increase in broad-spectrum insecticide use, disrupting previous IPM programs, increasing costs for the growers and causing negative consequences to the environment and consumers (Leskey et al. 2012; Maistrello et al. 2017).

Due to the high reproductive potential and overlapping of different instars and generations during summer (Costi et al. 2017), high mobility of all instars (Lee et al. 2014; Wiman et al. 2015; Lee et al. 2015) and high polyphagy, management of *H. halys* is particularly challenging. Research is currently focusing on surveying natural antagonists in the invaded areas (Abram et al. 2017; Costi et al. 2019; Shanovich et al. 2020; Bulgarini et al. 2020) and on the potential of biocontrol strategies using native and exotic agents (Conti et al. 2020; Stahl et al. 2019). Approaches have been developed with the aim to reduce the use of insecticides, like the behaviour-based strategies Integrated Pest Management-Crop Perimeter Restructuring (IPM-CPR) (Blaauw et al. 2015), trap-crop (Mathews et al. 2017) and “attract and kill” (Morrison et al. 2019), or prevention by means of exclusion netting (Candian et al. 2020). However, none of these strategies proved to be both fully successful and easily applicable in different contexts.

In the view to develop sustainable strategies to manage this pest, one approach is to consider the use of natural substances, in particular those with a repellent action, to be eventually used in combination with other techniques. Essential oils (EOs) are mixtures of volatile organic substances produced by plants as secondary metabolites, composed mainly of hydrocarbons, such as monoterpenes and sesquiterpenes both oxygenated and not, and other oxygenated compounds, and are usually responsible for the distinctive odours of some plants (Dhifi et al. 2016; Guenther 1972; Nerio et al. 2010; Regnault-Roger et al. 2012). Botanical EOs could be widely used for insect control, as they are able to significantly affect different aspect of insects’ life cycle. Indeed, EOs can interfere with insects’ behaviour and physiology, delaying their development, adult emergence and fertility, and affecting oviposition (Regnault-Roger et al. 2012; Zhang et al. 2013; Werdin et al. 2011). Bio-pesticides based on EOs are considered more eco-friendly than synthetic molecules, as they have a low persistence in the environment and are usually considered nontoxic to humans (Terriquez et al. 2013). Being available in large quantities, some EOs are commonly used as fragrances or in the food industry (Isman 2006).

Repellents are substances that can act locally or with action across great distances, preventing insects, or arthropods in general, from coming into contact with a surface of interest by creating a vapour barrier (Nerio et al. 2010). EOs extracted from different plant families showed strong repellent properties, in particular against pests of medical interest (Terriquez et al. 2013; Trongtokit et al. 2005) and of stored food products (Regnault-Roger et al. 2012; Ukeh et al. 2009). Focusing on agricultural pests, the white-spotted stink bug *Eysarcoris ventralis* (Westwood, 1837) (Hemiptera: Pentatomidae) is repelled by a compound isolated from the green foxtail’s endophyte, whereas *Origanum vulgare* (Linnaeus, 1753) (Lamiaceae) (oregano) and *Thymus* *vulgaris* (Linnaeus, 1753) (Lamiaceae) (thyme) were able to repel *Nezara viridula* (Linnaeus, 1758) (Heteroptera: Pentatomidae) in laboratory trials (Werdin et al. 2011; Nakajima et al. 2010). In a study by Zhang et al. (2014), several EOs were used to test their ability to prevent adults and nymphs of *H. halys* from entering the traps baited with the attractive lures based on aggregation pheromones. Among the EOs tested, the ones of clove, ylang-ylang, lemongrass and spearmint were able to completely block the attraction of the lures, whereas the oils of wintergreen, geranium, pennyroyal and rosemary reduced effectiveness of the traps by 60-85%.

As previously reported (Rice et al. 2014), the main damage caused by *H. halys* involves primarily fruits and vegetables, and, among all developmental instars, adults are the ones that cause the most damage (Acebes-Doria et al. 2016). Adults are present throughout the year (Costi et al. 2017), and, as in most pentatomoids, they undergo different physiological-behavioural phases, i.e. specific seasonal adaptations that allow them to synchronize dormancy periods with the most unfavourable season of the year, and development and reproduction with the most favourable seasons, in terms of food and climate (Musolin & Saulich 2018). Specifically, during early spring, adults exit from overwintering shelters seeking host plants to recover water, nutrients and energies after a long period of starvation (5-7 months). Late spring-summer is the peak activity period, when they actively feed and mate on the many available host plants. In late summer/beginning of autumn, the adults of the new generation stop feeding and seek dry, protected shelters to overwinter, often aggregating in groups of dozens or hundreds. These large groups are also likely formed due to the attractive effects of the aggregation pheromone produced by adult males which is able to attract both sexes and all stages of development (Rice et al. 2014; Harris et al. 2015). As, in urbanized areas, these overwintering aggregations mostly occur in houses, warehouses and other man-made structures, *H. halys* is also considered a dwelling nuisance.

The purpose of this study was to verify the potential of EOs as eco-friendly tools to reduce the damage of *H. halys* to crops and to prevent overwintering adults from entering buildings. Specifically, the repellent activity of four EOs was evaluated on adults belonging to the different physiological-behavioural phases.

2. Materials and Methods

2.1 Essential oils and insects

The tested EOs were: ginger, *Zingiber officinale* (Roscoe, 1807) (Zingiberacaceae), turmeric, *Curcuma longa* (Linnaeus, 1753) (Zingiberacaceae), clove, *Syzygium aromaticum* (L.) (Myrtaceae), and vetiver, *Chrysopogon zizanioides* (L.) (Poaceae). Ginger and clove EOs were purchased at Erbamea Srl (Selci Lama di San Giustino, Perugia, Italy), whereas vetiver and turmeric at the Oshadhi Italian dealer (Verona, Italy). All EOs were 100% pure, with no adjuvants. Each EO was diluted in ethanol and tested at six different concentrations: 50%, 25%, 12%, 6%, 3% and 1%.

