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CICLO XXXVI

Ants and agricultural pests in the Mediterranean: exploring antagonism and mutualism paradigms for biological control

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Abstract

Ants are insects with an almost unparalleled ecological impact on terrestrial ecosystems, providing many important ecosystem services through their complex interactions with countless other organisms. Ant role in agroecosystems has drawn significant attention by humans for centuries but is still better investigated for the tropics as compared to temperate regions. Most ants are generalist predators, and their predatory abilities have also been appreciated for their antagonistic effects on many agricultural pest insects. At the same time, ants establish mutualistic relationships with several honeydew-producing hemipterans, forming keystone ecological associations that can also develop into a problem for agricultural settings in which some ant-mutualist hemipterans are important pests. We explore ecological and behavioral interactions between ants and agricultural pest insects through antagonism and mutualism paradigms using laboratory and field experiments, while also gathering baseline data on the diversity and distribution of ant species in agroecosystems across Italy. We present our results through eleven chapters grouped in three sections: *i)* Ants as biological control agents of insect pests; *ii*) Ants as mutualist partners of insect pests; *iii*) First data on ant diversity and distribution in Italian agroecosystems. In the first section, we describe for the first time the role of ants as natural enemies of key agricultural pests accounting both direct and direct interactions and analyzing potential conflicts with other natural enemies, while also presenting encouraging results on behavioral manipulation of ants through artificial nectaries. In the second section, we describe ecological and behavioral aspects of the interactions between ants, honeydew-producing mutualist hemipterans, and their natural enemies, emphasizing the variety of outcomes that the species-specific characteristics of the involved actors may produce. Furthermore, we present a comparative review of the methodologies developed to manage ant-hemipteran mutualism when this is responsible for significant pest outbreaks. In the third section, we present first data on ant diversity in Italian agroecosystems as well as the inclusion of the ant species studied in this thesis in the DNA barcoding library of European ants.

Riassunto

Le formiche sono responsabili di importanti servizi ecosistemici attraverso le complesse interazioni con innumerevoli altri organismi, rendendosi insetti dall'impatto ecologico quasi senza pari per quanto riguarda gli ecosistemi terrestri. Il ruolo delle formiche negli agroecosistemi ha attratto significativa attenzione da parte dell'uomo per secoli, ma è ancora meglio noto nelle regioni tropicali rispetto a quelle temperate. La maggior parte delle formiche sono predatori generalisti, e le loro abilità predatrici sono state apprezzate anche per il loro effetto antagonistico nei confronti di molte specie dannose di insetti. Allo stesso tempo, le formiche instaurano relazioni mutualistiche con molti emitteri produttori di melata, formando associazioni chiave di volta dal punto di vista ecologico che possono divenire problematiche nei contesti agricoli in cui alcuni emitteri mutualisti delle formiche hanno un ruolo importante come insetti dannosi. Abbiamo esplorato le interazioni ecologiche e comportamentali fra formiche e insetti dannosi in agricoltura attraverso i paradigmi di antagonismo e mutualismo, utilizzando esperimenti sia di campo che di laboratorio, e raccogliendo al contempo dati di base sulla diversità e distribuzione delle specie di formiche degli agroecosistemi italiani. Presentiamo i nostri risultati in undici capitoli, raggruppati in tre sezioni: *i*) Le formiche come agenti di controllo biologico di insetti dannosi; *ii*) Le formiche come partner mutualisti di insetti dannosi; *iii*) Primi dati sulla diversità e distribuzione delle formiche negli agroecosistemi italiani. Nella prima sezione descriviamo per la prima volta il ruolo delle formiche come nemici naturali di importanti insetti nocivi in agricoltura, tenendo conto sia delle interazioni dirette che di quelle indirette, analizzando possibili conflitti con altri antagonisti naturali e presentando risultati incoraggianti sulla manipolazione comportamentale delle formiche tramite nettàri artificiali. Nella seconda sezione descriviamo aspetti ecologici e comportamentali delle interazioni fra formiche, emitteri mutualisti produttori di melata, ed i loro antagonisti naturali, enfatizzando la varietà di risultati che possono scaturire dalle caratteristiche specie-specifiche degli attori coinvolti. Infine, presentiamo una revisione comparativa delle metodologie sviluppate per la gestione del mutualismo formiche-emitteri

quando questo è responsabile di significativi incrementi di insetti nocivi. Nella terza sezione, presentiamo primi dati sulla diversità delle formiche negli agroecosistemi italiani, e l'inclusione delle specie di formiche studiate in questa tesi all'interno della libreria di DNA barcoding delle formiche europee.

1. INTRODUCTION

1.1 Ants and their ecological role in terrestrial ecosystems

Ants (Hymenoptera: Formicoidea: Formicidae) are an eusocial insect family that evolved during the Cretaceous period, closely related to the Apoidea and Scolioidea superfamilies (Branstetter et al. 2022; Romiguier et al. 2022). Today, they count around 14,150 described species (Bolton 2023), being the largest of all eusocial animal groups (Wilson & Hölldobler 1990), while a large portion of their diversity is thought to be still undescribed (Kass et al. 2022). Latest estimates suggest there are around 20×10^{15} (20 quadrillion) ants on Earth, accounting for a remarkable biomass of 12 megatons of dry carbon, which is more than wild mammals and birds combined and around 20% of human biomass (Schultheiss et al. 2022). While their origins were most likely tropical, ants have become almost ubiquitous in all terrestrial habitats excluding the poles (Guénard et al. 2017), and in this wide geographic range, they have an important and multifaced ecological role (Wilson & Hölldobler 1990; Lach et al. 2010; Parker & Kronauer 2021).

Ancestral ants were probably ground-nesting predators and scavengers of tropical rainforests, and most of the extant species retain both characteristics (Wilson & Hölldobler 1990; 2008). While a large part of them diversified their diet by including food sources other than other small animals, almost all ants are at least occasionally predators and scavengers of other arthropods, and entire lineages are exclusively predatory, including several specialized predators (Wilson & Hölldobler 1990; 2008). The predatory activity of generalist and specialized ants has acted as a evolutionary pressure selecting ecosystem-wide defensive or evasive adaptations to coexist with ants across terrestrial arthropods (Parker & Kronauer 2021). Diet diversification, however, was especially important in the formicoid clade, which comprises most of the actual species diversity of ants, with honeydew and seed consumption becoming particularly important outside the tropics (Wilson & Hölldobler 2008). Both represent are the foundation of some of the most frequent interactions between ants and plants, which also encompass several other mechanisms, from indirect herbivory by fungus-growing Attini species to strong mutualistic relationships involving several different lineages of myrmecophytes or myrmecophilous plants (Wilson & Hölldobler 1990; Rico-Gray & Oliveira 2008). Ants eat the honeydew produced by several phytophagous insects, mostly aphids and scale bugs, often in exchange for protecting them against predators or parasitoids, pathogen fungi, and microbes, or adverse climatic conditions (Wilson & Hölldobler 1990). This type of mutualistic relationship between ants and honeydew-producing herbivores, known as trophobiosis, is considered an ecological keystone, being extremely successful across the world and part of larger multitrophic networks (Parker & Kronauer 2021). Other sugary liquid foods can be directly provided by myrmecophilous plants to their ant partners in the form of nectar produced by extrafloral nectaries (Rico-Gray & Oliveira 2008). On the other hand, seed consumption can be both beneficial or negative for different plant species, based on whether it contributes positively to seed dispersal (myrmecochory) as in the case of ant-adapted seeds with elaiosomes, and can have a deep influence over plant communities in some habitats (Rico-Gray & Oliveira 2008; Lach et al. 2010).

Most extant ant species build their nest on the ground, either digging in the soil, rotten wood, or living in rock crevices, but a considerable number of species build their nests on the trunk or canopy of trees and other plants (Wilson & Hölldobler 1990). In all cases, ants may act as important ecosystem engineers, chemically enriching the soil and participating in bioturbation processes, while also speeding the degradation of deadwood (De Bruyn & Conacher 1990; Lach et al. 2010). Ant nests are typically essential to the life of a plethora of myrmecophilous animals, mostly insects, and spiders, that have evolved special adaptations to live as inquilines or parasites of ant colonies, exploiting their resources and the protection the nests and colonies can offer (Parker & Kronauer 2021).

Ant communities, which may strongly vary in species richness and composition based on biogeographical and habitat characteristics, are structured along hierarchical structures based on behavioral dominance, colony size, foraging strategies, and other key biological characteristics, and can be used as a monitoring tool to track environmental changes (Lach et al. 2010).

1.2 The intricate relationship between ants and agriculture

Ants play a significant but ambivalent role in agricultural settings, where they offer both services and disservices, in a complex balance that is overall favorable in most cases but may significantly vary based on local ecological conditions and on agricultural management (e.g., Styrsky & Eubanks 2007; Anjos et al. 2022).

The service ants provide that has attained the most attention is the control of numerous insect pests (Way & Khoo 1992; Choate & Drummond 2011; Offenberg 2015; Anjos et al. 2022). At the very least since 304 A.D., farmers in Asia appreciated the favorable role of ants as enemies of phytophagous insects to the point of making active use of them by placing weaver ant (*Oecophylla smaragdina* Fabricius, 1775) nests on the canopy fruit trees (Huang & Yang 1987; Van Mele 2008; Offenberg 2015). The earliest reference is contained in the flora text *Nanfang caomu zhuang* (南方草木狀, Plants of the Southern Regions), attributed to the Chinese botanist Ji Han (嵇含, 263-307), who describes how farmers in southern China would by weaver ant nests in local markets and place them on their citrus tree, and how this was believed to be the only way to protect the fruits from the attacks of insects (Huang & Yang 1987; Van Mele 2008; Offenberg 2015). The use of weaver ants is a traditional farming practice, and the Chinese testimony represents the earliest known case of biological control. It has been the subject of extensive research efforts and is still exercised in several regions of Asia and some parts of Africa (Van Mele 2008; Lach et al. 2010; Offenberg 2015). However, while weaver ants possess some unique characteristics, they share with most other ant species across the world several key traits that are favorable for biological control (Risch & Carrol 1982; Way & Khoo 1992; Philpott & Foster 2005; Benckiser 2010; Choate & Drummond 2011; Offenberg 2015): *i)* being polyphagous generalist predators in most cases, and often territorially aggressive, they can prey upon a wide range of pests while still displacing others; *ii*) their permanent colonies can withstand periods of food shortage by building food stocks, and at the same time respond to pest outbreaks with mass recruitment capabilities; *iii*) their activity can be manipulated by transferring colonies and offering alternative food sources in specific sites. Most of the existing knowledge about ants' role as natural enemies of agricultural pests comes from the tropics, although knowledge from temperate systems is improving (Anjos et al. 2022).

However, when it comes to the control of phytophagous pest insects, ants are at the same time very well known as a nuisance in several cases due to their mutualism with several major honeydew-producing hemipteran pests, leading to pest outbreaks, especially in temperate agroecosystems. This is the most frequently reported disservice ants may cause to agricultural systems (Way and Khoo 1992; Anjos et al. 2022), only followed by indirect phytophagy by American fungus-growing ants, which however, represent a biologically unique group (Della Lucia et al. 2014). Ant attendance can lead to significant increases of the abundance of hemipteran pests beyond acceptable thresholds, with often a prominent role of invasive alien ants in this sense (e.g., Banks & Macaulay 1967; Kaplan and Eubanks, 2002; Daane et al. 2007; Cocco et al. 2021; Delabie et al. 2021; Wang et al. 2021). Among the different protective effects that ants may grant to their partners and can be responsible to pest outbreaks, the better studied is direct interference against parasitoids and predators that are exploited in biological control (e.g., Mgocheki and Addison 2009; Cheng et al. 2015; Plata et al. 2023), although other effects may involve sheltering the hemipterans during unfavorable seasons or protecting them from pathogen outbreaks caused by uncollected honeydew (Queiroz & Oliveira 2001; Giannetti et al. 2021). Interestingly, the presence of hemipteran pests may still prove overall beneficial when it attracts ants that prey upon more damaging herbivores (Styrsky & Eubanks 2007).

Considering ant role in soil bioturbation and enrichment in natural settings, they are believed to be an important soil component in agroecosystems, although additional research on this topic is particularly desirable (De Bruyn 1999). There is also growing evidence that, by producing antifungal and antibiotic substances, ants can have a significant influence in reducing the incidence of plant pathogens on wild and cultivated plants (Offenberg & Damgaard 2019; Offenberg et al. 2021). Finally, the seed consumption activity of ants can have an ambivalent effect, causing losses when affecting seeds of cultivated plants, but also contributing to the control of weeds (Baraibar et al. 2011a,b).

1.1 Aims

Our goal has been to study the role of Mediterranean ants in the biological control of agricultural pests. In the Mediterranean region, agriculture has a long history that produced a rich diversity of cultural systems and varieties, and at the same time, the region is one of the terrestrial biodiversity hotspots of the world, including for what concerns ants (Kass et al. 2022). In this complex and ecologically diverse region, the relationship between ants and agricultural activities has started attracting significant attention only during the last few decades after being long overlooked. In respect to biological control, Mediterranean ants have been shown to be able to have significant positive or detrimental influences based on context (e.g., Campolo et al. 2015; Castracani et al. 2017; Borbély & Nagy 2022; Plata et al. 2023). We wished to explore the relationship between Mediterranean ants and biological control of agricultural pest insects by working on three main topics that are here presented as sections of this thesis:

- The role of ants as natural enemies useful for the biocontrol of native and exotic agricultural pests through predation or displacement (*Section I – Ants as biocontrol agents of insect pests*).
- The role of ants as mutualist partners of hemipteran pests, its potential drawback on biological control, and the management options that can be adopted (*Section II – Ants as mutualist partners of insect pests*).
- Gathering first data on the diversity and distribution of ants in Italian agroecosystems, intended as an important fundamental basis to understand ant role in this region (*Section III – First data on ant diversity and distribution in Italian agroecosystems*).

