Searching for new predators of the invasive *Halyomorpha halys*: the role of the black garden ant *Lasius niger*

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Abstract

In recent years, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae, Cappaeini) has become an invasive pest in North America and Europe, where it 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* (L.) (Hymenoptera: 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* under laboratory conditions. The results indicate that *L. niger* significantly reduces the survival of the second and third nymphal instars by 56 and 58%, respectively, but it is unable to reduce the egg hatching and the survival of the first, fourth, and fifth instars. Our preliminary results obtained in laboratory conditions suggest a possible role of the ant *L. niger* in controlling *H. halys* invasion mainly acting on the smaller and more mobile nymphal stages. 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 is discussed.

Introduction

The brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae), is native to China, Japan, Taiwan, and South Korea, and from the late 1990s it has become an invasive pest in Europe and North America (Leskey & Nielsen, 2018). It has been present in North America since 1996 (Hoebeke & Carter, 2003) and by the end of 2020 it has been reported in 46 states of the USA and in four provinces of Canada (StopBMSB, 2020). Recently, it was also detected 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 to be established throughout 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 nature of the overwintering adults, which hide inside structures and packaging of any kind and are carried all over with trade and movements 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 spatial model based on its spatiotemporal dynamics suggested its possible first entry in the country was as early as 2009 (Maistrello et al., 2018). Currently, *H. halys* is established throughout the Italian peninsula and Corsica (Maistrello et al., 2018). Genetic analysis showed a high biodiversity of haplotypes, indicating that the Italian populations are the result of multiple invasions from native and invaded countries (Cesari et al., 2018), thus confirming the hitchhiking abilities of this species.

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

Currently, its management relies mainly on the use of broad-spectrum insecticides; however, these compounds also kill non-target insects, including potential pest antagonists, and disrupt the most innovative integrated pest management (IPM) strategies, causing both economic and ecological damage (Leskey et al., 2012; Maistrello et al., 2017). Factors that make management of this pest especially difficult include specific biological, behavioural, and ecological traits that include: (1) a wide range of host plants (Rice et al., 2014), such as many fruits, vegetable, and row crops, as well as many ornamental and spontaneous shrubs and trees that are usually found on the wooded areas surrounding the crops; (2) the great mobility of both adults (Lee & Leskey, 2015; Wiman et al., 2015) and nymphs (Lee et al., 2014); (3) the high rate of population growth (Costi et al., 2017); and (4) the absence of specific natural antagonists in invaded regions (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 preyed on at quite low percentages, and eggs showed greater damage due to predators with chewing mouthparts rather than predators with piercing-sucking mouthparts (Cornelius et al., 2016; Ogburn et al., 2016; Shanovich et al., 2020). These studies have demonstrated the existence of potential predators but often 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 prey on *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 instars are preyed on by Nabidae and by the pentatomid *Podisus maculiventris* (Say) (Pote & Nielsen, 2017). The pentatomid *Euthyrhynchus floridanus* (L.) also showed potential as a predator of all developmental stages, including adults (Arellano et al., 2019).

In Europe, a 3-year field survey conducted by Costi et al. (2019) in northern Italy with fresh sentinel egg masses showed low rates of parasitism (<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 preyed on by *Adalia bipunctata* (L.) (Coccinellidae), *Eupholidoptera chabrieri* (Charpentier) (Tettigoniidae), and *Nagusta goedelii* (Kolenati) (Reduviidae); first instars were preyed on by *E. chabrieri*, *Chrysoperla carnea* (Stephens) larvae (Chrysopidae), *Himacerus mirmicoides* (OG Costa) (Nabidae), *N. goedelii*, and *Rhynocoris iracundus* (Poda) (Reduviidae), and second instars were preyed on by *E. chabrieri* and *R. iracundus*. The latter species also preyed on *H. halys* adults (Bulgarini et al., 2021). From this study, the tettigoniid *E. chabrieri* and the reduviid *R. iracundus* appeared to be the most effective predators, each preying on at least three instars of *H. halys* (Bulgarini et al., 2021).