Adults of three different physiological-behavioural phases were tested: i) individuals exiting overwintering (EXOV, March-May); ii) adults active during summertime (SUMM, June-August), field collected during tree-beating sessions on mixed shrubs in urban parks of Reggio Emilia (northern Italy); and iii) adults entering overwintering (ENOV, mid-September-mid October) collected from overwintering sites in houses and barns. The EXOV individuals were collected during the previous autumn from barns, garden sheds and buildings and maintained in wooden boxes (24 x 26.5 x 19 cm) filled with cardboard panels in order to simulate overwintering sites. The boxes, positioned outdoors inside a plastic box (50 x 40 x 25 cm) with fine mesh windows on all sides and sheltered from atmospheric precipitation, were provided with a slit allowing perception of outdoor temperature, photoperiod and the possibility to exit. For the trials, only the individuals that came out naturally and were found actively walking in the plastic box were tested. The trial started on the same day as the exit from the wooden boxes.

2.2 *Experimental apparatus*

The apparatus for the two-choice bioassay (Figure 1) was built using a transparent tube (50 cm length, 3 cm internal diameter), composed of five 50 ml plastic vials properly cut. The three central vials were fixed, while the two end vials, called A and B, were removable, washable and interchangeable. A small hole in the centre of the tube was used to insert the insects to be tested (one at a time), by temporarily removing the cap that normally covered the hole. Both ends of the tubes were connected to transparent cylindrical plastic boxes (13 cm height, 10.5 cm diameter) also called A and B. Each box was provided with a transparent lid (9 cm diameter) with an insect-proof net to allow airflow.

Ten apparatuses were available for the trial, for a maximum of ten insects tested each day. For the EXOV and SUMM tests, the same amount of fresh organic food and water was placed inside each of the two boxes as follows: two green beans, two chunks of carrots, two peanuts and a cotton wick soaked in water. For the ENOV test, alongside the food, three adult males were also added in each box, in order to simulate the typical aggregation conditions mediated by the male-emitted pheromone (Harris et al. 2015). Each one of the removable ends that protruded towards the boxes was plugged with a circular filter paper disk (diameter: 6.5 cm), secured with an elastic band. Some small cuts were made on each paper disk to allow the volatile compounds from the food (and the males in the case of the ENOV test) to disperse out of each box. The cuts were such that their edges were folded back to facilitate the eventual entry of the tested bugs inside the box and, in the case of ENOV tests, at the same time, prevent the male adults enclosed together with the food from leaving the box and entering the test tube. The end-tube A was always used as control side (i.e. filter paper wetted with 50 µl of ethanol), while the end-tube B was used as treatment side (i.e. filter paper wetted with 50 µl of EO diluted in ethanol at the different concentrations).

In order to avoid any alteration in behaviour due to laboratory conditions, the experimental apparatuses were always placed outdoors under a roof, exposed to the seasonal temperature and photoperiod, but sheltered from direct sunlight or rain. The position of the apparatuses was randomized with respect to the light source.

2.3 *Repellency bioassay*

Each disk was treated by pipetting either 50 µl of ethanol (A) or 50 µl of diluted EO (B) all over the surface. After treatment, the disks were air-dried for 40 minutes. Meanwhile, each box was filled with fresh food (and three adult males for the ENOV tests) as described previously. After a fasting period of 24h, the adults were individually inserted inside the middle tube of apparatus. The EXOV insects skipped the 24h starvation phase, as they had been fasting since the previous autumn. Observations were performed after 1h, 6h and 24h, recording the position of the individuals (A or B). A choice was established if an individual was inside the box, or in the end of the tube towards the box. The outcome was considered as “no-choice” if the individual remained in the central part of the tube (10 cm length), in proximity of the (plugged) hole used as entrance. Individuals that had not made a choice at the end of the experiment were discarded. After each trial, every part of each apparatus was thoroughly washed with ethanol and tap water, to remove any trace of food, EOs or semiochemicals emitted by the tested insects. The use of detergents was avoided in order to limit any disturbance related to chemical substances. Each combination of EO, concentration and physiological phase was tested independently 10 times (using 5 males and 5 females), for a total of 720 individuals tested. Data are available in a specific public repository (Maistrello 2020).

2.4 *Statistical analysis.*

To calculate the repellency, a Percentage of Repellency (PR) index was calculated using the following formula:

$$PR = ((Nc - Nt) / (Nt + Nc))*100$$

where Nt is the number of individuals in the treated section and Nc is the number of individuals in the control section. A criterion was associated with each PR to highlight the intensity of repellency/attraction, depending on its value (modified after Rinaldi et al., 2016):
PR ≥ 80: very high repellency
60 ≤ PR < 80: high repellency
40 ≤ PR < 60: medium repellency
20 ≤ PR < 40: low repellency
20 ≤ PR < -20: neutral
-20 ≤ PR < -40: low attraction
-40 ≤ PR < -60: medium attraction
-60 ≤ PR < -80: high attraction
PR ≥ 80: very high attraction.

For statistical analyses, the software R 3.6.3 GUI 1.65 Snow Leopard build (6784) (R Core Team 2019) was used. A generalized linear model (glm) with a binomial error structure (logit link function) was used to compare the mean repellency (expressed as the ratio between the number of repelled individuals and the number of individuals that made a choice). An analysis of deviance of the fitted glm model with Wald statistics χ^2 was performed to compare the repellency: i) among the three time checks (1h, 6h, 24h); ii) between the two sexes, and iii) among the four oils. Analyses were performed separately, without considering the other variables, and in case of significant differences, a t-test was used to compare the effect of time checks, sex, oils.

A t-test was used to compare mean repellency among the concentrations for each physiological phase (EXOV, SUMM, ENOV) and then to compare mean repellency among the three physiological phases for each concentration and each oil.

3. Results

The PR values (Table 1) showed that the highest level of repellency after 24h was observed principally in SUMM with clove at 12% and 3%, vetiver at 25% and turmeric at all concentration except 1%, and in ENOV with ginger at 50% and turmeric at 12%. All the oils showed low repellency or were neutral at 1% in any physiological phase.

Ginger was repellent at concentrations equal or higher than 12%, especially in SUMM and ENOV, and highly repellent to EXOV at 3%. Clove was always repellent especially in SUMM and ENOV at all concentrations equal or higher than 3%.

Vetiver was repellent in SUMM at all concentrations equal or higher than 3%, but it showed high attraction in EXOV at 25%. Turmeric showed very high repellency in SUMM at all concentrations equal or higher than 3%.