2. SECTION I:

ANTS AS BIOCONTROL AGENTS OF INSECT PESTS

This first section contains five chapters. Chapter 1 illustrates a field study that we carried out in Northern Italy by increasing ant activity in a pear orchard through the provision of a sugary liquid by special dispensers, the “artificial nectaries”. The experiment, in which plants with increased ant activity were compared to plants with normal and reduced ant activity, demonstrated a positive role of ants in pear orchards, reducing the incidence of the pear scab *Venturia pyrina* Aderh. (1896) on the leaves and the attacks on the fruits by the codling moth *Cydia pomonella* (L.) – a polyphagous fruit pest of global relevance. In Chapter 2, we illustrate laboratory experiments aimed at elucidating the ability of ants as direct predators of *C. pomonella*, by employing two model ant species and studying their appetite for *C. pomonella* eggs and 1st instar larvae which are those that have not yet damaged the fruits. The results suggest that ants may act as predators of young larvae but are unlikely to have any effect on eggs. While previous studies had demonstrated how ants could prey upon *C. pomonella* at the pupation stage, after damage to the fruits already occurred, our results suggest that it will be worth investigating if ants can also discourage adult moths from laying their eggs on fruits visited by ants, to reduce the chance that the vulnerable 1st instar larvae are killed before digging into the fruits.

Chapter 3 analyzes the interactions between different ant species and the ambrosia beetle *Xylosandrus compactus* (Eichoff, 1875), an invasive alien species recently introduced to Europe that can strongly impact wild and cultivated species. We conducted laboratory experiments testing direct interactions and indirect interactions mediated by semiochemicals. Our results suggest that certain ants can effectively limit

X. compactus, with a special attention to small-sized arboreal-nesting ant species that may be able to enter *X. compactus* nests.

In Chapters 4 and 5, we conducted laboratory experiments investigating ant predators of stink bugs and their interactions with the specialized egg parasitoids that are often used in the biological control of the stink bugs, to explore the possible interference between different biocontrol agents. In Chapter 4, we studied the interactions between the acrobat ant *Crematogaster scutellaris* (Olivier, 1792), which Castracani et al. (2017) demonstrated to be a predator of the invasive alien brown marmorated stink bug *Halyomorpha halys* (Stål, 1855), with two *Trissolcus* parasitoids used in its biological control. The results showed interesting behavioral differences between the two parasitoids, but overall revealed a remarkable lack of aggression on the part of ants towards the parasitoids, suggesting no interference of the two in biological control. In Chapter 5, we explored for the first time the role of Mediterranean ants as potential predators of another stink bug pest, the southern green stink bug *Nezara viridula* (L.), and their interactions with its main parasitoid, *Trissolcus basalis* (Wollaston, 1858). These experiments revealed a similar pattern, with ants increasing the mortality of stink bug nymphs without interfering with the egg parasitoid.

Chapter 1

New tools for conservation biological control: testing ant-attracting artificial nectaries to employ ants as plant defenders

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Article

New Tools for Conservation Biological Control: Testing Ant-Attracting Artificial Nectaries to Employ Ants as Plant Defenders

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Abstract: Knowledge of the role of ants in many agroecosystems is relatively scarce, and in temperate regions the possibility to exploit ants as biocontrol agents for crop protection is still largely unexplored. Drawing inspiration from mutualistic ant–plant relationships mediated by extrafloral nectaries (EFNs), we tested the use of artificial nectaries (ANs) in order to increase ant activity on pear trees and to evaluate the effects on the arthropods, plant health and fruit production. While EFNs secrete a complex solution mainly composed of sugars and amino acids, ANs were filled with water and sucrose only. The results suggest that ANs can be used as manipulative instruments to increase ant activity over long periods of time. High ant activity was significantly linked to lower incidence of the pathogen fungus *Venturia pyrina* (pear scab) on pear leaves, and of the presence of *Cydia pomonella* (codling moth) caterpillars on pear fruit production. These results further encourage exploring underrated possibilities in the development of new tools for conservation biological control (CBC).

Keywords: Integrated Pest Management (IPM); Conservation Biological Control (CBC); ant-plant relationships; multitrophic interactions; applied myrmecology; agroecology; mutualism; indirect defense; *Pyrus* orchard; plant health; pear trees

1. Introduction

Plants have evolved very complex relationships with ants, and have been fundamental to the rise of many modern lineages of these insects [1–4]. Some of these relationships are strictly antagonistic, revolving around herbivory in a few New World species and more commonly around seed predation [5]. Many of the other relationships are beneficial to plants, and encompass several different aspects from seed dispersal and soil processing to rare cases of pollination [6,7], while the vast majority are based on an ant's appetite for sugary liquids [1], which is produced in the form of honeydew by sap-feeding insects (mainly heteropterans) or directly by plants in the form of nectars. Ants may protect sap-feeding insects in exchange for honeydew [8–11]. Although ants may indirectly damage plants through this relationship, its cost–benefit ratio may still be beneficial overall for the plant if ant presence displaces more damaging herbivores [12]. Some plants produce nectars, other food rewards or offer shelter to ants in exchange for protection against herbivory, competing plants or even pathogens [13–17]. These rewards may also be aimed at distracting ants from tending sap feeders [18–21]. Several cases of these ant-plant mutualisms are mediated by extrafloral nectaries (EFNs), which have been described for 4017 plant species of over 450 evolutionary lineages (21% of vascular plant families), and are expected to be found in about 8000 species [22,23]. EFNs are both a food reward for ants and manipulative tools to maximize plant advantages in the ant–plant relationship [24–26]. They produce complex solutions in which sugars and amino acids are the main components, while less abundant constituents such as secondary metabolites may also play an important role [25,26]. Moreover, in some plants EFNs can be an induced defense in response to herbivory [27]. However, plant defense by ants against herbivores can also be mediated by other adaptations, such as certain volatiles [28–32]. Although the latter mechanism has only been documented in a few cases, it is easy to speculate that ants may act as plant defenders by attacking herbivores much more often than currently reported.

An urgent need to develop more-sustainable agriculture is widely recognized, and Integrated Pest Management (IPM) should reduce the use of pesticides by favoring biological control techniques [33–35]. The use of ants as biocontrol agents is overlooked in comparison to other insect groups, and their use has been limited mainly to equatorial and tropical regions. Nonetheless, the oldest case of biological control mediated by insects (304 A.D.) attests to the use of the ant genus *Oecophylla* Smith, F., 1860 as a biocontrol agent [36]. Today, *Oecophylla* spp. are still used in more than 20 countries of Africa, Asia and Oceania to control about 50 different pest species [37,38]. Although they are considered bioindicators of soil function and habitat quality in rural environments [39,40], both their distribution and their role in most agroecosystems are still insufficiently documented. However, ants have several characteristics that make them good candidates for biocontrol agents [5,38,41–46]. First, they may be generalist predators, whose polymorphism often enhances their polyphagy. Second, many ants are often territorial and aggressive, thus chasing away intruders unsuitable as prey. In addition, recruitment allows a quick reaction to increases in prey. Moreover, colonies can withstand periods of food shortage and without becoming particularly susceptible to satiety because they build food stocks and rear immature stages. Finally, ant activity in agroecosystems can be easily manipulated, for example, by transferring colonies or offering additional food sources and nest sites (e.g., [47,48]). In addition, the manipulation of ant foraging pathways may redirect foragers towards target pests, and can be conducted using plants with EFNs [49].

Ant–sap feeder interactions are usually the main cause of concern about the role of ants in some agroecosystems. For example, in vineyards and citrus orchards, a number of studies have shown that ant presence increases populations of mutualistic aphids and coccids and decreases those of some of their natural enemies (e.g., [50–56]). As a result, additional research has focused on the control of ant populations in these agroecosystems, either by employing chemical substances (e.g., [57–64]) or distracting tending ants by providing sugary substances (e.g., [21]). The latter method may allow the positive effects of ant presence (see [12]). Nevertheless, other studies have found that the presence of ants in citrus and vine groves was weakly related or not related at all to the presence of sap feeders [21,65]. In addition, some natural predators of aphids and coccids may even increase under ant presence in vineyards [51]. Moreover, not all economically important sap-feeding pests are mutualists, and many ant species are not mutualists either. Even in citrus and vine groves where the phenomenon has attained particular attention, only a small proportion of ant species has been linked to pest outbreaks. In fact, of the 123 ant species inhabiting South Africa citrus orchards, only about 25 tend aphids and only three or four are considered responsible for pest outbreaks [66,67].

In Europe, the use of ants in biological control has mostly developed around wood ants (*Formica rufa* group). These have usually been employed in forest ecosystems [68,69], and much more rarely in agroecosystems, to which they had to be transported [48,70]. Only a few studies have dealt with ants in Italian agroecosystems (e.g., [71–77]). However, native ant species common in Italy and in the Mediterranean basin could be effective control agents against highly problematic pests such as the exotic brown marmorated stink bug *Halyomorpha halys* (Stål, 1855) [78], fungi and other herbivores [79]. In some cases, common harvester ants may have a positive role in the control of weeds [80]. Many of the above-mentioned ant characteristics considered promising for biological control were also documented for Italian ants (e.g., [7,68,74,75,81–84]).

Our paper reports on the role of ants and their use in conservation biological control (CBC) [85,86], and our experiment was carried out in a pear agroecosystem. Pear trees have been cultivated for at least 3000 years, and currently continue to be important for fruit production in wide regions of the planet, from Eurasia to North America [87]. Moreover, in Italy, a remarkable diversity of wild and cultivated forms has been documented [88], none of which possess EFNs or other structures aimed at attracting ants. Finally, pear orchards host a rich community of pest insects, whose control costs about USD 14 million each year worldwide, and does not usually employ ants [89]. However, ant presence in pear agroecosystems is not commonly associated with pest outbreaks. Conversely, *Crematogaster subdentata* Mayr, 1877 was reported to suppress a population of the San Jose scale *Quadraspidiotus perniciosus* Comstock, 1881 [90]. Moreover, *Formica neoclara* Emery, 1893 was considered to be a promising biocontrol agent of the pear psylla, *Cacopsylla pyricola* (Foerster, 1848) [91–93]. Although other authors suggest some ants may instead favor the latter [94,95], no clear evidence was provided. Finally, there is other evidence suggesting that *Cacopsylla* spp. primary parasitoids are even favored when ants are present, because they are more effective at keeping away the hyperparasitoids [96].

We introduced artificial nectaries (ANs) to study the impact of ants in a pear agroecosystem. Inspired by EFNs, ANs are manipulative tools designed to increase ant activity on trees by dispensing a liquid made of water and sugar to attract ants. Although a few studies have already tested the use of ANs or other food sources to attain agroecological benefits (e.g., [20,21,97–100]), none of them have studied ant effectiveness in defending plants, focusing instead on distracting ants from mutualistic sap feeders. Thus, the aim of our study was to evaluate both the functionality of ANs and the impact of different levels of ant activity on arboreal arthropodofauna, plant health and fruit production.

2. Materials and Methods

2.1. Study Area

Sampling was conducted in a 1-hectare organic orchard located near Pontescodogna, Parma, Italy (44.7378, 10.1954) and part of the Regional Natural Park “Boschi di Carrega”. The orchard consisted of 430 fruit trees (mainly pear, but also apple, apricot, cherry, fig, peach and plum trees), arranged in 15 rows and partially surrounded by a deciduous broadleaved forest. The orchard management was limited to periodical lawn mowing.

2.2. Artificial Nectaries (ANs)

The artificial nectary used in the experiment was made of a 1l plastic bottles (nectary tank) connected to an infusion set (Figure 1). Two holes were drilled in the bottle: one on its basal surface, later used to add or refill the liquid, and one on its cap, to connect the infusion set consisting of a flow controller and a dispenser releasing the liquid at the AN distal end. The AN created a direct slow, steady and adjustable flux of artificial nectar (set at 15–20 drops per minute) to a focus area on the plant (Nectar-releasing points). The content of the ANs consisted of a liquid solution made of 10g of sucrose in 1l of water and was refilled whenever needed (usually once a week) in order to provide an uninterrupted operation.

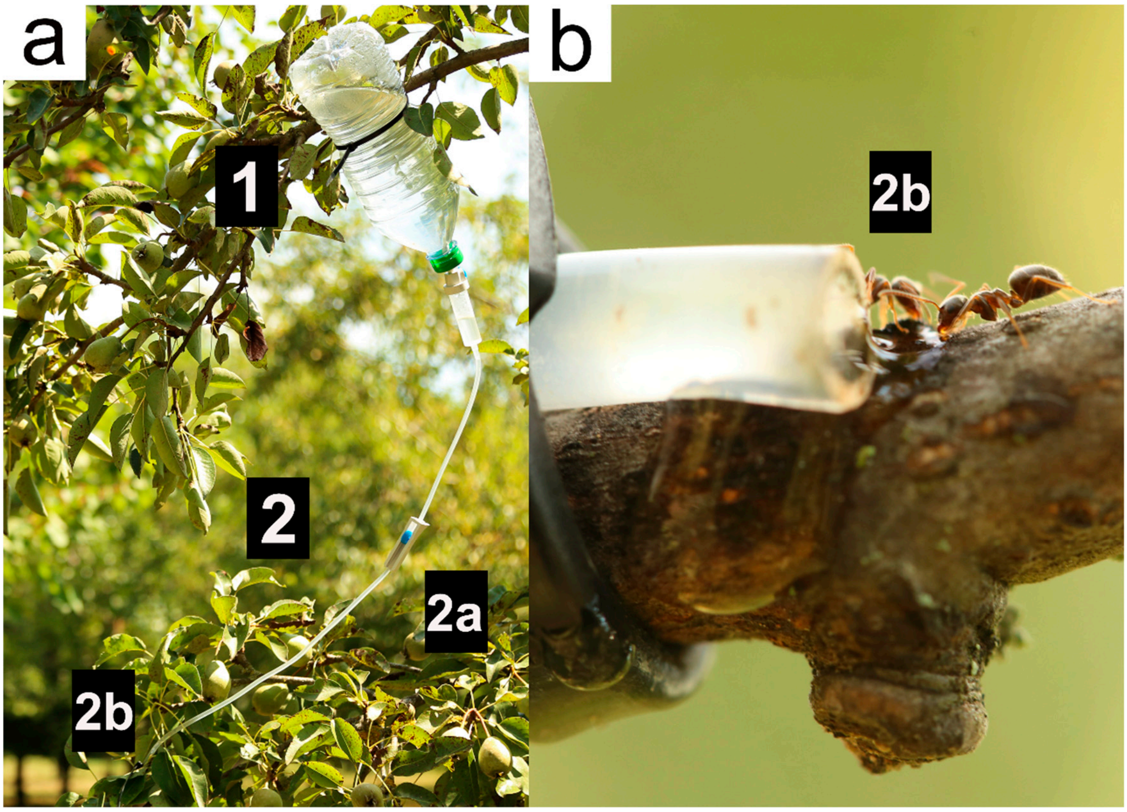

Figure 1. Artificial nectary (AN) installed on a pear tree. In pictures (**a**) and (**b**), the main components of the artificial nectary are shown: the tank (1) and the infusion set (2). The infusion set is made of the flow controller (2a) and the dispenser (2b). In (**b**), two *Lasius niger* workers are seen drinking at the nectar released by the dispenser.