Ants are globally diverse and abundant, comprising a large fraction of animal biomass in most terrestrial communities, and are key contributors to a range of ecosystem functions. They generally form very populous colonies, large and often permanent foraging trails, and they defend extended territories from intruders (Hölldobler & Wilson, 1990; Grasso et al., 1998, 1999, 2005). Owing to their abundance, stable populations, interactions with both biotic and abiotic factors, and a variety of food habits, ants are involved in the dynamics of multitrophic interactions as well as in improvement of soil and the nutrient cycle (Lach et al., 2010; Castracani et al., 2015; Gibb et al., 2017). Among the various trophic roles, they are also predators of pest species (Cerdá & Dejean, 2011). This may represent an important service for plants hosting ants, for those that are more or less routinely 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) showed that this ant is able to significantly prey on all the 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: (1) they respond quickly to changes in pest density (Hölldobler & Wilson, 1990; Maňák et al., 2013); (2) they can be abundant even when prey is scarce; (3) they are able to store food and continue preying even if it is not immediately necessary (Hölldobler & Wilson, 1990); (4) in cases of prey too large to kill they can drive them away with the use of chemical repellents (Way & Khoo, 1992; Goheen & Palmer, 2010); and (5) they occupy a wide range of habitats and trophic levels in many terrestrial ecosystems (Hölldobler & Wilson, 2009;

Castracani et al., 2010; Lucky et al., 2014). These characteristics render some mechanisms of defense by prey ineffective, 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 an increase in the number of pests. This also leads to the protection of plants from low-density pests (e.g., Way & Khoo, 1992; Giannetti et al., 2019; Schifani et al., 2020).

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 instars of *H. halys* as prey. On the basis of a previous field survey carried out in pear orchards in northern Italy, it emerged that *Lasius niger* (L.) (Hymenoptera: Formicidae), a species known to be resistant to anthropogenic impacts such as mowing, fertilization, and mechanical stress of the soil (Seifert, 2018), was the most abundant ant species both on trees and on the ground (Schifani et al., 2020). *Lasius niger* is an aggressive and territorial species, a generalist omnivore that tends to feed on any underground, epigeal, or arboreal nutrient source, including other invertebrates, when they are available and manageable (Seifert, 1992, 2018). The aim of the present study was to collect information on the potential of the autochthonous ant *L. niger* as a control agent of *H. halys* and evaluate under controlled conditions the ant's predatory effectiveness on eggs and all five instars of this invasive pest.

Materials and methods

Prey rearing

Adults of *H. halys* were reared in mesh cages (30 × 30 × 30 cm) inside climatic chambers at 26 °C, 60% r.h., and L16:D8 photoperiod. They were fed on organic carrots (*Daucus carota* L.), tomatoes (*Solanum lycopersicum* L.), green beans (*Phaseolus vulgaris* L.), and raw peanuts (*Arachis hypogaea* L.). A bottle cap with water-soaked cotton was used to supply water. 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 cages were checked daily for freshly laid eggs. Eggs were transferred to Petri dishes containing a wet piece of cotton and a bean pod. Immediately after molting from the first instar, second instars were transferred to a new cage (30 × 30 × 30 cm) and reared under the same conditions as the adults.

Predator rearing

During May-September 2020, 23 colony fractions (ant nest soil, debris, and ant specimens within) of *L. niger* were collected from various nests located in orchards located in the province of Reggio Emilia (northern Italy). Ants (from 200 to 500 workers, without queens or eggs) were collected together with nest materials and placed inside plastic containers (29 × 28 × 39.5 cm), one container for each nest, which were maintained outdoors under 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, and a few *Tenebrio molitor* L. larvae (previously killed by freezing) as protein source. The soil was periodically wetted with a sprayer and the food was changed twice a week.

Experimental arenas

Two types of containers were used for the trials. The first type served as the nest, the other was used as the testing arena. The nest arena was a rectangular plastic box (10 × 13 × 21 cm) closed with a lid. The testing arena was a cylindrical plastic box (20 cm diameter, 11 cm high) without lid. The two arenas were connected through a removable transparent plastic tube (2.5 cm diameter, 7 cm long). In the center of the testing arena, a bean plant (*P. vulgaris*) with at least two well-developed leaves was placed. The plant pot (8 cm diameter) was wrapped in fine mesh fabric (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 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.