According to the statistical analyses, no significant differences emerged comparing the three time checks ($\chi^2 = 2.99$; df = 2; $p = 0.22$) and between males and females ($\chi^2 = 0.93$; df = 1;

p = 0.33). On the contrary, a significant difference was detected comparing the four oils (χ^2 = 17.14; df = 3; $p < 0.001$), indicating that clove and turmeric are the most repellent EOs (Figure 2).

By comparing the effect of the different concentrations in the different physiological-behavioural phases (Figure 3), it emerged that: i) individuals exiting overwintering showed low repellence (less than 0.7) to all concentrations, and 25% and 1% were the least repellent; ii) in summer, all concentrations equal or higher than 3% show a high repellency (higher than 0.75) with almost no differences among each other; iii) individuals entering overwintering exhibited a wider range of responses, with repellence decreasing as the concentration of the EOs decreases, with 50% being the most effective, and 3% and 1% the least repellent concentrations.

Comparisons among the different physiological-behavioural phases for each EO concentration showed that, in most cases, there were significant differences, especially with concentrations equal or higher than 3% (Figure 3).

Considering ginger, significantly higher repellency was recorded in ENOV at 50% and in SUMM and ENOV at 25%; EXOV and ENOV individuals were significantly less repelled than other phases at 25% and 3%, respectively; EXOV were the least repelled individuals, especially at 50%, 25% and 1%.

For clove, significant differences among the phases were detected only at 12% and 3%, where SUMM individuals were significantly more repelled at 3% (with maximum repellency), and EXOV were significantly less repelled at 12%.

Vetiver was significantly more repellent in SUMM individuals at 25%, 6% and 3% whereas the lowest repellency was recorded in EXOV individuals at 25% and 12%.

Turmeric elicited significantly higher repellency in both SUMM and ENOV individuals at 50% and 12%, and in SUMM at 25%, 6% and 3%, whereas EXOV individuals were usually less repelled.

4. Discussion

In the present study, we evaluated the repellent activity of four EOs (ginger, clove, vetiver and turmeric) on *H. halys* adults in different physiological-behavioural phases, exposing them for a total of 24 hours to attractive stimuli from fresh food and, in the case of the adults entering overwintering, also to adult males emitting the aggregation pheromone. Results showed that all the tested EOs elicited a repellent response at concentrations equal or higher than 3%, and that repellency did not differ in the time checks along the 24 hours. This indicates that, at all but the lowest concentration, all the tested compounds were bioactive for at least one day, demonstrating a potential in the protection of susceptible target crops as well as to prevent the entrance of overwintering bugs in buildings in autumn. In every trial of our study, males and females were repelled in the same way by the EOs, suggesting the two sexes respond in the same way to stimuli in feeding and aggregation behaviours. Similarly, a coupled gas chromatography-electroantennographic detection (GC-EAD) showed no differences in the olfactory responses of females and males exposed to various essential oils and some of their individual volatile compounds (Zhang et al. 2014). In addition, absence of sexual dimorphism in antennal structure was confirmed by Ibrahim et al. (2019), showing the same morphological features and abundance of sensilla in the two sexes.

According to our results, turmeric and clove were the most repellent EOs at the majority of the tested concentrations, whereas ginger and vetiver showed on average a medium-low repellency. Remarkably, significant differences emerged among the physiological-behavioural phases, with the individuals collected during summer showing a greater repellency to many of the tested concentrations, and individuals exiting overwintering being overall the least susceptible to these substances. These results could be a consequence of the higher temperature during summer that might intensify the release rate of volatile compounds, thus increasing the repellent response. Besides, the increased response to the EOs observed in summer could also be related to the fact that, during the full activity period, these polyphagous bugs have plenty of choices to feed upon and are likely more sensitive to semiochemicals, in order to be able to choose the most suitable feeding substrates. On the contrary, the bugs that exit overwintering after 5-7 months of starvation, being in strong need of water and nutrients in a season with a much lower availability of useful food items, are apparently less susceptible to compounds potentially able to deter feeding. The individuals that enter overwintering start to lose interest in food, but are apparently still sensitive to aggregation pheromones, and their response to the EOs was somewhat intermediate when compared to that of the other phases, with ginger and turmeric apparently quite effective in contrasting the attractive stimuli. A change in the response to volatiles according to the physiological phase was also observed in other phytophagous insects, namely the psyllids. Females of *Cacopsylla pruni* (Scopoli, 1763) (Hemiptera: Psyllidae), which migrate between *Prunus* spp. for reproduction and conifers for overwintering, showed a different susceptibility to different volatile blends while being in their reproduction phase or in the overwintering one (Gallinger et al. 2020). Our experiment did not allow us to separate the effect of the increased temperature on the volatiles from the possible specific changes occurring in the physiology and behaviour of the insects during the seasons. An experiment should be appropriately designed to better understand these responses. In any case, taking into account the different responses to repellents in relation to the physiological-behavioural phase is a crucial point for the development of tools that contain these substances.

Clove EO elicits a medium-high repellency for at least 24 hours (2 “Very high” PR and 4 “High” PR, with a total PR of 807). Thus, our findings confirm the results obtained on *H. halys* by Zhang et al. (2014), where clove was among the six EOs that totally blocked the entrance of the individuals inside traps baited with aggregation pheromones. Clove EO proved to be repellent also for mosquitoes, such as *Aedes aegypti* (Linnaeus, 1762) (Diptera: Culicidae) for 2h and *Culex quinquefasciatus* (Say, 1823) (Diptera: Culicidae) and *Anopheles dirus* (Peyton & Harrison, 1979) (Diptera: Culicidae) from 2 to 4h (Trongtokit et al. 2005), as well as for the stored product pest *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) at very low concentrations (from 0.2% to 1% in 1 mL acetone) within 10 min (Abo-el-Saad et al. 2011).

Turmeric EO also showed a quite strong percentage of repellency in our study (6 “Very high” PR and 1 “High” PR, with a total PR of 769). This EO is already used in India and Pakistan as an insect repellent against foodstuffs pests, and is proven also to interfere with the reproduction and development of *T. castaneum* (Jilani et al. 1988) and *Rhyzopertha dominica* (Lesne, 1896) (Coleoptera: Bostrichidae) (Jilani & Saxena 1990). This EO was able to protect cardamom sprouts treated at concentrations of 0.5 and 1%, from *Pentalonia nigronervosa* (Coquerel, 1859) (Rhynchota: Aphididae) (Saju et al. 1998) and is repellent to mosquitoes, showing greater efficacy when combined with vanillin (Tawatsin et al. 2001).