2.3. Treatments

A total of 20 adult pear trees, homogenous in size (tree height: 2.5–3 m; trunk Ø: 30–40 cm), were selected for the experiment from two pear rows. They were chosen from among those plants standing far enough away from neighboring trees in order to avoid any contact and eventual passage of ants between them. Four treatment groups, each made up of five randomly selected trees, were then created. On each tree, two of the main branches departing from the trunk were selected as focal branches and used for data collection. Treatment groups differed according to the presence/absence of two different manipulations: ANs and ant-exclusion. Six ANs were placed in each tree in order to create six different release points: three points each per focal branch, one proximally to the trunk, one in the middle of the branch and the last distally to the trunk (Figure 2). Ant-exclusion consisted of the placement of sticky barriers at the base of the trunk in order to prevent soil-nesting ant access to the tree. Sticky barriers are a common system used for ant exclusion in field experiments [20,101,102]. Given the almost complete absence of arboreal-nesting species in the orchard, sticky barriers were expected to be effective in eliminating ants from targeted trees.

As a result, the following treatments were created:

- Treatment 1 (ANs+/Ants+): ANs were installed and ants were free to climb the tree
- Treatment 2 (ANs−/Ants+): no ANs were installed and ants were free to climb the tree (trees with no manipulations)
- Treatment 3 (ANs+/Ants−): ANs were installed and ants were not free to climb the tree since sticky barriers were installed
- Treatment 4: (ANs−/Ants−): no ANs were installed and ants were not free to climb the tree since sticky barriers were installed

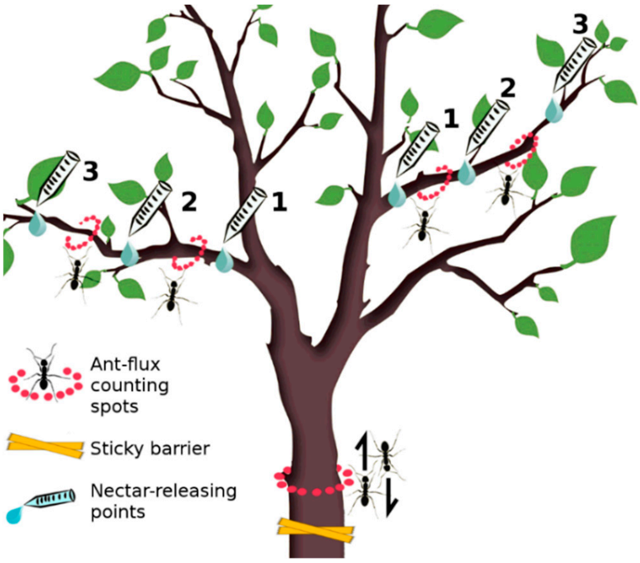

Figure 2. Positioning of the experimental apparatus on the trunk and the two focal branches of the tree. Ant-flux counting spots were present in all the trees (n = 20), whereas nectar-releasing points and sticky barriers were present only according to treatments: ANs+ for nectar-releasing points and Ants− for barriers.

2.4. Data Collection

Data were typically collected from 09:00 to 12:00 once a week during a two-month period from the beginning of July to the end of August 2018 (nine weeks). Data recording started one week after the beginning of manipulations.

2.4.1. Ant Activity

Data were collected with the aim of monitoring both abundance and diversity of ants active on the trees according to the four treatments. In order to avoid specimen collection during data sampling, a checklist of ant species in the orchard was compiled before the beginning of the experiment (see Table S1). In June 2018, ants were collected by direct sampling and identified under a ZEISS Stemi 508 stereoscopic microscope. Measurements were taken with the aid of an Axiocam Erc 5 s mounted on the microscope and ZEISS Zen core Software. Taxonomic identifications were mainly made following Radchenko and Elmes [103] and Seifert [104].

For each tree, five ant-flux counting spots were selected to record ant activity: on the trunk (50 cm above the ground) (1), on each focal branch between the first and second (2, 3) and between the second and the third nectar-releasing points (4, 5). At each spot, the species and number of ants were recorded during one-minute samplings (ant flux: N ants/min). Ants were counted if crossing (both directions) an imaginary circumference of the trunk/branch at each spot (Figure 2).

2.4.2. Arthropod Abundance

In order to evaluate the effect of the presence of ants and ANs on the arthropod fauna, the number of all non-ant arthropods (herein simply "arthropods") was recorded. When possible, individuals were identified at species rank, and, if necessary, a few individuals were collected from adjacent trees (not involved in the samplings) in order to minimize any possible influence on the experiment. Taxonomic aids to identification were Chinery, Leraut and the website Araneae—Spiders of Europe [105–107]. Arthropods were counted and identified on both focal branches of each tree through direct observation, by inspecting each branch from side to side. We decided to concentrate on focal branches since the maximum effect of ANs was expected at this location.

In addition, in order to produce a checklist of the arthropods of the area, data from focal branches were integrated with other direct observations on arthropod presence recorded by chance while working on the experiment.

2.4.3. Leaf Damages

A preliminary assessment of the most common types of damage affecting the leaves in the study area was conducted during June. These types of damage may be linked to herbivory or pathogens, which are damage sources potentially affected by ant activity (e.g., [48]). As a result, four damage categories were established and a damage scale was set for each category (also see Figure 3):

- Scab (S): presence of distinct black spots on the leaf surface, attributed to the fungus *Venturia pyrina* Aderh. (1896). Scores: 1 = absent; 2 = low (less than half leaf surface interested); 3 = medium (half of the leaf surface interested); 4 = high (more than half leaf surface interested)
- Necrosis (N): presence of extended necrotic areas on the leaf (surfaces larger than spots and with different shapes). Scores: 0 = absent; 1 = present
- Holes (H): presence of holes due to missing parts of tissue far from the edges. Scores: 0 = absent; 1 = present
- Damaged Edge (DE): presence of altered leaf profile due to missing parts of tissue at the edges; Scores: 1 = absent; 2 = low (less than half leaf edge interested); 3 = medium (half of the leaf edge interested); 4 = high (more than half leaf edge interested)

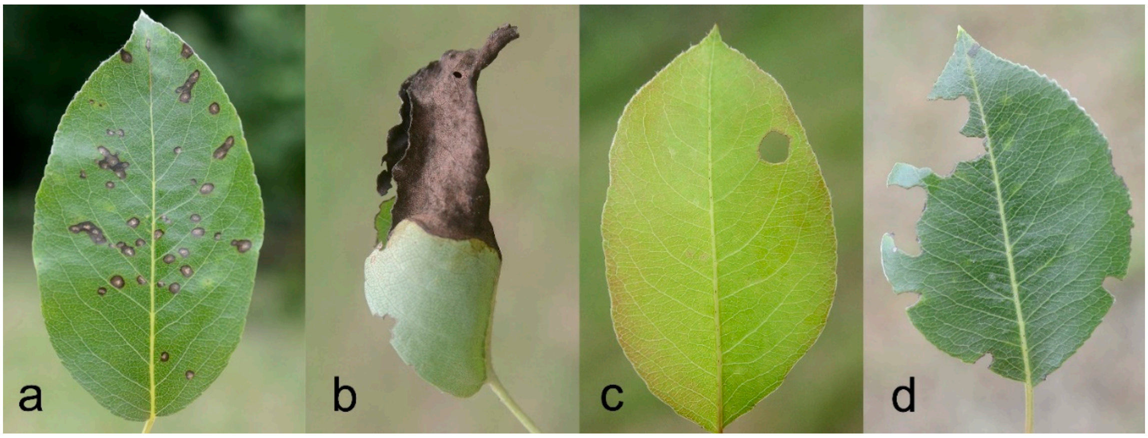

Figure 3. Pear leaves showing the main characteristics of the four damage categories used to evaluate their condition: Scab—S (**a**), Necrosis—N (**b**), Holes—H (**c**), Damaged Edge—DE (**d**). Leaf in picture (**b**) also shows signs of Holes and Damaged Edge.

The damage level of the leaf was always inferred by visual inspection. Prior to the beginning of the sampling, 15–25 leaves per focal branch were selected and monitored for the duration of the experiment recording for each sampling, damage category and score.

2.4.4. Fruit Damages

In order to describe fruit damage, four hypothetical damage categories were established:

1. Damage caused by sucking or chewing phytophagous insects (e.g., see [108] for *H. halys*)
2. Holes produced by codling moth *Cydia pomonella* (Linnaeus, 1758) caterpillars (Lepidoptera, Tortricidae)
3. Damage caused by fungi (e.g., *V. pyrina*)
4. Other damage

Immediately after being harvested, all fruits of each tree were counted and their statuses labeled as damaged or not damaged for all categories.

2.5. Statistical Analyses

If not specified, data were analyzed using IBM SPSS Statistics software (Italian version 25). Analyses and results are presented according to the nomenclature reported in [109].

In order to test the effect of treatments and time on ant activity, a mean ant flux ($n = 5$) was calculated for each tree and week and was used as the dependent variable in a repeated measures ANOVA ($n = 180$: 5 trees × 4 treatments × 9 weeks). Weeks 1–9 were considered a within-subjects factor (repeated measures design) and treatments (ANs+/Ants+; ANs−/Ants+; ANs−/Ants−; ANs+/Ants−) were used as a between-subjects factor (independent design). For the repeated measures, if assumption for sphericity was violated (Mauchly's test), Greenhouse-Geisser correction was used. A repeated contrasts procedure was used to compare weeks and Tukey post hoc tests were used for treatments.

To test the effect of treatments on arthropods, and because of a data distribution clearly deviating from normality, a non-parametric approach was chosen. For each recorded taxon, a Kruskal-Wallis test on abundances was run ($n = 360$: 2 branches × 5 trees × 4 treatments × 9 weeks). All possible pairwise comparisons ($n = 6$) with adjusted p-values (Bonferroni) were run only in cases of statistically significant results. Taxa with a total number of less than 10 specimens were excluded from the analysis.

Data on leaf damage were treated as follows: for each leaf damage category (S, N, DE and H) a mean score ($n = 15$–25) was calculated for each branch and week and used as a dependent variable in a repeated measures ANOVA ($n = 360$: 2 branches × 5 trees × 4 treatments × 9 weeks). Weeks 1–9 were considered as a within-subjects factor (repeated measures) and treatments (ANs+/Ants+; ANs−/Ants+; ANs−/Ants−; ANs+/Ants−) were used as a between-subjects factor (independent design). For the repeated measures, if the assumption for sphericity was violated (Mauchly's test), Greenhouse-Geisser correction was used. A repeated contrasts procedure was used to compare weeks and Tukey post hoc tests were used for treatments.

To test the effect of the treatments on fruit damage, two separate analyses were run: one on data from focal branches, and the other on data from the rest of the whole tree. In the case of focal branches, a generalized linear model (GLM) with binomial error structure (logistic regression) was run on the status of the fruit (damaged vs not damaged) ($n = 271$ fruits with an average of 13.55 fruits per tree ± 1.92 SE). Treatments (ANs+/Ants+; ANs−/Ants+; ANs−/Ants−; ANs+/Ants−) were considered as a fixed factor. For these analyses, the glm() function of the R statistical package was used [110]. The analysis was repeated to include a random factor for Tree-id using the glmer() function of the lme4 R package.

The same logistic regression model was used for the analysis of fruit damage on the rest of the whole tree ($n = 7699$ fruits with an average of 384.95 fruits per tree ± 81.97 SE). We found no evidence of over-dispersion in either analysis.

3. Results

3.1. Ant Activity

A total of 19 ant species were recorded during the preliminary assessment (Table S1). Among these species, 10 were observed on the experimental trees (Table 1). As concerns the total number of trees where ants were allowed to climb ($n = 10$), *Formica cunicularia, Camponotus piceus* and *Plagiolepis pygmaea* were respectively recorded in 100%, 90% and 70% of the trees. As concerns the ant flux observations ($n = 450$, trunk + branches), the most frequently observed species were *Lasius paralienus*, *Lasius niger* and *F. cunicularia*, which were observed in 22.44%, 16.44% and 12.89% of records, respectively. The most widespread species were *F. cunicularia, C. piceus* and *P. pygmaea*, which were recorded in 100%, 90% and 70% of the trees where ants were allowed to climb ($n = 10$), respectively. The most abundant species were *L. paralienus*, *P. pygmaea* and *L. niger*, with 553, 368 and 335 individuals were counted

($n = 450$), respectively. Only *L. paralienus* and *P. pygmaea* were sporadically able to climb trees despite the sticky barriers (Figure 4).

Data on the "ant flux" revealed a significant main effect of weeks ($F_{(3.77–60.28)} = 4.24$, $p = 0.005$). Repeated contrasts revealed a general decrease from week 1 to week 9. There was also a significant main effect of treatments ($F_{(3–16)} = 11.83$, $p < 0.001$). Tukey post hoc tests revealed a decreasing gradient of ant flux from ANs+/Ants+ to ANs+/Ants− treatments.

Results according to treatments and weeks are presented in Figure 4, where differences among treatments are shown, including a decreasing trend from week 1 to week 9 that was mainly evident for the treatments with no ant exclusion (ANs+/Ants+; ANs−/Ants+).

Table 1. List of the ant species most frequently observed on the trees during the experiment. For each species, the percentages of visited trees and of presence records are provided for Ants+ treatments.