Experimental protocol

One hundred 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 to 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 24 h, the 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 24 h, the potential 'prey item' was placed on the leaves of the bean plant. Prey items consisted of a single egg mass with at least 21 eggs or six individuals of each juvenile (nymph) instar (N1, N2, N3, N4, N5) of *H. halys*. Fresh (<24 h old) egg masses laid on the substrate (either paper or bean leaf) were collected from the *H. halys* rearing cage and attached to one of the leaves of the bean plant in the testing arena using paper

clips. Once the prey item was positioned, the connection between the two arenas was re-established and the ants were given free access to the plant. Observations were performed after 1, 24, and 48 h to record the number of dead nymphs and check whether the eggs were removed or damaged (i.e., whether the egg shell was pierced/opened). Once the tests were finished, the egg masses were placed in Petri dishes inside a thermostatic chamber for 5 days, after which the hatched eggs were counted. There were 10 replicates for each type of prey item. For each experiment, a control trial was set up at the same time, consisting of a testing arena provisioned with a bean plant and prey stages as above but not connected with an ant nest arena.

Statistical analysis

All statistical analyses were performed using R v.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 after 48 h (obtained from the ratio between the number of surviving prey and the initial number of prey) between the treatment (ants present) and the respective control (ants absent) at the end of the tests. 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/absence of ants), an analysis of deviance of the fitted model with Wald χ^2 statistics was performed.

To calculate the effectiveness of predation over time, a one-way ANOVA was carried out for each type of prey item (N1-N5), comparing the survival rate of the prey after 1, 24, and 48 h, and considering only the survival rates of the treatment (ants present). For egg survival, the number of hatched eggs was used for both the control and the treatments, but as hatching necessarily took place after 48 h, the eggs were excluded from this analysis. In cases of significant differences, means were separated using Tukey's honestly significant difference (HSD) test.

Results

### Survival difference between control and treatment

During the tests, it was observed that nymphs from the second instar onwards tended to drop from the plant when threatened by the ants, and that *L. niger* attacked, killed, and in some cases dragged into their nest all stages of prey items with exception of the eggs. This dragging behaviour significantly affected the survival of the prey items in some but not all cases compared with controls. *Lasius niger* did not affect egg hatching [prey survival rate, treatment vs. control = 92 $\pm$ 2 vs. 87 $\pm$ 3% (mean $\pm$ SE); χ^2 = 3.05, d.f. = 1, P = 0.08]. For instars, *L. niger* affected the survival of N2 (treatment vs. control = 56 $\pm$ 9 vs. 91 $\pm$ 4%; χ^2 = 15.47, P<0.001) and N3 stages (59 $\pm$ 5 vs. 81 $\pm$ 7%; χ^2 = 9.46, P<0.01, both d.f. = 1). No differences were seen for N1 (75 $\pm$ 9 vs. 81 $\pm$ 5%; χ^2 = 0.74, P = 0.38), N4 (66 $\pm$ 4 vs. 81 $\pm$ 7%; χ^2 = 3.30, P = 0.06), and N5 (80 $\pm$ 5 vs. 76 $\pm$ 6%; χ^2 = 0.55, P = 0.45, all d.f. = 1) (Figure 1).