In our study ginger EO was a mild repellent to all *H. halys* phases (1 “Very high” PR and 4 “High” PR, with a total PR of 554) whereas, in a vertical olfactometer, to *Bemisia argentifolii* (Gennadius, 1889) (Hemiptera: Aleyrodidae) this EO was highly repellent at concentrations of 0.5% - 1% when dipped on a tomato leaf (Zhang et al. 2004). Ginger isolated as a vacuum distillate is capable of repelling *Sitophilus zeamais* (Motschulsky, 1855) (Coleoptera: Curculionidae) in laboratory tests (Ukeh et al. 2009), while its oil repels *Myzus persicae* (Sulzer, 1776) (Rhynchota: Aphididae) at a dose of 10 µl but also at the lowest dose of 0.1 µl, and in experiments using an olfactometer, also the aphids *Aphis craccivora* (CLKoch, 1854) (Hemiptera: Aphididae), *Macrosiphum euphorbiae* (Thomas, 1878) (Hemiptera: Aphididae) and *Capitophorus formosartemisiae* (Takahashi, 1921) (Hemiptera: Aphididae). Ginger EO is less effective than rosemary, but its use decreased the spread of potato virus Y necrotic strain (PVYT) (of which *M. persicae* is a vector) in a tobacco field in the same way (Hori 1999).

Vetiver oil was overall the least effective repellent in our trials (1 "Very high" PR and 3 "High" PR, with a total PR of 499) but it was unique in determining strikingly different behavioural responses according to the physiological phase. In particular, at 25%, although not all individuals made a clear choice, it elicited a high repellency in the summer individuals, a low repellency in the ones entering overwintering and a high attraction in those exiting overwintering. This ambiguous response might be due to the specific blend of components of vetiver oil, and on their different volatility at different temperatures. It is probable that some components are able to trigger attraction in the beginning of spring, which might be functional to find specific host plants after the long winter starvation. On the other side, strong repellency during summer might be activated by other components, which are likely perceived as dominant, due to the higher temperatures. This point definitely warrants a specific study using GC-EAD investigations. A GC-EAD analysis identified 20 active compounds from 8 EOs, that are repellent to *H. halys* (Zhang et al. 2014). This work did not focus on the characterization of the four tested EOs, but their composition could play an important role in their perception and, consequently, in their repellency activity to *H. halys*. Previous works identified the profile of these EOs (Raina et al. 2001; Adams et al. 2004; Asghari et al. 2010; Ravi et al. 2013) and further studies might clarify their mechanism of action on the response of *H. halys*.

Vetiver EO is known to repel several species of mosquitoes and a higher efficacy, always against mosquitoes, is proven when combined with other oils such as citronella and hairy basil oil (Nuchuchua et al. 2009). Vetiver EO was effective at low concentrations against *Coptotermes formosanus* (Shiraki, 1909) (Blattodea: Rhinotermitidae), both repelling it and negatively affecting its behaviour, completely inhibiting tunnelling activity when mixed with sand at 0.0025% (Zhu et al. 2001a). Among the components of vetiver oil there is nootkatone, which is able to kill the fire ants *Solenopsis invicta* (Buren, 1972) (Hymenoptera: Formicidae), reducing invasion, and it can itself repel termites and cockroaches (Zhu et al. 2001b; Henderson et al. 2005).

In our study the EOs were considered individually. A combination of them could increase their effectiveness, as suggested in the study by Zhang et al. (2014). They demonstrated that a mixture of three EOs showed a nearly complete repellency to *H. halys*. The use of a mixture might be advantageous due to a synergic (or additive) effect of volatile compounds. The possibility to create formulations, dispensers or spray products based on repellent EOs offers potential for their application in numerous scenarios; from open fields to post-harvesting storages, from gardens and houses to other buildings. This variability of application makes EOs a potentially effective tool to protect both agricultural products and public or private buildings. In particular, repellents based on essential oils could be even more effective in controlling *H. halys* when combined with specific attractants, such as the aggregation pheromone, to drive the pest away from the field, implementing a push-pull strategy (Zhang et al. 2014).

The responses of *H. halys* to the tested EOs were the same after 1, 6 and 24 hours indicating the repellent effect was immediate and persistent for at least one day. This short-range behavioural assay was useful to verify the potential of EOs and identify the best candidates. However, EOs are very volatile, most effective just after their application and losing their activity over time. In the view to develop specific applications, field trials with longer exposure of the insects to these EOs are therefore needed to understand the insect behaviour outdoors and to estimate the life-time of the EOs.

In conclusion, the use of EOs as insect repellents might represent a sustainable and safe tool to be integrated in strategies to protect fruits and vegetables in the field as well as to prevent entrance of dwelling pests inside buildings. Indeed, EOs have a low impact on non-target species, are biodegradable with low accumulation in the environment, and act with various mechanisms of action. Our findings show the possibility to use turmeric, clove, and eventually also ginger and vetiver EOs in strategies to prevent/reduce the damages caused by *H. halys* to agricultural products and prevent the overwintering adults from sneaking inside buildings. A combination of EOs and a fixative mixture could improve their activity and persistence over time. An in-depth investigation on the response of *H. halys* to the volatiles could improve the formulation and combination of EOs for the implementation of specific tools useful for improve the sustainability in the management of this challenging invasive pest.