Taxon	Subfamily, Tribe	Trees (n = 10)	Records (n = 450)	Counted Individuals (n = 450)
Camponotus piceus (Leach, 1825)	Formicinae, Camponotini	90%	11.3%	79
Camponotus vagus (Scopoli, 1763)	Formicinae, Camponotini	30%	0.9%	4
Dolichoderus quadripunctatus (Linnaeus, 1771)	Dolichoderinae, Dolichoderini	10%	6.4%	113
Formica cunicularia Latreille, 1798	Formicinae, Formicini	100%	12.9%	105
Lasius niger (Linnaeus, 1758)	Formicinae, Lasiini	40%	16.4%	335
Lasius paralienus Seifert, 1992	Formicinae, Lasiini	60%	22.4%	553
Myrmica sabuleti Meinert, 1861	Myrmicinae, Myrmicini	50%	2.0%	21
Plagiolepis pygmaea (Latreille, 1798)	Formicinae, Plagiolepidini	70%	9.1%	368
Tapinoma subboreale Seifert, 2012	Dolichoderinae, Tapinomini	10%	0.2%	1
Temnothorax italicus (Consani, 1952)	Myrmicinae, Crematogastrini	10%	0.2%	1

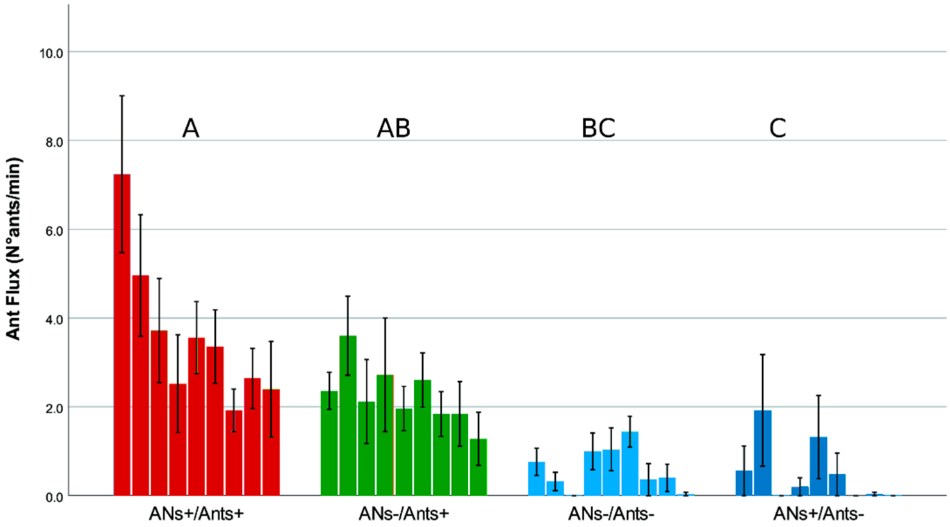

Figure 4. Effects of treatments and weeks on ant flux. Bars represent the mean number of ants crossing (both directions) an imaginary circumference of the trunk/branch at each spot during a single minute. For each treatment, bars correspond to the nine weeks of the experiment (left to right). For each bar, the SE interval is provided. Bars are lumped according to treatment and treatments with the same letter are not statistically different (mixed-design ANOVA and Tukey post hoc tests were used on treatments, see text for further details).

3.2. Arthropods Abundance

Arthropods found on the trees during the whole experiment were classified as belonging to 70 taxa: 23 species, 16 genera, 21 subfamilies/families and 10 orders (Table S2). The analysis of arthropod abundance on focal branches revealed the presence of spiders (Arachnida, Aranea) and seven orders of insects: Coleoptera, Dermaptera, Hemiptera, Hymenoptera, Lepidoptera and Neuroptera (Table 2). The most abundant species were *Hyphantria cunea* (Drury, 1773) (Lepidoptera, Erebidae) and *Stephanitis* *pyri* (Fabricius, 1775) (Hemiptera, Tingidae), but they were only recorded in high numbers during two single events and on trees where ants were not allowed to climb. Over 330 caterpillars of the fall webworm *H. cunea* eliminated almost all the leaves of one focal branch, while about 100 individuals of the pear lace bug *S. pyri* were counted on another.

Table 2. List of the arthropods scanned weekly on the two focal branches from the beginning of July until the end of August 2018 (nine weeks). "Total" represents the total number of individuals recorded during the whole sampling period. In *p*, statistically significant results are highlighted in bold. In "Treatment", treatments with the same letter are not statistically different. In "Mean" and "SE", results refer to *n* = 90.

Class	Order	Family/Species	Total	$H_{(3)}$	*p*	Treatment	Mean	SE
Arachnida	Araneae		29	6.268	0.099	ANs+/Ants+	0.04	0.02
						ANs−/Ants+	0.03	0.02
						ANs+/Ants−	0.14	0.05
						ANs−/Ants−	0.10	0.03
Insecta	Coleoptera		33	1.759	0.624	ANs+/Ants+	0.07	0.03
						ANs−/Ants+	0.09	0.07
						ANs+/Ants−	0.13	0.05
						ANs−/Ants−	0.08	0.03
	Dermaptera		1	-	-	-	-	-
	Diptera		35	0.727	0.867	ANs+/Ants+	0.07	0.03
						ANs−/Ants+	0.13	0.05
						ANs+/Ants−	0.09	0.03
						ANs−/Ants−	0.10	0.04
	Hemiptera	*M. pruinosa*	65	3.402	0.334	ANs+/Ants+	0.03	0.02
						ANs−/Ants+	0.19	0.08
						ANs+/Ants−	0.17	0.06
						ANs−/Ants−	0.33	0.22
		S. pyri	192	9.094	0.028	ANs+/Ants+	0.00	0.00
						ANs−/Ants+	0.40	0.20
						ANs+/Ants−	1.73	1.19
						ANs−/Ants−	0.00	0.00
		others	32	2.348	0.503	ANs+/Ants+	0.08	0.03
						ANs−/Ants+	0.08	0.03
						ANs+/Ants−	0.03	0.01
						ANs−/Ants−	0.08	0.03
	Hymenoptera	*Vespula* sp.	37	37.749	<0.001	(A)ANs+/Ants+	0.14	0.04
						(B)ANs−/Ants+	0.00	0.00
						(A)ANs+/Ants−	0.27	0.06
						(B)ANs−/Ants−	0.00	0.00
		others	4	-	-	-	-	-
	Lepidoptera	*H. cunea*	631	9.050	0.029	ANs+/Ants+	0.00	0.00
						ANs−/Ants+	0.00	0.00
						ANs+/Ants−	0.00	0.00
						ANs−/Ants−	7.01	4.92
		others	4	-	-	-	-	-
	Neuroptera	Chrysopidae	7	-	-	-	-	-
		Chrysopidae (eggs)	40	6.642	0.503	ANs+/Ants+	0.02	0.02
						ANs−/Ants+	0.10	0.04
						ANs+/Ants−	0.11	0.04

As concerns the effect of treatments on arthropod abundances, a Kruskal-Wallis test was performed on the following taxa: Araneae, Chrysopidae eggs, Coleoptera, Diptera, *Vespula* spp. (Hymenoptera, Vespidae), *Metcalfa pruinosa* (Say, 1830) (Hemiptera, Flatidae), *Stephanitis pyri* (Fabricius, 1775) (Hemiptera, Tingidae), other Hemiptera and *Hyphanthria cunea* (Drury, 1773) (Lepidoptera, Erebidae). The remaining taxa were excluded from the analysis due to their low abundances (Table 2). The Kruskal-Wallis test found statistically significant differences in *Vespula* spp. ($H_{(3)} = 37.749$, $p < 0.001$), *S. piryi* ($H_{(3)} = 9.094$, $p = 0.028$) and *H. cunaea* ($H_{(3)} = 9.050$, $p = 0.029$). For *Vespula* spp., pairwise comparisons showed the presence of two groups that differed according to the presence of ANs, with a higher abundance in treatments where ANs were present (Table 2). For *S. piryi* and *H. cunaea*, pairwise comparisons showed no significant differences between groups in all the possible comparisons. Finally, the Kruskal-Wallis tests found no statistically significant differences in Coleoptera ($H_{(3)}$ = 1.759, p = 0.624), Araneae ($H_{(3)}$ = 6.268, p = 0.099), Diptera ($H_{(3)}$ = 0.727, p = 0.867), *M. pruinosa* ($H_{(3)}$ = 3.402; p = 0.334), other Hemiptera ($H_{(3)}$ = 2.348, p = 0.503) and Chrysopidae eggs ($H_{(3)}$ = 6.642, p = 0.084) (Table 2).

3.3. *Leaf Damage*

As concerns Scab, there was a significant main effect of weeks on damage scores ($F_{(3.58–121.82)}$ = 51.61, $p < 0.001$). Repeated contrasts revealed a general increase of scores from week 1 to week 9. There was also a significant main effect of treatments ($F_{(3–34)}$ = 4.36, p = 0.011). Tukey post hoc tests revealed that ANs+/Ants+ treatment had lower scores than both treatments with ant exclusion (ANs+/Ants− and ANs−/Ants−), which did not differ from one another. ANs−/Ants+ treatment was associated with scores in-between the two previous groups (Figure 5).

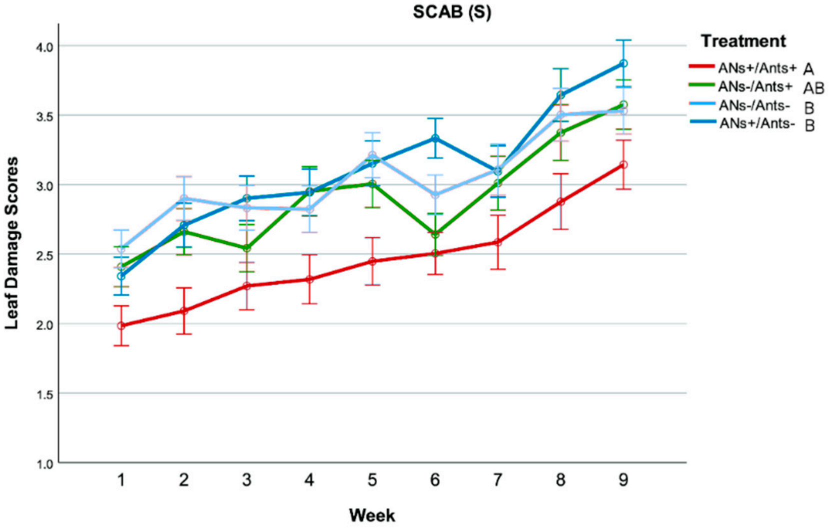

Figure 5. Effects of treatments and weeks on leaf damage for the Scab category. Points represent the mean score per week and treatment. For each point, whiskers show the SE interval. Lines with the same letter are not statistically different (mixed ANOVA and Tukey post hoc tests, see text for further details).

The analysis on the Necrosis category showed that there was a significant main effect of weeks on damage scores ($F_{(5.04–272)}$ = 49.00, $p < 0.001$). Repeated contrasts revealed a general increase of scores from week 1 to week 9. There was also a significant main effect of treatments ($F_{(3–34)}$ = 4.39, p = 0.010). Tukey post hoc tests revealed that ANs+/Ants+ treatment had lower scores than ANs−/Ants− treatment. ANs−/Ants+ and ANs+/Ants− treatments did not differ from one another and had scores in-between the two previous groups (Figure 6).

As concerns the Damaged Edge category, scores were generally very low with very few records encompassing a score of 2, meaning that damage was absent for less than half the leaf margin. There was a significant main effect of weeks on damage scores ($F_{(5.89–200.20)}$ = 4.48, $p < 0.001$). Repeated contrasts revealed no differences between week 2 and week 8, lower scores for week 1 compared to week 2 and higher scores in week 9 as compared to week 8. No significant main effect of treatments was found ($F_{(3–34)}$ = 0.23, p = 0.87).

The analysis on the Holes category showed generally very low scores with very few records encompassing a score of 0.4, meaning that holes were usually absent. No significant main effects were found on the damage scores of weeks ($F_{(4.18–142.19)}$ = 1.66, p = 0.16) and treatments ($F_{(3–34)}$ = 1.12, p = 0.36).

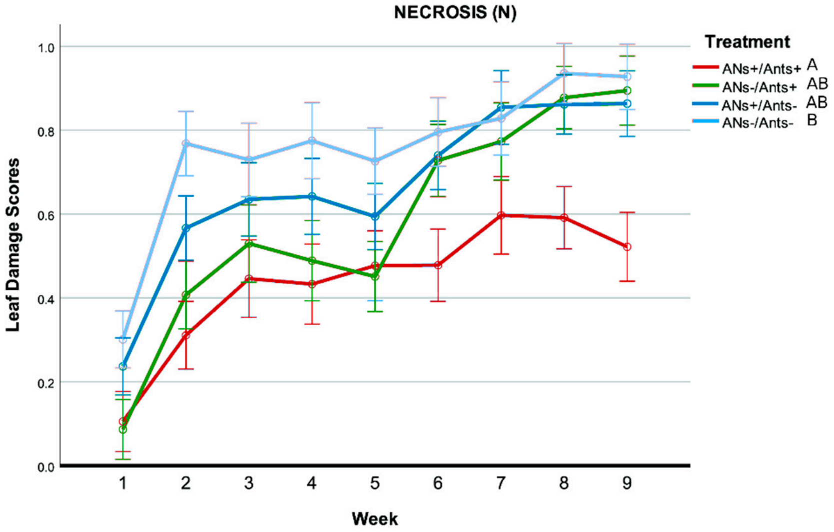

Figure 6. Effects of treatments and weeks on leaf damage for the Necrosis category. Points represent the mean score per week and treatment. For each point, whiskers show the SE interval. Lines with the same letter are not statistically different (mixed ANOVA and Tukey post hoc tests, see text for further details).

3.4. Fruit Damage

As concerns fruit damage, only holes produced by caterpillars of the codling moth *C. pomonella* were recorded and used for a statistical analysis (Figure 7). Only 3% of the fruits of the whole fruit production from ANs+/Ants+ trees were damaged by *C. pomonella*, whereas this value increased for the other treatments (ANs−/Ants+: 23%; ANs−/Ants−: 28%; ANs+/Ants−: 11%) (Table S3).