Survival difference over time

Time (interval) had an effect on survival 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
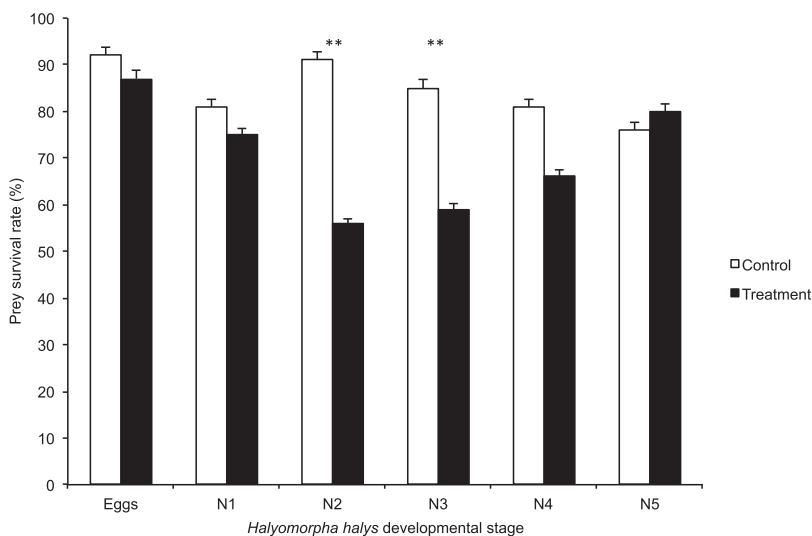
Figure 1 Mean (+ SE) survival rate (%) of eggs and juvenile stages (N1-5) of *Halyomorpha halys* after 48 h with ants present (treatment) or absent (control). Asterisks indicate a significant difference between control and treatment (glm: **0.01<P<0.001).
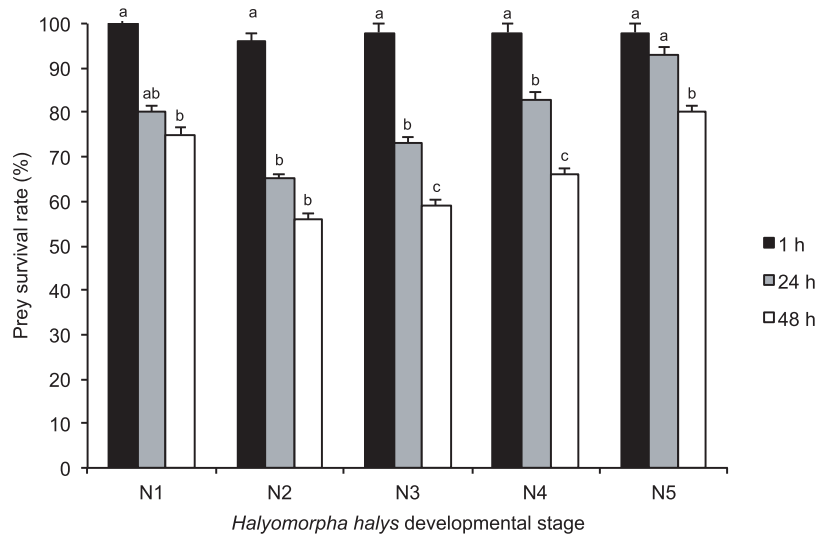
Figure 2 Mean (+ SE) survival rate (%) of *Halyomorpha halys* juvenile stages (N1-5) over three time intervals (1, 24, and 48 h). Means within a developmental stage capped with different letters are significantly different (Tukey's HSD: P<0.05).

($F_{2,27}$ = 18.90, P<0.001), and N5 ($F_{2,27}$ = 5.85, P = 0.007). Survival of each nymphal stage decreased over time (Figure 2). N3 and N4 survival rates significantly decreased for each interval of time. The N2 survival rate significantly decreased only after 24 h, whereas survival rate of the N1 and N5 was reduced only after 48 h (Figure 2).

Discussion

After 48 h of exposure to various instars of the invasive stink bug, *L. niger* was able to reduce the survival of the second and third instars by up to 56 and 59%, respectively. Very little predatory efficacy was demonstrated against eggs and the other three instars, despite attempts to attack, kill, and drag some individuals into the nest that were observed during the experiments.

In a previous study, Castracani et al. (2017) showed that the arboreal European acrobat ant *C. scutellaris* was able to prey on the instars of the pest but left the eggs untouched. In the only comparable study available from 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, in this case single individuals were tested, a condition that strongly limits the predation potential of the ants that often rely on group foraging and cooperative predation. Thus, the few studies available indicate that egg hatching of *H. halys* seems to not be affected by the presence of the ant species tested so far. This is probably due to several factors, including the size and strength of the mouthparts of these ants, which may be too weak or too small to damage the eggs. In fact, among the potential predators tested, the ones able to consistently damage *H. halys* eggs are mainly tettigoniids and carabids,

insects equipped with very strong and large mouthparts (Morrison et al., 2016; Bulgarini et al., 2021). Other possible explanations of the limited vulnerability of the eggs are as follows: (1) *H. halys* may deposit chemicals on the eggs to interfere with the ant predatory ability (Schatz & Hossaert-McKey, 2010; Cerdá & Dejean, 2011); (2) the lack of chemical cues (chemical insignificance) eliciting ant interest towards eggs as food items; (3) the natural position of the egg mass (maintained during the experiments), which is typically on the underside of the leaf, might deter detection; and/or (4) efficient manipulation and ingestion by the predators. Combinations of these hypotheses are also possible. Further studies on both the physical and communication features of *H. halys* eggs singly and in egg masses are needed to clarify the nonpredation by ants and other arthropods.