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Data Availability Statement

Data used for this work are available in a public repository (Knowledge Network for Biocomplexity), identified with doi:10.5063/W957K1

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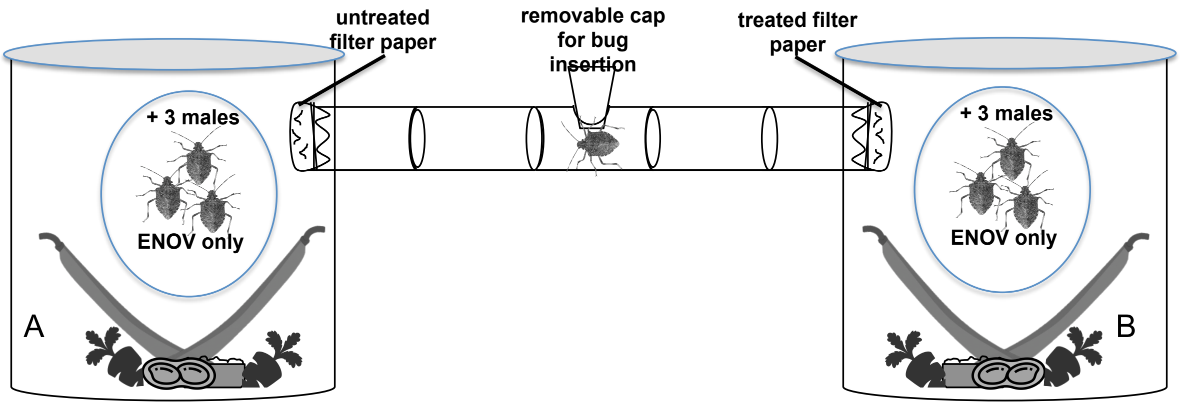

Fig 1. Schematic representation of the experimental apparatus. Both plastic boxes contained fresh organic vegetables, peanuts and a wet cotton wick. Three adult males were added in both boxes only for the trials with Individuals entering overwintering (ENOV). Both filter papers had small cuts that allowed eventual entrance of the tested bug inside either one of the boxes

Phase	Concentration	Oil	PR	Repellence level	No choice (%)
EXOV	50%	Ginger	33	Low	0
		Clove	53	Medium	0
		Vetiver	53	Medium	0
		Turmeric	20	Low	0
	25%	Ginger	-3	Neutral	4
		Clove	20	Low	0
		Vetiver	-76	High attraction	14
		Turmeric	26	Low	0
	12%	Ginger	46	Medium	14
		Clove	10	Neutral	4
		Vetiver	-17	Neutral	4
		Turmeric	33	Low	10
	6%	Ginger	14	Neutral	7
		Clove	40	Medium	0
		Vetiver	4	Neutral	17
		Turmeric	0	Neutral	34
	3%	Ginger	60	High	17
		Clove	0	Neutral	0
		Vetiver	18	Neutral	10
		Turmeric	24	Low	4
	1%	Ginger	-21	Low attraction	7
		Clove	30	Low	14
		Vetiver	-6	Neutral	17
		Turmeric	16	Neutral	20
SUMM	50%	Ginger	53	Medium	0
		Clove	73	High	0
		Vetiver	60	High	0
		Turmeric	86	Very high	0
	25%	Ginger	61	High	14
		Clove	41	Medium	20
		Vetiver	90	Very high	27
		Turmeric	83	Very high	0
	12%	Ginger	60	High	0
		Clove	80	Very high	0
		Vetiver	40	Medium	0
		Turmeric	93	Very high	0
	6%	Ginger	6	Neutral	0
		Clove	33	Low	0
		Vetiver	73	High	0
		Turmeric	86	Very high	0
	3%	Ginger	26	Low	0
		Clove	100	Very high	0
		Vetiver	71	High	7
		Turmeric	80	Very high	0
	1%	Ginger	-3	Neutral	4
		Clove	20	Low	0
		Vetiver	24	Low	4
		Turmeric	6	Neutral	0

ENOV	50%	Ginger	88	Very high	0
		Clove	73	High	0
		Vetiver	40	Medium	0
		Turmeric	66	High	0
	25%	Ginger	72	High	27
		Clove	53	Medium	0
		Vetiver	38	Low	14
		Turmeric	18	Neutral	10
	12%	Ginger	46	Medium	0
		Clove	66	High	0
		Vetiver	40	Medium	10
		Turmeric	86	Very high	0
	6%	Ginger	-20	Neutral	0
		Clove	68	High	17
		Vetiver	18	Neutral	10
		Turmeric	33	Low	0
	3%	Ginger	-3	Neutral	10
		Clove	26	Low	0
		Vetiver	17	Neutral	4
		Turmeric	23	Low	14
	1%	Ginger	39	Low	24
		Clove	21	Low	7
		Vetiver	12	Neutral	0
		Turmeric	-10	Neutral	4

Tab 1. Percentage of Repellency (PR), repellency level and percentage of individuals that did not make a choice for each combination of physiological-behavioural phase, concentration and oil after 24h. Phases: EXOV= exit from overwintering, SUMM= summer activity; ENOV= enter overwintering. A colour code was associated with different levels of repellency (dark orange = very high; light orange = high; yellow = medium; green = low; grey = neutral; light blue = low attraction; darker blue = high attraction)