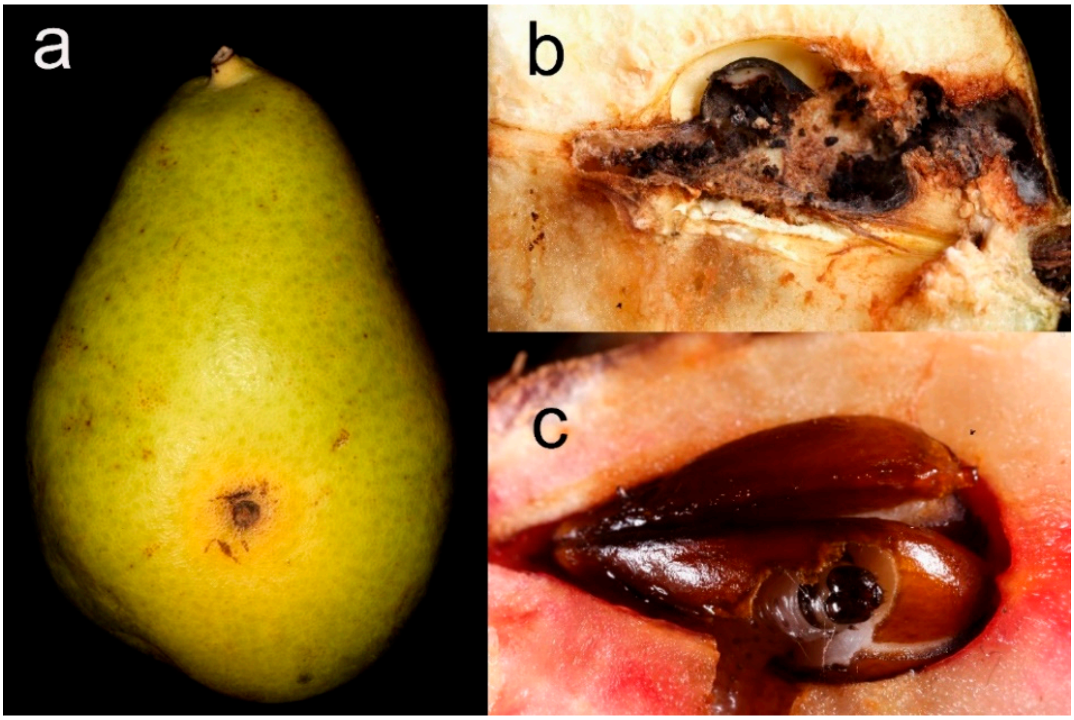

Figure 7. Damage produced by caterpillars of the codling moth *Cydia pomonella* on pear fruits. A small entrance hole (**a**) is usually visible on its side or near the stamen if the fruit is attacked. The larvae penetrate inside, often eating both the pulp and seeds (**b**,**c**), and may create favorable conditions for other organisms such as fungi to grow within the fruit (**b**).

The logistic regression analysis showed a significant association between treatments and fruit status (damaged vs not damaged) on the focal branches. Fruits from ANs+/Ants+ trees were 10 times less likely to be damaged than the control group (ANs−/Ants+), whereas no differences were detected between the control group and the other treatments (ANs+/Ants−; ANs−/Ants−) (see Table 3 and Figure 8). The addition of the random factor Tree-id to the model did not change previous results.

Table 3. Logistic regression of treatments (ANs+/Ants+; ANs−/Ants+; ANs+/Ants−; ANs−/Ants−) on fruit status (damaged vs not damaged). The coefficients of treatments are contrasts with the control group (ANs−/Ants+). ** $p < 0.01$; *** $p < 0.0001$.

Variable	B	SE	Odds Ratio	Sig.
Constant	−1.04	0.25	−4.18	<0.001 ***
ANs+/Ants+	−3.06	1.04	−2.95	0.003 **
ANs+/Ants−	−0.80	0.41	−1.96	0.05
ANs−/Ants−	−0.12	0.43	−0.29	0.76
χ^2	22.18, *df* = 3, $p < 0.001$ ***			

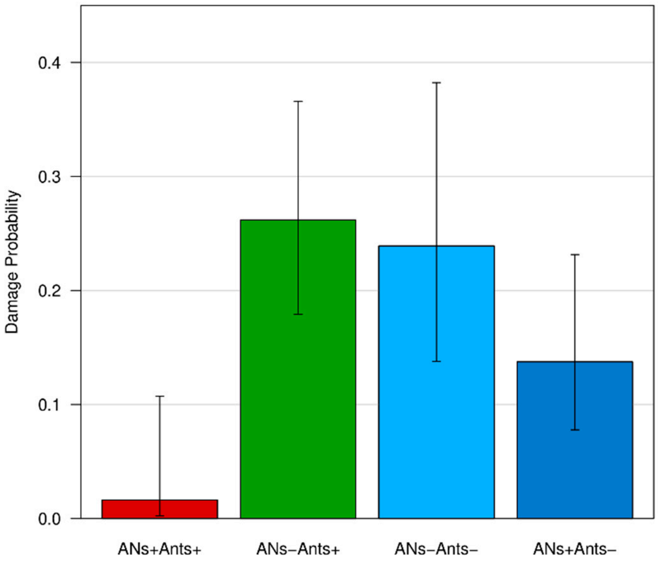

Figure 8. Effects of treatments on fruit damage by *C. pomonella* for focal branches. Bars represent the probability of a fruit to be damaged according to the treatment (logistic regression model). For each bar, a confidence interval is provided.

The logistic regression analysis on data from the whole tree (focal branches excluded) showed similar results to those on the focal branches, highlighting a significant association between treatments and fruit status (damaged vs not damaged). Fruits from the ANs+/Ants+ trees were 3.47 times less likely to be damaged than the control group (ANs−/Ants+). Given the higher number of fruits with respect to the previous analysis, the differences between the control group and the other treatments (ANs+/Ants−; ANs−/Ants−) were also significant (see Table 4 and Figure 9). Specifically, the fruits from ANs−/Ants− trees, where ants were excluded, were 1.32 times more likely to be damaged than the control group (ANs−/Ants+). The addition of the random factor Tree-id to the model did not change the previous results.

Table 4. Logistic regression of treatments (ANs+/Ants+; ANs−/Ants+; ANs+/Ants−; ANs−/Ants−) on fruit status (damaged vs not damaged). The coefficients of treatments are contrasts with the control group (ANs−/Ants+). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

Variable	B	SE	Odds Ratio	Sig.
Constant	−1.93	0.08	0.15	<0.001 ***
ANs+/Ants+	−1.34	0.13	0.26	<0.001 ***
ANs+/Ants−	−0.23	0.11	0.80	0.03 *
ANs−/Ants−	0.33	0.11	1.39	0.002 **
χ^2	231.09, $df = 3$, $p < 0.001$ ***			

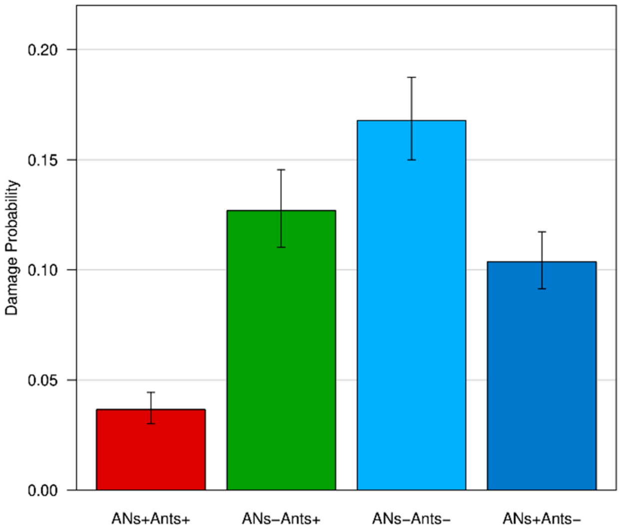

Figure 9. Effects of treatments on fruit damage by *C. pomonella* for the whole tree (except focal branches). Bars represent the probability of a fruit to be damaged according to the treatment (logistic regression model). For each bar, the confidence interval is provided.

4. Discussion

Results demonstrated that ANs can be used to manipulate ant presence on pear trees. Moreover, new data on the role of ants in pear agroecosystems and their impact as plant defenders were obtained. To the best of our knowledge, the list of ant species observed and their relative abundances may represent the first assessment of this kind in a pear orchard worldwide. The scarcity of arboreal-nesting species in the orchard is noteworthy, including the almost complete absence of ecologically and behaviorally dominant ants (see [104,111]) such as *Crematogaster scutellaris* (Olivier, 1792) (present in about 2.5% of the orchard trees) or *Lasius fuliginosus* (Latreille, 1798) (absent in the orchard), which were clearly more abundant in the surrounding areas. A lack of suitable nesting sites within the orchard trees may have contributed to this difference. The most representative species we found in the orchard are commonly recorded in the Padan Plain [112].

A fundamental result of this study is the success of ANs at attracting ants, helping to create a gradient in activity of ants among treatments. The existence of different ant activity levels among treatments through the use of ANs and sticky barriers was the fundamental premise to the success of the rest of the experiment, allowing an evaluation of how varying rates result in varying effects on other arthropods and on plants. Notably, these differences were successfully maintained throughout the season. An interesting high peak of ant activity, correlated with the presence of ANs, was recorded during the first week of monitoring (note that ANs were installed a week earlier). This could be explained as the result of a "novelty effect", or of a latency before some degree of saturation of the colony "social stomach", or by a change in dietary needs throughout the season (e.g., switching from carbohydrates to a protein-rich diet as more brood are produced) [113,114]. In any case, ANs should be further studied as possible tools to direct ant activity toward target areas over significantly long periods of time.

While a rich arthropod community was observed, most of the typical pests of pear orchards [89] were either absent or only few individuals were present. The San Jose scale, *Q. perniciosus*, against which the predatory activity of certain ants may be very effective [90], was absent. Similarly, *Cacopsylla* sp., which ants may effectively control or, according to others, favor [91–93], were only present in low numbers. Wasps (*Vespula* sp.) were the sole group showing significant differences in their presence on trees according to treatments: they were very poorly represented overall, but slightly more common in trees with ANs. This pattern seems to be the result of wasp interest in the artificial nectar (seldom observed in European hornets, *Vespa crabro* Linnaeus, 1761, too), which apparently grew throughout the period of the experiment. In a few instances, *Lasius* spp. were observed aggressively preventing the wasps from landing to drink at the AN dispensers they had occupied, and wasps usually aimed to land at dispensers without ants. Differences in the presence of other arthropod taxa were expected (e.g., [101,102,115]), but perhaps they would have required a much heavier sampling effort to be numerically appreciated. However, during the second month of the experiment, some cases of pest outbreaks were noted on a few trees with sticky barriers preventing ant presence. The fact that no increase in sap feeders was observed in conjunction with increased ant activity is notable. It is possible that ANs also decreased the importance of honeydew producers as nectary sources for ants, thus disrupting their protective tending behavior ([20,21,97–100]—but see also [116]). However, the ant–sap feeder association did not appear relevant in the study area.

Interesting differences were found regarding leaves among different treatments. The first two damage categories showed very similar trends, suggesting they both may be mainly attributed to the same pathogen (*V. pyrina*). In both cases, trees with a high ant activity were significantly less affected, which may indicate the role of ants in limiting the spread of this epiphytic fungus. Indeed, effects on plants by ant antimicrobial secretions have already been documented in several cases, but the ecological weight of these interactions—not to mention their possible applications in agriculture—is far from being understood and in need of investigation [17,48,79]. The ability of ants to reduce the damage by apple scab (*Venturia inequalis* (Cooke) G. Winter (1875)) was recently documented [48]. The other two damage categories had no significance in relation to ant activity. However, leaves were already quite damaged at the beginning of data sampling. As a consequence, an experiment specifically aimed at further evaluating the effects of an increase of ant presence on pear leaves may benefit from starting earlier in the season, in conjunction with the appearance of the first leaf gems.

Moreover, interesting data were obtained in relation to fruit production. Contrary to leaves, fruit were still in an early stage of development and mostly intact at the beginning of the experiment. By the time they were collected, a large percentage had been attacked by the codling moth (*C. pomonella*), which is considered to be one of the most important pear pests worldwide [89]. Such attacks tend to compromise both pulp and seeds, making fruit unsuitable for the food market and seed production, and damaging plant fitness. However, in trees in which ant activity was increased by ANs, the attacks were much less problematic. The two analyses carried out separately on focal branches and on the rest of the fruit production depicted very similar results. This suggests that, although ANs were placed on only two branches per tree, they increased ant activity on an unexpectedly larger area of the plant. We can speculate that ant activity may have limited the action of the codling moth through different, non-exclusive mechanisms, such as via a dissuasive effect on egg-laying activity (possibly mediated by semiochemicals), or by direct predation on the moth eggs and/or caterpillars. Ants are known to prey on both the eggs and larvae of different lepidopterans [48,71,117–119], and they also prey on other frugivorous insect larvae such as those of fruit flies [120–122]. Moreover, they may inhibit fruit fly egg deposition due to their secretions, and Tephritidae may even avoid landing on fruit previously exposed to ants [123]. Predation on the pupal stage in the soil may also have occurred (e.g., as concerns flies [124]). It is remarkable to note that none of the few biological control techniques currently employed against the codling moth are able to target eggs and larval stages [90,125–127]. Moreover, pesticide treatments against this pest face increasing difficulties due to the emergence of resistance mechanisms [128,129]. Additional investigation should clarify in detail how ants can affect the moths and their activity on plants, possibly leading to new control methods.

Both the ANs and nectar solution used during the experiment were very basic, and may be improved in the future, perhaps by further drawing inspiration from natural systems such as more elaborate nectar solutions with specific carbohydrates/nitrogen ratios or proteins, which could possibly enhance ant predatory attitude (e.g., [20,26,130,131]). Moreover, in comparison with a recent experiment of inoculation biological control employing ants in an apple orchard [48,71], we obtained somewhat similar beneficial results by using the native ant-fauna, while we did not witness sap feeders outbreaks.

5. Conclusions

Although a relatively simple protocol was employed, very encouraging results on fruit production and plant health were achieved, many of which solicit the need for additional investigation. Future efforts may focus on the mechanisms eventually adopted by ants to contain *V. pyrina* and *C. pomonella*, and on how the activity of different ant species may impact plants and their pests. Overall, significant results were observed when ant activity was increased due to the ANs, while no significant differences were detected between control trees in which ant activity was untouched and those where it was artificially reduced. Moreover, no negative side effects of AN use were found. In conclusion, these results further encourage studying the role of ants and the employment of ANs in agroecosystems and suggest it is worth continuing to explore unchecked possibilities in the development of new tools for CBC.