In previous studies, some insects were shown to effectively prey on first instars, including tettigoniids, nabids, reduviids, and the ant *C. scutellaris* (Castracani et al., 2017; Pote & Nielsen, 2017; Bulgarini et al., 2021). This most likely occurred because N1 is the smallest instar with the softest body, and therefore is much easier to handle and subjugate. 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 while they acquire the symbionts left by the mother on the eggs (Taylor et al., 2014). In our experiments, although the first instars were placed on the plant separated from their parent egg masses, they still tended to remain aggregated under the leaf during the test. This behaviour probably reduced the detection of these nymphs and/or their effective manipulation by *L. niger* that is not specialized on an arboreal habit.

The second and third instars are more mobile than the first, and their location by the ants is more likely. Moreover, from the second instar onwards, *H. halys* tends to drop from the leaves when threatened, as observed during these experiments. This behaviour is a defense mechanism that can help to escape from individual predators. However, in the case of ants, colonies may rely on several workers patrolling the foraging area at various levels (below/above ground, on plants) depending on the species, and this may also result in intercepting prey eventually dropping from the trees (Way & Khoo, 1992; Campolo et al., 2015). The fourth and fifth instars 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 requires a greater number of ants to kill them, and also to their higher mobility, which favours a faster escape from the predators.

The tests of mortality over time showed that during the 1st h of exposure to *L. niger*, the ants did not have any effect on the nymphs. The mortality of the various instars increased over time, reaching the highest value after 48 h, when the ants had likely fully explored the arena and had also begun to patrol the plant, increasing the probability of interaction with the prey. This was particularly evident for the second and third instars. The difference between the second instar, whose survival dropped drastically after 24 h, and the third instar, whose survival dropped more gradually over time, is probably due to the difference in size, which makes the second instar easier to kill compared to the third instar, which is faster and probably better able to escape from predators.

These results are partially in line with findings on the arboreal ant *C. scutellaris*, which failed to impact *H. halys* eggs, but, unlike *L. niger*, successfully preyed on all instars (Castracani et al., 2017). This difference may be due to physical and behavioural adaptations of the two species linked to their respective ecology. In particular, the head of *C. scutellaris* is larger (also wider) than that of *L. niger* (Seifert, 2018). In ants, a larger and/or proportionally wider head generally comes with bigger 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 our observed results. Another possibility is that *L. niger* is less prone to search for 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, but not necessarily to prey on them (Seifert, 2018). This

interference and disturbance may have important effects also in agroecosystems, contributing to reduced crop damages in the case of phytophagous pests.

A possible concern for 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 in with sap-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, the net cost–benefit balance may be beneficial (Rosumek et al., 2009). In order to protect their source of honeydew, or because of their predatory and territorial habit, ants could provide services to the plant, such as warding off other insects or pathogens that could be much more noxious to the plant (Giannetti et al., 2019; Schifani et al., 2020).

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) had a greater impact (Castracani et al., 2017). This points out the need to consider the diversity in the behaviour and ecological roles of the various ant species as natural pest managers in agroecosystems (Hölldobler & Wilson, 1990; Way & Khoo, 1992; Maňák et al., 2013; Schifani et al., 2020). Ant diversity and ecology, different feeding habits, behavioural ecology, and impact on the dynamics of species assemblages and community structure, as well as specific climate and environmental factors, suggest the importance of considering both single species and community effect on herbivores in agroecosystems to better plan programs of conservation biological control and IPM involving ants (Gibb et al., 2017; Arnan et al., 2018; Castracani et al., 2020). *Lasius 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 location 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 used to manipulate ant behavior helping them to encounter food items. This suggests that an association of various species of predators could lead to greater results in reducing pest population. Hence, a better understanding of the specific roles of ants as control agents of pests in agroecosystems, as well as of the interactions among ant species, is crucial for providing further tools for IPM strategies, where different approaches complement each other and lead to better pest control.

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