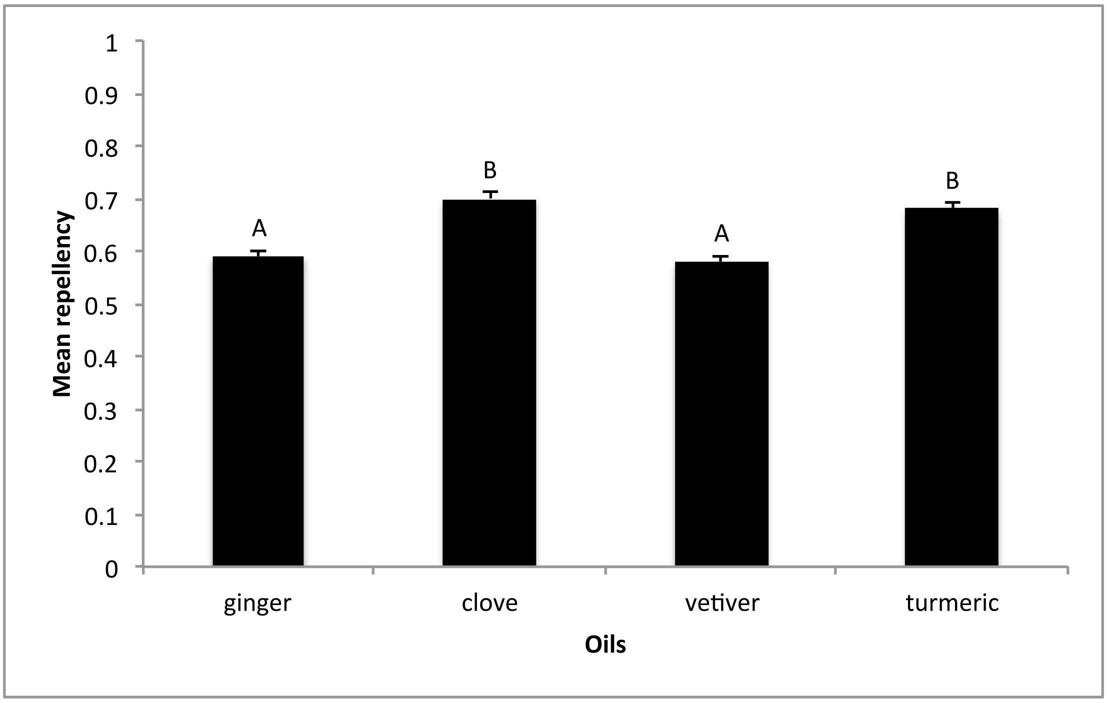

Fig 2. Mean repellency (+SE), expressed as the ratio between the number of repelled individuals and the number of individuals that made a choice, shown for each essential oil after 24h, without considering the other variables. Different letters indicate statistically significant differences between the oils, based on t-test ($p < 0.05$)

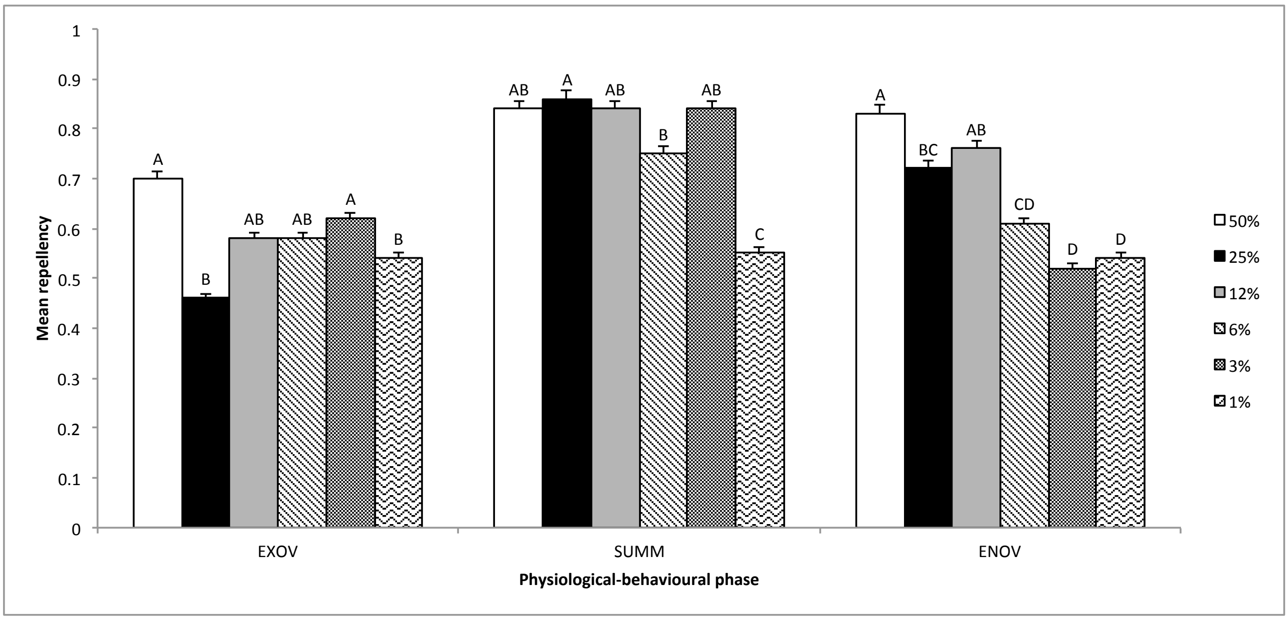

Fig 3. Mean repellency (+SE), expressed as the ratio between the number of repelled individuals and the number of individuals that made a choice, shown for each concentration in each physiological phase after 24h, without considering the other variables. Different letters indicate statistically significant differences between the concentrations in each physiological phase, based on t-test ($p < 0.05$)