Supplementary Materials: The following are available online at http://www.mdpi.com/2075-4450/11/2/129/s1, Table S1: List of ant species recorded during the preliminary assessment of the ant fauna of the study area, Table S2: List of arthropods (ants excluded) observed on the trees during the experiment, Table S3: Fruit production of the experimental trees and damage inflicted by the codling moth caterpillar (*Cydia pomonella*).

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Section I - Ants as biocontrol agents of insect pests

Chapter 2

Predatory abilities of two Mediterranean ants on the eggs and larvae of the codling moth *Cydia pomonella*

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Communication

Predatory Abilities of Two Mediterranean Ants on the Eggs and Larvae of the Codling Moth *Cydia pomonella*

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Simple Summary: Ants are widespread across terrestrial ecosystems, including agroecosystems, where they take part in several important processes. They often can act as predators of a wide range of insect pests in agricultural fields, which should be considered by management programs, and can sometimes be actively exploited to promote sustainable biological control strategies. In a recent experiment conducted in Europe, pear trees visited by larger numbers of ants suffered fewer attacks to their fruits by the codling moth, a small lepidopteran, which is a significant economic pest worldwide, especially in apple, pear, and walnut orchards. However, the exact form of the interaction between the ants and codling moths remained unclear. While ants were already known to prey upon mature larvae or pupae in the soil, this new evidence suggested they could also control the eggs or newly hatched larvae that had not yet attacked the fruits, which are the two stages whose removal would directly prevent fruit damage. We conducted laboratory experiments to determine whether two common European ants could prey upon these stages. Our results suggest that these ants are effectively able to kill newly hatched larvae, while the eggs do not appear directly vulnerable to predation. Further investigation under field conditions would be needed to assess whether ants may also interfere with the oviposition by adult moths.

Abstract: The predatory ability of ants (Hymenoptera, Formicidae) against insect pests can offer an important service to agricultural activities and may sometimes be directly exploited in biological control strategies. The codling moth *Cydia pomonella* (Lepidoptera, Tortricidae) is a major agricultural pest of fruit orchards, whose biological control is complicated by the fact that the larvae spend most of their life protected within the fruits they damage. In a recent experiment in Europe, pear trees in which ant activity was artificially increased by the addition of sugary liquid dispensers (artificial nectaries) suffered less damage caused by the larvae to their fruits. While some ants were already known to prey upon the mature larvae or pupae of *C. pomonella* in the soil, prevention of fruit damage would require predation upon eggs or newly hatched larvae, which have not yet excavated into the fruits. We verified whether two different Mediterranean ants frequently observed in fruit orchards, *Crematogaster scutellaris* and *Tapinoma magnum*, were able to prey upon *C. pomonella* eggs and larvae in laboratory conditions. Our experiments demonstrated that both species similarly attacked and killed young *C. pomonella* larvae. On the other hand, the eggs mostly attracted the attention of *T. magnum* but were never damaged. Further field assessments are required to understand whether ants may also interfere with oviposition by adults or whether larger ant species, although generally rarer in orchards, may also prey upon eggs.

Keywords: biological control; pest management; Formicidae; Tortricidae; Lepidoptera; *Crematogaster scutellaris*; *Tapinoma magnum*

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1. Introduction

Ants (Hymenoptera, Formicidae) are among the most successful insect groups, and their widespread presence in terrestrial habitats has significant ecological consequences [1,2].

Their relationship with plants is of particular interest from both an evolutionary and an applied perspective [2,3]. One of the most important services that ants may provide to plants in these relationships is protection from a range of different herbivore insects that ants may prey upon or at least displace [4,5]. In addition, ants in agriculture may also play important roles in soil enrichment and bioturbation, as well as control of weeds and certain plant pathogens [6–8]. Ants' ability to protect certain honeydew insect pests must be acknowledged; at the same time, their generalist predatory ability against several phytophagous arthropods promotes their recognition as biological control agents across different agricultural contexts [4,5,9]. This is especially well known in the tropics and comparatively less studied in temperate regions [4].

The codling moth, *Cydia pomonella* (Linnaeus, 1758) (Lepidoptera, Tortricidae), is a key polyphagous fruit pest whose economic relevance is particularly significant in apple, pear, and walnut orchards [10–12]. Its control is complicated by the development of resistance against pesticides and baculoviruses [12–14], while pesticide usage may disrupt the control of secondary pests [15]. Biological control strategies normally focus on last instar larvae that seek a shelter to pupate, on pupae, or on adults, using predators, parasitoids, and viruses [16–21]. In addition, pheromones or the sterile insect technique can be used in mating disruption strategies [22–24]. However, few biological control agents are known to target eggs or younger larvae, which spend almost their entire life protected inside the fruit they consume except for a short window after hatching (usually within 24 h), during which they may travel for up to a few meters searching for some fruit to dig into [10,25]. Predatory heteropterans and earwigs are the only known predators of eggs [26–28], which are very small (1–1.2 mm long), may be laid directly on the surface of fruits or on nearby areas of the plants, and hatch in about 5–12 days [10,25].

Among the different generalist predators that may play a role in the control of *C. pomonella* [29], ground-dwelling ants can prey upon last instar larvae and pupae [18]. More recently, field data suggested that trees visited more intensively by ants may suffer less damage to their fruits by the moths [30].

While this result suggested an effect of ants on the activity of *C. pomonella* before its larvae dig into the fruits, it remained unclear whether ants affected the eggs and/or the newly hatched larvae [30]. We aimed to test whether two Mediterranean ants that are common in fruit orchards and agroecosystems, *Crematogaster scutellaris* (Olivier, 1792) and *Tapinoma magnum* Mayr, 1861 [31,32], may act as predators of *C. pomonella* eggs and/or newly hatched larvae by documenting their behavioral interactions in laboratory experiments.

2. Materials and Methods

All experiments were conducted during June 2022. Four days before the experiments, fragments of *C. scutellaris* and *T. magnum* of at least 500 workers each [33] were taken from Parma University Campus (northern Italy) and temporarily reared under laboratory conditions (T: 25 ± 1 °C, RH: 60 ± 0.5%, photoperiod 12:12 L:D; honey provided as food). Commercially available *C. pomonella* eggs were obtained from Andermatt Biocontrol (Grossdietwil, Switzerland) and kept under the same laboratory conditions. *Cydia pomonella* eggs and first-instar larvae (hatched in the previous 2–8 h) were used in the experiments alongside ant workers randomly selected from the colony fragments.

In each trial, we introduced into a petri dish (∅ = 9 cm) either an ant and a group of 6 *C. pomonella* eggs laid on a 1 cm × 1.5 cm paper or an ant and a single *C. pomonella* larva. The *C. pomonella* eggs or larva were initially put at the center of the petri dish and the ant was introduced one minute later. When the ant was introduced, the petri dish was filmed for 10 min using a camera to record the behavioral interactions. Insects used for an experimental trial were not reused in any following trial. A total of 12 trials were conducted for each ant species to study its interaction with *C. pomonella* eggs (n = 24), while 15 trials were conducted to study the interaction of each ant species with *C. pomonella* larvae (n = 30).

The videos were subsequently analyzed using the software, Solomon Coder 19.08.02, to evaluate behavioral interactions. We recorded the following behaviors performed by the ants towards the larvae:

(i) Antennation: the ant touches the eggs/larva with its antennae while slowing or stopping nearby.
(ii) Mandible opening: the ant opens its mandibles in front of the eggs/larva without biting.
(iii) Biting: the ant bites the eggs/larva with its mandibles.
(iv) Chemical attack: the ant uses its chemical repellent to the eggs/larvae (this behavior is performed by applying the venom topically, using the spatulate stinger in *C. scutellaris* and by short distance spraying in *T. magnum*).
(v) Transportation/feeding: the ant starts to feed on the eggs/larva or transport them with its mandibles—this is considered as predation and/or as a proxy of food retrieval to the nest.
(vi) Walking over: the ant walks over eggs/larva.

The frequency of each behavior was recorded in each experimental trial. Biting and transportation/feeding were always displayed together in our observations and were therefore treated as a single behavior for the purpose of statistical analyses. Furthermore, at the end of each trial, we inspected under a stereoscopic microscope whether the eggs appeared damaged and whether the larvae were dead or injured.

We used a generalized linear model (GLM) followed by Tukey's post hoc tests to analyze the frequency of the behavioral interactions between the ants and the eggs, according to the identity of the ant species and of the behavior, considering their possible interactions. Differences between the two ant species concerning single behaviors performed were analyzed using Mann–Whitney U tests. We used a GLM with binomial distribution followed by Tukey's post hoc tests to analyze the frequency of the behavioral interactions between the ants and the larvae, according to the identity of the ant species and of the behavior, considering their possible interactions. Differences between the two ant species for single behaviors performed were then analyzed using the chi-square test. The data were analyzed using the software R 4.2.2 and RStudio [34,35].

3. Results

In the interactions between the ants and the eggs, only three behaviors were observed: antennation, mandible opening, and walking over (see Supplementary File S1). We found no significant difference in the frequency of the different behaviors ($0.864 \leq p \leq 0.997$), while *T. magnum* interacted more frequently with the eggs as compared with *C. scutellaris* ($p = 0.009$). All three behaviors were more frequently expressed by *T. magnum* as compared to *C. scutellaris* (Antennation: W = 37, $p = 0.038$; Mandible opening: W = 39.5, $p = 0.021$; Walking over: W = 29.5, $p = 0.009$; Figure 1, Supplementary File S2). No eggs were harmed by the ants during the trials.

In the interactions between the ants and the larvae, four behaviors were observed: antennation, biting and transportation/feeding, and mandible opening (see Supplementary File S1). Each behavior was observed only once per experiment. Mandible opening was performed significantly less frequently than antennation ($p = 0.023$), while no significant differences were detected between the frequency of the interaction by the two ant species ($p = 0.705$) nor between the frequency of individual behaviors (Antennation: $\chi^2 = 0.14$, $p = 0.705$; Biting and Transportation/feeding: $\chi^2 = 0.13$, $p = 0.712$; Mandible opening: $\chi^2 = 2.16$, $p = 0.142$; Figure 2, Supplementary File S2). Biting and transportation/feeding always implied that the larvae were dead by the end of the experiment; so, 43% of the larvae were killed during the 10-min trials.

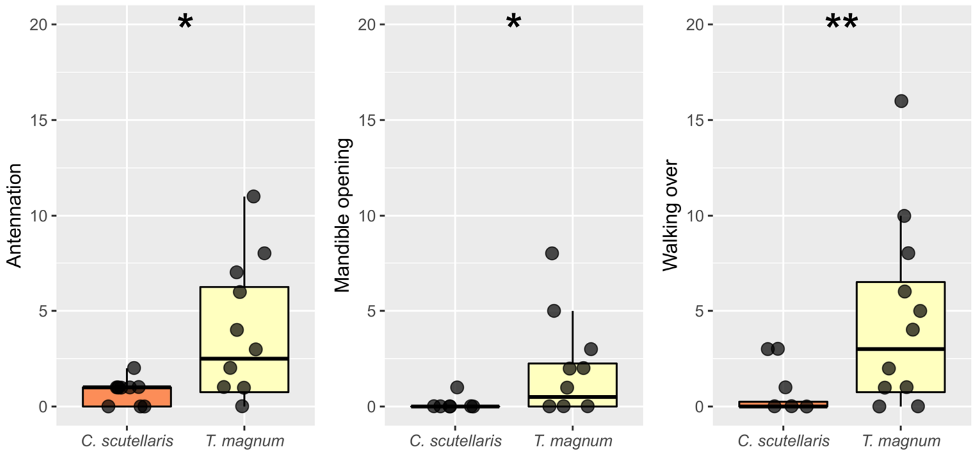

Figure 1. The interactions observed between the ants (*Crematogaster scutellaris* and *Tapinoma magnum*) and the *Cydia pomonella* eggs. Asterisks represent the significance level of the differences between the two species (*, $p \leq 0.05$; **, $0.001 < p \leq 0.01$).

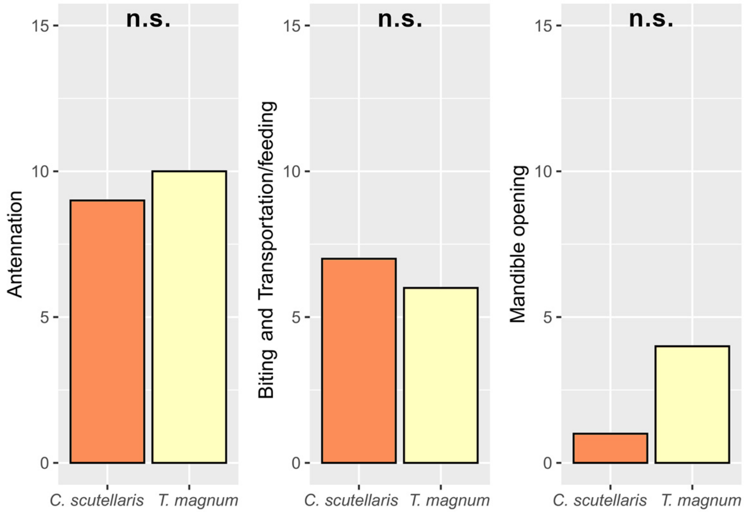

Figure 2. The interactions observed between the ants (*Crematogaster scutellaris* and *Tapinoma magnum*) and the *Cydia pomonella* larvae. No statistically significant differences (n.s.) between the two ant species were detected.

4. Discussion

Our data revealed that common Mediterranean ants may act as predators of newly hatched *C. pomonella* larvae. Newly hatched larvae are particularly vulnerable to predators, as well as temperature variation and rainfall, until they can locate and excavate into fruit, which may take from 10 min to a few hours to accomplish [25,36]. In our experiments, both *C. scutellaris* and *T. magnum* behaved similarly towards the larvae, killing them in approximately half of the short trials by repeatedly biting their soft parts and then immediately feeding on them or transporting them with their mandibles. Detection through antennation was typically followed by attacks, while in most trials in which no attacks were recorded, the larvae remained undetected. We can speculate that very small newly hatched larvae may be a more attractive and more easily encountered item for smaller ants. While both species did not attack the eggs, these attracted the attention of *T. magnum* significantly, as the workers were repeatedly observed performing stereotyped mandible threats and often kept antennating or walking over them several times. Eggs may offer little foothold to the ants' mandibles and can adhere strongly to the substratum of leaves and fruits, thus becoming physically invulnerable at least to the species we tested [37,38]. Larger ants with stronger and larger mandibles may be more capable of damaging or feeding on the eggs, but they are often less frequent in agroecosystems [32]. While we cannot entirely discard other possible mechanisms of protection (e.g., chemical repellency or insignificancy), the eggs appeared to be attractive for ants during our experiments (especially in the case of *T. magnum*), which may at least increase the chances that ant workers can take advantage of the moment they hatch to prey upon the larvae. In our experiment, whenever the ants attempted to attack a larva, the larva was always successfully killed. However, even if young larvae manage to escape ants, any delay in their effort to find and excavate fruit is expected to result in significantly higher mortality rates [36]. Based on the evidence of other ant–plant–phytophagous interactions, it is also possible that the excavation behavior by the young larvae releases semiochemicals that are attractive to ants [39].