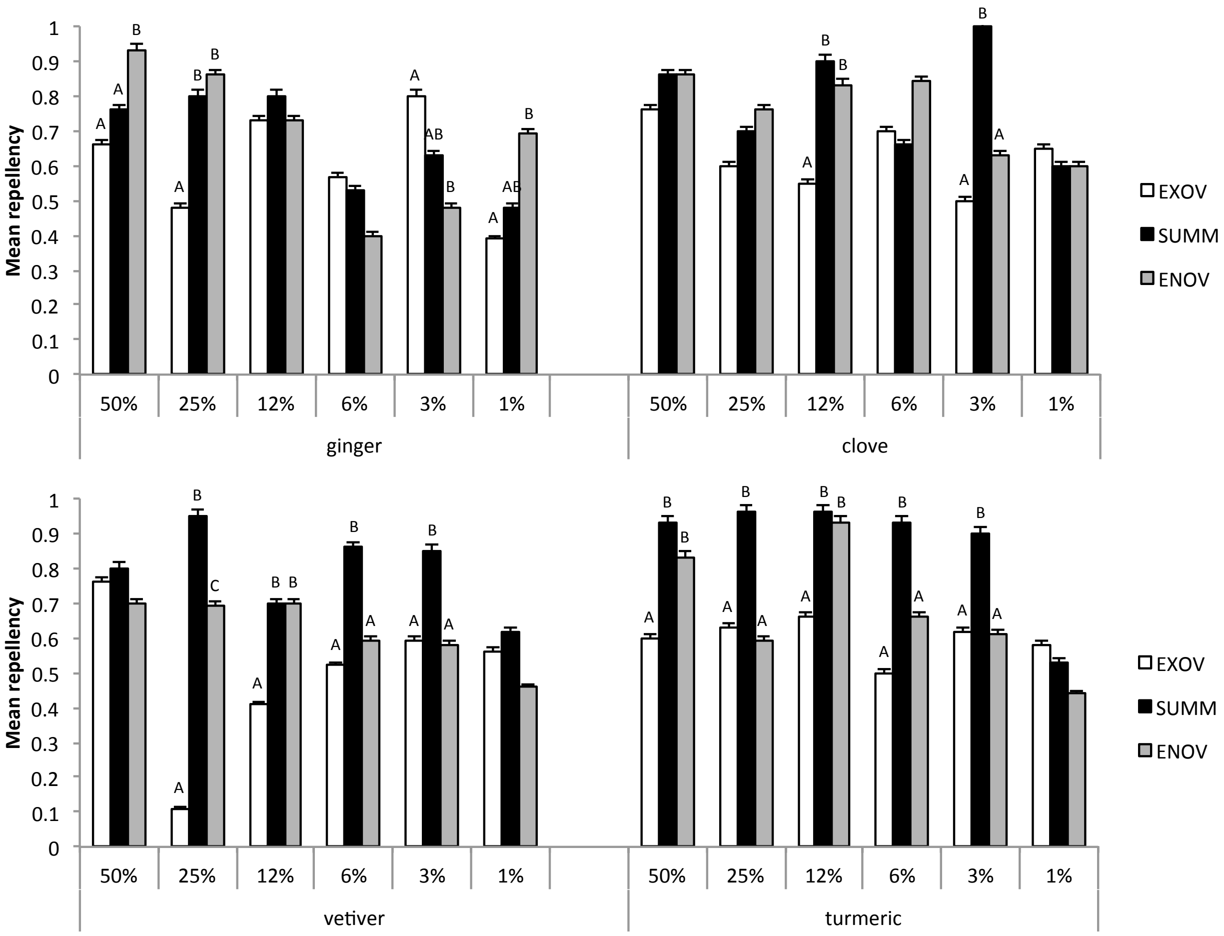

Fig 4. Mean repellency (+SE), expressed as the ratio between the number of repelled individuals and the number of individuals that made a choice, shown for each combination of essential oil, concentration and physiological phase after 24h. For each concentration of each EO, different letters indicate statistically significant differences between the physiological-behavioural phases, based on t-test ($p < 0.05$)

CHAPTER 7

CONCLUDING REMARKS

The build-up of the population of an invasive alien species that causes severe damage to agriculture, triggers a response by farmers, which usually consists in an increased use of pesticides as an ultimate, desperate attempt to save the crop, and the income. The higher the host plant range, climatic adaptability, reproductive potential and intrinsic mobility of the invasive pest, the higher the damage and the more difficult is the management. Besides, if the pest has behavioural features that make it a stowaway on inanimate objects during its overwintering period, its spread inside an invaded country and among continents is virtually unstoppable, since it is highly facilitated by trade and other human activities.

This thesis focused on an invasive agricultural pest that has all these features, the brown marmorated stink bug *Halyomorpha halys*. It was first officially detected in Italy in 2012 in the Province of Modena, Emilia Romagna (northern Italy) (Maistrello et al. 2016), one of the most important for fruit production in Europe (EUROSTAT 2017), and it was quickly recognized as a key pest of fruit orchards when damage to pear crops reached 60% in 2015 (Maistrello et al. 2017). Its spread in the country was extremely fast (Maistrello et al. 2018) and in 2019 the estimated losses to the fruit production in northern Italy were € 588 million (CSO Italy 2020). According to the Italian plant protection officers, *H. halys* is by far one of the most important phytosanitary emergency of the country in the recent years (Maistrello, pers. comm.), also because it radically switched backward the orchard management, towards an impressive increase in the use of broad spectrum insecticides. For example, according to the IPM guidelines of Emilia Romagna region, the total number of authorized insecticide treatments on pear crops was 5/year (4 organophosphates and 1 neonicotinoid) in 2014 and became 10/year in 2019 (5 organophosphates, 3 neonicotinoids and 2 pyrethroids) (Regione Emilia Romagna 2019).

In order to restore and improve overall sustainability, a better knowledge of the invasive pest and its ecology in the invaded areas is necessary, together with the implementation of biological control and alternative techniques, especially those based on prevention and behavioural manipulation.

A field survey was performed in urban parks and hedgerows surrounding fruit orchards in Emilia Romagna over three years (2014-2016), where fresh sentinel egg masses exposed for a few days were later checked for evidence of predation and parasitoid emergence (Costi et al. 2019). According to this survey, about 6% of the sentinel eggs showed signs of predation by either chewing or sucking mouthparts, indicating the existence of generalist predators able to exploit the eggs of the invasive stink bugs as food source. However, this study could not provide any indication on the identity of the predatory species, and it was specifically limited to potential egg predators.

Results in Chapter 2 and 3 were obtained by means of "no choice" laboratory bioassays, where potential predators, chosen among solitary and social insect species, underwent a period of starvation and their predatory ability was tested during 48 hours in a tridimensional apparatus by placing them in turn with either fresh *H. halys* eggmasses or groups of young nymphs (N1 and N2). From the trials shown in Chapter 2, it emerged that: i) the eggs are preyed upon by three predators, namely the coccinellid *Adalia bipunctata*, the orthopteran *Eupholidoptera chabrieri* and the reduviid *N. goedelii*; ii) the first instar nymphs are eaten by five species: the neuropteran *Chrysoperla carnea* (larvae), *E. chabrieri*, the reduviids *Rhynocoris iracundus* and *N. goedelii*, and the nabid *H. mirmicoides*; and that iii) the second instar nymphs are predated by *E. chabrieri* and *R. iracundus*. *R. iracundus* proved also to be able to significantly prey on the adults, making it one of the best candidates for a possible biological control program, taking into account that *E. chabrieri* is an omnivorous species, which could also be harmful to crops. Besides, it must be considered that the eggmasses used in the bioassay were all freshly laid, and possibly this might have negatively affected the predation attempts by the tested specimens.

Results presented in Chapter 3 were obtained testing a social insect, the ant *Lasius niger*, in an apparatus similar to the one used with solitary predators, properly adjusted to host a small colony fraction of the ant (less than 200 individuals). It emerged that during the 48 hours of the trial, this ant species predated only on the second and third instar nymphs of the invasive stink bug, which are the smaller and more mobile ones. However, since only a small portion of the colonies was used in the experiments, it is possible that using whole colonies and/or longer times of exposure, the results might be different.