Both ant species we used in our experiments are known to be able to act as predators of many other agricultural pest insects [31,40–42]. Potentially problematic relationships with aphids or coccids are also possible in some cases [43,44]. Manipulation of nesting site availability and trophic resource may be crucial to maximizing the benefits of these ants in biological control strategies [30,32]. Further efforts should focus on interactions between *C. pomonella* and ants in the field [18,30]. For instance, potential interference between ants and ovideposing adults has so far not been investigated but may contribute to explaining the reduction in damaged fruits in ant-visited plants [30]. In fact, in several ant species, more or less specialized workers may function as a constant "presidium", exploring and patrolling even large areas in search for suitable resources [1,45,46].

If predation of *C. pomonella* larvae in the field is confirmed to be significant, it is possible that adult moths prefer to avoid laying their eggs in ant-visited fruits even without coming into direct contact with the ants, as observed in similar interactions with fruit flies or scolytid beetles, which are mediated by semiochemicals [42,47]. In fact, it is well known that, apart from chemical trails, both arboreal and ground-dwelling ants may lay additional markers on patrolled and defended areas [48–50].

In conclusion, the predatory role of ants in temperate agroecosystems is for the most part still little understood [30,31,51,52], but due to their ubiquitous presence and generalist feeding habits, ants are likely to play a significant yet overlooked role in the control of the populations of several pest insects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects14020097/s1, Supplementary File S1: Video documentation of behavioral interactions; Supplementary File S2: Behavioral data and GLM output.

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Section I - Ants as biocontrol agents of insect pests

Chapter 3

Native European ants can discourage host colonization and reduce reproductive success of the invasive ambrosia beetle *Xylosandrus compactus*

Giannetti, D., Schifani, E., Gugliuzzo, A., Zappalà, L., Biondi, A., & Grasso, D.A. (2022). Native European ants can discourage host colonization and reduce reproductive success of the invasive ambrosia beetle *Xylosandrus compactus*. *Biological Control*, 174, 105032. https://doi.org/10.1016/j.biocontrol.2022.105032

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Native European ants can discourage host colonization and reduce reproductive success of the invasive ambrosia beetle *Xylosandrus compactus*

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HIGHLIGHTS

- Four European ant species exhibited potential in predating *Xylosandrus compactus* juveniles.
- *Temnothorax mediterraneus* is able to enter galleries and prey beetles.
- Dispersing beetle females avoid infesting twigs previously patrolled by ants.
- Biocontrol services provided by the tested ants need consideration in further field studies.

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ABSTRACT

Due to their ubiquity and their nature as generalist predators, ants have long been used as biological control agents in forest and agricultural systems. Several exotic ambrosia beetles (Coleoptera: Curculionidae: Scolytinae, Platypodinae) are considered emerging widespread pests of various trees and shrubs growing in forests, nurseries, orchards, and urban areas. Among them, the Scolytinae *Xylosandrus compactus* (Eichhoff) is an invasive fungus-farming species native to Asia and able to cause serious damage to a broad range of natural and cultivated plants worldwide, exerting significant ecological and economic costs. Its biology makes conventional control strategies often ineffective, while little is known about natural enemies. We explored the potential of native European predators as natural enemy of this pest, conducting laboratory tests with four widespread ant species using chestnut and laurel as beetle hosts. In particular, we evaluated the interactions between *X. compactus* and four species of native Euro-Mediterranean ants that usually forage on plants: *Crematogaster scutellaris* (Olivier), *Tapinoma magnum* Mayr, *Temnothorax affinis* (Mayr), and *Temnothorax mediterraneus* Ward, Brady, Fisher & Schultz. Results indicate that ants may significantly limit the reproductive success of *X. compactus*, increasing the mortality of the beetle foundresses and reducing their offspring. Smaller ant species may also invade *X. compactus* nests, killing larvae, pupae and adults, while female beetles avoid nesting in twigs previously visited by ants. These results encourage to explore possible applications of ants in the biological control of *X. compactus* and the ecological implications of these interactions in the field.

1. Introduction

Ants (Hymenoptera: Formicidae) are a highly diverse, successful and widespread insect family, whose overwhelming majority of species are opportunistic generalist predators (Hölldobler and Wilson, 1990; Parker and Kronauer, 2021). As such, they have been employed by humans for biological control in agricultural or forest ecosystems since at least the 3rd century CE and are still widely exploited for this service today (Way and Khoo, 1992; Peng and Christian, 2010; Offenberg, 2015). The polyphagous habits, territorial aggressiveness, ability to sustain starvation and the manipulability of their behavior are useful traits for ants acting as biocontrol agents of various insect pests, including beetles (Peng and Christian, 2010; Choate and Drummond, 2011; Offenberg, 2015).

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Xyleborine ambrosia beetles (Curculionidae: Scolytinae: Xyleborini) are invasive pests able to rapidly spread and establish in non-native habitats (Lantschner et al., 2020; Hulcr et al., 2021; Urvois et al., 2021a). Several non-indigenous species of this group are causing serious damage to a broad range of host trees growing in the newly invaded environments (Dzurenko et al., 2021; Mendel et al., 2021; Wang et al., 2022; Pureswaran et al., 2022). Among them, various species belonging to the genus *Xylosandrus* are considered highly successful invaders, including *Xylosandrus compactus* (Eichhoff), also known as the black twig borer (Greco and Wright, 2015; Gugliuzzo et al., 2020; Urvois et al., 2021b). This beetle, native to subtropical Asia, has become an emerging pest of several plants of the Mediterranean maquis (Vannini et al., 2017; Gugliuzzo et al., 2019a, 2019b) as well as of a wide variety of nursery ornamental trees and shrubs and cultivated plants (Greco and Wright, 2015; Gugliuzzo et al., 2019a).

Adult females of *X. compactus* commonly attack the xylem of twigs and small branches of woody plants causing necrosis, sap flow, wilting, and canopy dieback (Greco and Wright, 2015). However, unusual and heavy pest infestations of carob (*Ceratonia siliqua* L.) large branches and trunks have been reported in southern Italy (Gugliuzzo et al., 2019c). Females of this ambrosia beetle rapidly colonize the host plant by digging galleries in the xylem and inoculating mutualistic ambrosia fungi (i.e, *Ambrosiella xylebori* Brader ex Arx) which represent the food source for the developing progeny (Biedermann and Vega, 2020; Gugliuzzo et al., 2020). This tunnelling activity interrupts the transmission of water and nutrients within the plant and contributes together with secondary pathogens and fungal symbionts to the host dieback (Greco and Wright, 2015). Each foundress female remains in the entry tunnel of the brood chamber, protecting the developing progeny, and leaves the gallery about five days earlier than adult offspring (Ngoan et al., 1976; Hara and Beardsley, 1979).

The broad host range, the cryptic habit inside the host wood and its rapid spread makes conventional control strategies of this fungus-farming insect often ineffective (Gugliuzzo et al., 2021). Moreover, little is known about potential biocontrol strategies against this invasive pest. Various opportunistic predators have been reported as able to feed on different *X. compactus* biological stages but none of them have shown the ability to effectively suppress the pest populations (Sreedharan et al., 1992; Egonyu et al., 2015; Greco and Wright, 2015; Ogogol et al., 2017; Brill et al., 2021). Among them, *Callimerus* spp. (Coleoptera: Cleridae) and *Cryptamorpha desjardinsi* (Guérin-Méneville) (Coleoptera: Silvanidae) were able to feed on larvae of *X. compactus* infesting coffee (*Coffea canephora* Pierre ex A. Froehner) (Sreedharan et al., 1992; Greco and Wright, 2015). Moreover, *Carthartus quadricollis* (Guérin-Méneville) (Coleoptera: Silvanidae) and *Leptophloeus* spp. (Coleoptera: Laemophloeidae), have been reported as fortuitous predators of the black twig borer in coffee and macadamia nut (*Macadamia* spp.) crops (Brill et al., 2021). The ant *Plagiolepis* sp. (Hymenoptera: Formicidae) was reported as an indigenous predator of *X. compactus* in Uganda, able to colonize over 18 % of beetle galleries (Egonyu et al., 2015). The big-headed ant, *Pheidole megacephala* (Fabricius) (Hymenoptera: Formicidae), was also observed feeding on all *X. compactus* biological stages in laboratory bioassays, but individuals of this species were unable to enter infested galleries. However, the presence of *P. megacephala* on coffee twigs reduced beetle presence by almost 22-fold in field trials, very likely due to predation on beetle adult females before they bored into twigs (Ogogol et al., 2017).

We investigated in the laboratory the interactions between *X. compactus* and four species of native Euro-Mediterranean ants that usually forage on plants: *Crematogaster scutellaris* (Olivier), *Tapinoma magnum* Mayr, *Temnothorax affinis* (Mayr) and *Temnothorax mediterraneus* Ward, Brady, Fisher & Schultz. All species, but *T. magnum*, habitually nest in dead wooden parts of living trees (Galkowski and Cagniant, 2017; Prebus, 2017; Seifert, 2018), and *T. mediterraneus* was occasionally found inside empty *X. compactus* galleries during field survey in Italy (AG unpublished data). On the other hand, *T. magnum*, despite building its nest in the soil, often visits trees based on the habitats it lives in: it can inhabit both habitats with open vegetation (such as meadows) and habitats with trees, including fruit orchards (Campolo et al., 2015; Seifert, 2018). Workers of *C. scutellaris* are monomorphic and averagely larger in size than those of the other species, while *T. magnum* is a polymorphic species with a great size variation, and *Temnothorax* workers are monomorphic and smaller (Seifert, 2018). We carried out laboratory experiments using two plant species attacked by *X. compactus*, the chestnut *Castanea sativa* Mill. and the laurel *Laurus nobilis* L., with the aim of determining whether native ants in Europe may provide the plant they visit a defensive service against *X. compactus*, directly or indirectly interfering with the beetles.

2. Materials and methods

2.1. Origin and rearing of the beetles

Adults were caught in the field from the invaded area in Ragusa, Sicily (Gugliuzzo et al., 2022). Laboratory rearing was conducted using twigs of *C. sativa* or *L. nobilis.* Leafless twigs were collected from trees (from Fornoli (Tuscany, Italy) and Catania (Sicily, Italy)), selecting those having a diameter of 7–15 mm, and cut into sections of length 12–15 cm. Segments were soaked in a 10 % EtOH solution for 2 h soon after being cut. Then, a Parafilm® cover was applied to the two ends to minimize drying. Afterwards, they were left to dry out for 30 min. Soaking the twigs in ethanol was meant to make them more attractive to the female beetles (Castrillo et al., 2016; Gugliuzzo et al., 2022). Each twig was put into a glass tube (25x250 mm Ø) with a wet cotton plug alongside 5–8 coetaneous *X. compactus* females emerged from maternal galleries 3–5 days earlier. Tubes were kept under the following rearing conditions: T: 25 ± 1 °C, R.H.: 70 ± 5 %, 16:8-hrs light/dark photoperiod. Beetles used in the experiments were selected among those having left their nest 1–2 days before.

2.2. Origin and rearing of the ants

Ants were collected from different areas in Italy and were identified according to the keys provided by Galkowski and Cagniant (2017) and Seifert (2018). The identification of *T. magnum*, within the cryptic *nigerrium* complex was tentatively based on its geographic distribution and ecology (Seifert et al. 2017). Colonies of *C. scutellaris* (workers and queen) were collected in Sicily (Corleone) and Tuscany (Fornoli) (N = 16). Large groups of workers (>1,000) from *T. magnum* polygynous supercolonies were collected in Sicily (Biancavilla) and Emilia-Romagna (Parma) (N = 2). Colonies of *Temnothorax* spp. (workers and queen) were taken from *Andricus* oak galls in Sicily (*T. mediterraneus* from Corleone, N = 14) and Emilia-Romagna (*T. affinis* from Parma, N = 6). Ants were kept in plastic boxes and fed with honey and insect proteins (*Drosophila melanogaster* Meigen or *Tenebrio molitor* L.) under the following rearing conditions: T: 25° ± 1 °C, R.H.: 55 ± 10 %, 16:8-hrs light/dark photoperiod. Before being used in the experiments, colonies were kept under laboratory conditions for at least seven days and went through a 48-hr starvation period, in order to increase their willingness to accept food.

2.3. Behavioral interactions during 1vs1 encounters

One ant worker and one beetle were placed in a 35 × 10 mm arena and their interactions were recorded for 3 min by HD cameras. Tests were performed with each of the four ant species and the three development instars of the beetles (larvae, pupae and adult females). Thirty replicates per treatment were carried out ($N = 4 \times 3 \times 30 = 360$) using different randomly selected ant individuals in each replicate. Videos were analyzed using the software Solomon coder (version 17.03.22), and the following behaviors were recorded from the ants: *i*) antennal contact with the beetle; *ii*) threat posture with open mandibles towards the beetle; *iii*) bites directed against the beetle; *iv*) aggressive interactions performed with the gaster (gaster flexing/extension, stinger use or chemical spraying). A beetle was considered preyed by the ant if the latter carried it away in its mandibles.