The results shown in Chapter 4 allowed to obtain an overview on how the invaded ecosystem is responding to the introduction of the invasive pest, identifying arthropod species that are able to exploit *H. halys* as a food source, using the innovative approach of the gut content analysis. Real-time PCR with specific primers was used to identify *H. halys* DNA within the gut content of arthropod species collected in the field during two summers, sharing the same habitat of *H. halys*. This work, which is the first one to use this approach on arthropod predators of *H. halys*, shows that 46 samples out of 190 were positive to *H. halys* DNA, and allowed to identify among the predators four species of Orthoptera (*Arachnocephalus vestitus*, *Phaneroptera falcata*, *Tylopsis liliifolia* and *Yersinella raymondi*), three of Coleoptera Coccinellidae (*Harmonia axyridis*, *Hippodamia variegata*, *Oenopia conglobata*), two of Hemiptera (the Reduviidae *Nagusta Goedelii* and the Nabidae *Himacerus mirmicoides*), one of Dermaptera (*Forficula auricularia*), as well as two species of Opiliones (*Mitopus morio* and *Opilio canestrinii*) and four genera of Araneae (*Anyphaena*, *Araneus*, *Philodromus* and *Calositticus*). These results partially confirm results of Chapter 2 with *N. goedelii* and *H. mirmicoides* among the positive predators, showing that these predators are capable of preying on *H. halys* also in the wild. Most of the sampled predators, such as the ladybugs, the dermapteran, the nabid, the opilionids and spiders (that were small in size) probably fed on the eggs and/or on the first juvenile instars, while the orthopterans could have also fed on the larger stages, considering their bigger and stronger mouthparts. The number of analysed predators was necessarily limited by the degradability of DNA (detectable only before the digestion by the predator is completed) and by the sampling time and technique, performed only in the morning and catching the arthropods occurring no higher than 3 m above the ground. It is likely that a higher number of predators could be discovered with a bigger sampling effort, to catch even the ones with nocturnal habits and/or those occupying the top of the trees. Furthermore, this type of genetic analysis did not allow to understand the quantity of prey eaten and/or at what stage it was preyed, suggesting the need to integrate with other techniques to obtain this type of information, in the view to develop a conservation biological control program able to take full advantage of the predatory abilities of the different species.

Overall, the results from the laboratory tests and the gut content analysis contributed to the identification of species of generalist predators able to exploit the invasive *H. halys* as prey in the invaded areas in Emilia Romagna, and are thus representative of southern Europe, a territory where this stink bug is currently spreading very fast, threatening many agricultural productions. Maintaining and protecting these predators in the ecosystems could therefore help limit the population and the damage of the invasive stink bug. In particular, ants are often considered as pests, due to the association of some species with aphids, but both *L. niger* and *Crematogaster scutellaris* (Castracani et al. 2017) have also proved to be useful as biocontrol agents against this bug. The increase in the number of these generalist predators, which could be possible under the development of a conservation biological control program, besides being potentially useful to reduce the invasive stink bug population, would probably not alter negatively the ecosystem. In fact, these predatory species are all native to southern Europe (with the only exception of the coccinellid *H. axyridis*) as well as their own natural antagonists, which co-evolved with them, and could therefore be able to regulate them, preventing their uncontrolled expansion.

The results presented in Chapter 5, obtained during the abroad experience in Ontario, Canada, showed that the jumping spider *Phidippus audax* is attracted to the aggregation pheromone of *H. halys* in fall, probably because it uses it as a track to identify a potential prey before the winter season, or to overwinter together with a potential prey to feed on when spring begins. This jumping spider is an effective generalist predator very common and abundant in North America, and laboratory tests showed also its predatory ability on the eggs of *H. halys* (Poley et al. 2018). Currently, *H. halys* is present in four Canadian provinces, where it is considered a nuisance rather than a crop pest (Stopbmsb 2020). However, precisely because it is not yet a problem, it would be useful to implement containment and prevention measures to limit its expansion as much as possible. *P. audax* is therefore a potential good candidate for a conservation biological control project aimed at reducing the invasive stink bug population, especially towards the end of the summer.

Among the IPM strategies, the most sustainable ones are the behaviour-based ones, since usually the reduction of the population of the pest below the economic threshold level is obtained just by manipulating the pest behaviour, without the use of toxic chemicals, or limiting their use to the minimum in space and time. Particularly interesting is the push-pull strategy, where attractive and repellent cues are used simultaneously, with a synergistic effect, to drive the target pest away from a specific objective/area to be protected, and attracting it where it is not noxious (Cook et al. 2007). In the case of *H. halys*, the resources to protect are the crops during summer, as well as the houses and warehouses during early autumn, since these bugs use man-made structures as overwintering sites in urbanized areas, becoming a dwelling nuisance and a threat when exporting goods. With *H. halys*, the aggregation pheromone could be used as an attractant, capable of attracting both sexes and all immature instars. Essential oils extracted from plants could be used as repellent, eco-friendly cues, and many of them are already being used to repel various insects, including mosquitoes and stored product pests (Trongtokit et al. 2005; Jilani et al. 1988). According to the results presented in Chapter 6, the essential oils of clove and turmeric are the most effective in repelling *H. halys* adults within 24h. The substances were tested in different physiological-behavioural phases to verify in which periods the pest would be more sensitive to their use. It emerged that *H. halys* was more sensitive during summer, when the stink bugs are actively feeding on the crops, and in early autumn, when the bugs begin to aggregate to enter overwintering. Essential oils, in particular those of clove and turmeric, are therefore promising candidates to be used in repellent formulations (with fixatives to ensure effectiveness over time) to protect the crops in the summer period, as well as to prevent the entrance of overwintering bugs in buildings in autumn. The combination with aggregation pheromones would likely synergize the effects in appropriately developed push-pull strategies.

Summarizing, the results presented in this thesis are the outcome of different approaches used to identify the species and the role of some native predators, both solitary and social, that are able to exploit *H. halys* as a food source in the invaded ecosystem. These results are especially useful for conservation biological control programs. On the other side, this thesis provided also the identification of repellent substances that could be exploited in push-pull strategies for the prevention of damage to crops as well as entrances in buildings. Overall, the presented findings provide useful contributions for the development of specific integrated strategies towards an increased sustainability in the management of the invasive *H. halys*.

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HOST INSTITUTIONS

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. Department of Biology, The University of Western Ontario, London, On N6A 5B7, Canada, during the period: May 27th2019 – November 25th2019.