2.4. *Exposure of beetle nests to ant colonies*

The experimental apparatus consisted in a 21 × 15 × 7 cm arena. Except for the control treatment, the arena was occupied by a group of ants (*C. scutellaris* or *T. magnum*, 60 workers) or a colony (*Temnothorax* spp., 60 workers and one queen). In each arena, we placed a twig colonized 15 days earlier by 6 female beetles because, at the rearing conditions used in the experiments, 15 days were enough to ensure the presence of developing progeny inside infested galleries (Antonio Gugliuzzo unpublished data). The experiment started after the twig was put in the arena and lasted 35 days. To observe ants' behavior, after the start, we performed a 2-hr long continuous sampling followed by 10 min continuous sampling 6-hr later, and by two scan samplings per day (at 10:00 and 16:00) on each of the following days. During each observation, we recorded the number of ants in a 1 cm range from each of the beetles' nests, the number of ants entering the nests and the number of *X. compactus* individuals outside the nests (dead or alive). Finally, we also quantified the number of beetle offspring reaching the adult stage within each nest. We conducted a number of 6 replicates per each ant species and each of the two plant species (using *T. affinis* in trials with *C. sativa* and *T. mediterraneus* in trials with *L. nobilis,* N = 6 × 3 × 2 = 36). In addition, 18 control groups, with no ant for each of the two plant species (N = 18 × 2 = 36), were carried out.

2.5. *Chemical attractiveness of infested twigs to ants*

We tested the olfactory response of *T. mediterraneus*, *C. scutellaris* and *T. magnum* to different volatiles related to *X. compactus* infestation via dual-choice experiments using a Y-tube olfactometer with the characteristics illustrated by Naselli et al. (2017). Considering that its performance was extremely similar to *T. mediterraneus* in the other experiments, *T. affinis* was not tested in this experiment. Each ant worker was individually introduced into the entry arm of the Y-tube and observed until it reached the end of one of the two arms (making a choice) or until five minutes passed (no choice made). For each of the three ant species and three beetle stages, different worker individuals belonging to at least six different colonies were tested, and a minimum of 30 choices were recorded. The bioassays were conducted between 08:00 and 20:00, at 23 ± 2 °C and 60 ± 10 % R.H.. Three combinations of odor sources were tested: *i*) laurel twig section predominantly containing beetle larvae vs control (not infested); *ii*) laurel twig section predominantly containing pupae vs control; *iii*) laurel twig section predominantly containing adults vs control. Twig sections were prepared as described in the rearing section above, but they were 5 cm long and infested by a single female. Based on data collected under the same rearing conditions, we used twigs infested 18–21 days before for larvae, 27–30 days before for pupae and 36–39 days before for adults. After each trial, twigs were replaced with new ones, and were dissected to verify their content: if this did not coincide with the expected, the trial was repeated.

2.6. *Effects of chemical residuals of ant patrolling on twig colonization by beetles*

The experimental apparatus consisted in a small arena (21 × 15 × 7 cm) where two twigs were put on the two opposite sides while a group of four beetle foundresses was placed in between (10 cm away from either twig). One of the twigs was previously exposed to patrolling ants, while the other was not (control), and the experiment lasted 24 hrs starting when the beetles were introduced. Then, the number of beetles who bored either twig was recorded. Twigs were prepared according to the same procedure described in the rearing section. Tests were conducted for all the four ant species and with both *C. sativa* and *L. nobilis* twigs, but *T. affinis* was only tested with *C. sativa* and *T. mediterraneus* only with *L. nobilis* (N = 12 × 3 × 2 = 72). To expose them to patrolling ants, they were put in an arena with an ant colony for 48 hrs. During this time, we assessed the number of ants walking on each twig with scan samplings (one every hour for 16 hrs and then one after 24 hrs and one after 48 hrs). As a result, we quantified this number as following (given as mean ± sd): on *C. sativa* twigs, 143 ± 12 for *C. scutellaris*, 227 ± 9 for *T. magnum*, and 24 ± 14 for *T. affinis*; on *L. nobilis* twigs, 90 ± 10 for *C. scutellaris*, 118 ± 9 for *T. magnum*, and 28 ± 5 for *T. mediterraneus*.

2.7. *Data analysis*

For each behavior (concerning the behavioral interactions during 1*vs*1 encounters, section *2.3.*), differences in its frequency between different ant species were analyzed using Kruskal-Wallis tests followed by Dunn post hoc tests whenever the firsts indicated significant differences.

Concerning the exposure of beetle nests to ant colonies (section 2.4), statistical analyses were carried out separately for *C. sativa* and *L. nobilis*. Data concerning the number of *X. compactus* foundresses found dead in the arenas in treatments with different ant species were compared through Kruskal-Wallis tests followed by Dunn post hoc tests (each value corresponded to an experimental arena). On the other hand, differences in the number of ant workers near the entrance of the beetle nests and in the number of beetle offspring per nest were compared using a linear mixed model in which the experimental arena where data were taken was set as the random factor (each value corresponded to an individual nest, with multiple nests per arena). These were followed by Tukey post hoc tests whenever significant differences between treatments with different ant species were detected.

Data concerning the chemical attractiveness of infested twigs to ants (section 2.5) were analyzed to evaluate the possible preference showed by the three ant species toward different *X. compactus* biological stages during Y-tube olfactometer bioassays. In particular, Chi-squared goodness-of fit tests were conducted in order to assess whether the ant response to different volatile sources was significantly different from a 50:50 distribution. Ant workers that did not make a choice were not considered in the analyses. Similarly, data concerning the effects of chemical residuals of ant patrolling on twig colonization by beetles (section *2.6.*) were analyzed by running separate Chi-squared tests comparing beetles' choice between ant-exposed and control twigs for each combination of plant and ant species utilized. Beetles who did not made a choice were not considered in the analyses. Analyses were carried out using the software R v4.1.1 (R Core Team, 2021) and RStudio v2021.09.0 351 (RStudio Team, 2021).

3. Results

3.1. *Behavioral interactions during 1vs1 encounters*

Interactions between ants and beetle larvae and pupae resulted in the latter being immediately taken and carried away as prey without any complex behavioral display on at least 90 % of tests. *Crematogaster scutellaris* preyed upon 90 % of both larvae and pupae, *T. magnum* 93 % of larvae and 97 % of pupae, *T. affinis* 90 % of both larvae and pupae, and *T. mediterraneus* 100 % of both larvae and pupae.

On the other hand, interactions between ants and adult beetles were characterized by a more complex behavioral pattern. Antennation, open-mandibles threatening and biting were the most frequently recorded behaviors (respectively recorded during 88 %, 69 % and 39 % of the trials). The frequency of exhibition of the three behaviors was statistically different among the four tested ant species (antennation: $H_3 = 25.92, p < 0.001$; open-mandibles threatening: $H_3 = 27.02, p < 0.001$; biting: $H_3 = 14.78$, $p = 0.002$), with *C. scutellaris* and *T. magnum* performing them more frequently. In particular, pairwise comparisons revealed that each species differed from the others for antennation frequency ($p < 0.001$). On the other hand, concerning open-mandibles threatening, no statistically significant differences were detected between *T. affinis* and *T. mediterraneus* ($p = 0.945$), nor between *C. scutellaris* and *T. magnum* ($p = 0.710$). However, differences were significant between the two groups ($0.006 < p < 0.001$). Finally, significant differences were detected for biting between *C. scutellaris* and *T. affinis* ($p = 0.046$), between *C. scutellaris* and *T. mediterraenus* ($p = 0.011$), and between *T. mediterraneus* and *T. magnum* ($p = 0.023$), but not in the other pairwise comparisons ($1.000 < p < 0.072$) (Fig. 1).

3.2. Exposure of beetle nests to ant colonies

Beetle nest entrances attracted workers of all ant species, and their attractiveness to different ant species differed significantly (*C. sativa*: $F_{2,15} = 98.24$, $p < 0.001$; *L. nobilis*: $F_{2,15} = 26.54$, $p < 0.001$). In particular, in both the *C. sativa* and the *L. nobilis* trials, *Temnothorax* spp. workers were more numerous around the nest entrances compared to those of *C. scutellaris* and *T. magnum* ($p < 0.001$), while *C. scutellaris* and *T. magnum* differed one from the other in *C. sativa* trials ($p < 0.001$) but not in *L. nobilis* trials ($p = 0.929$).

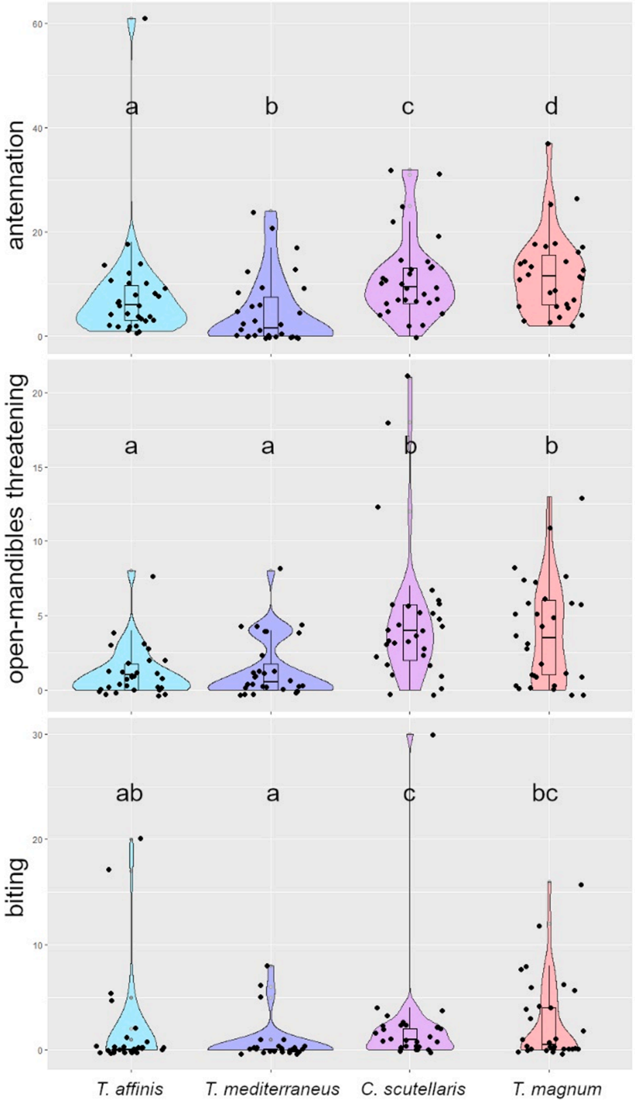

Fig. 1. Most frequently recorded behavioral interactions of ant workers in front of *X. compactus* adults. Groups with the same latter are not statistically different.

Nests showed significantly different mortality rates of beetle foundresses according to treatment (*C. sativa*: $H_3 = 27.77$, $p < 0.001$; *L. nobilis*: $H_3 = 31.57$, $p < 0.001$). The highest mortality scores were always recorded in the presence of *Temnothorax* spp., intermediate scores characterized tests with *C. scutellaris*, further lower scores were recorded in the presence of *T. magnum*, and the lowest mortality scores were recorded in the absence of ants (Fig. 2).

The beetles' reproductive success was on average greater in *L. nobilis* trials than in *C. sativa* trials (as mean ± sd: 20.5 ± 9.5 in *L. nobilis*; 7.1 ± 4.4 in *C. sativa*), but in both cases it was significantly affected by treatment (*C. sativa*: $F_{3,32} = 29.05$, $p < 0.001$; *L. nobilis*: $F_{3,32} = 78.42$, $p < 0.001$). In both, it was lowest in the presence of *Temnothorax* spp. and *C. scutellaris* (not different one from the other, $0.521 < p < 1.000$, and different from all the other treatments, $p < 0.001$), and in almost all cases highest in the absence of ants ($p < 0.001$). However, in treatments with *T. magnum*, the recorded reproductive success was either intermediate between that of treatments with other ants and the control group (in *L. nobilis* trials, $p < 0.001$), or not statistically different from the control group (in *C. sativa* trials, $p = 0.998$) (Fig. 2).

Finally, on two occasions during scan samplings, *T. mediterraneus* was observed entering the beetles' nests, its workers carrying away pieces of dead adult beetles and the whole colony transferring inside the nest in one case (Fig. 3) (Supplementary Video).

3.3. Chemical attractiveness of infested twigs to ants

Crematogaster scutellaris and *T. magnum* showed no significant preference for volatiles cues emitted by healthy stem sections nor those infested by the beetle (Fig. 4a). In particular, workers of *C. scutellaris* did not show any preference for volatiles emitted by stem sections infested by *X. compactus* larvae ($\chi^2 = 0.937$, $p = 0.333$), pupae ($\chi^2 = 0$, $p = 1$) or adults ($\chi^2 = 0.137$, $p = 0.711$) when compared with those of not infested stem sections. Similarly, volatiles related to the *X. compactus* infestation were not significantly attractive compared to those emitted by healthy laurel stem sections for *T. magnum* workers (larvae: $\chi^2 = 2.041$, $p = 0.153$, pupae: $\chi^2 = 0.082$, $p = 0.775$, adults: $\chi^2 = 0.657$, $p = 0.417$). By contrast, *T. mediterraneus* workers were significantly attracted by the volatiles emitted by laurel stem sections infested by beetle larvae compared to those of not infested stem sections ($\chi^2 = 9.766$, $p = 0.002$) (Fig. 4a). However, when the dual choice was between healthy stem sections and stem sections infested by beetle pupae, workers of this ant species did not show any preference ($\chi^2 = 0$, $p = 1$). Lastly, *T. mediterraneus* workers showed a significant preference for volatiles emitted by not infested stem sections when compared with those of stem sections infested by *X. compactus* adults ($\chi^2 = 14.063$, $p < 0.001$) (Fig. 4a).

3.4. Effects of previous ant patrolling on twig colonization by beetles

From 4 to 39 % of the beetle foundresses did not make any choice. Analyses of the choice data revealed a clear foundresses preference for twigs that were not previously exposed to ants ($p < 0001$) (Fig. 4b).

4. Discussion

The lack of coevolved natural predators can be a major facilitating factor in biological invasion processes (Pyšek et al., 2020; Yousuf et al., 2021; Desneux et al. 2022). Ubiquitous generalist predators, such as ants, may offer an important service in this regard, and their study as biocontrol agents in Europe is still little explored (Campolo et al., 2015; Castracani et al., 2017; Nielsen et al., 2018; Schifani et al. 2020; Bulgarini et al., 2021). Invasive exotic ambrosia beetles may cause alteration of native tree communities, reduced yields, and increased control costs (Grousset et al., 2020, Hulcr et al., 2021; Marchioro and Faccoli, 2021, Mendel et al., 2021). This is particularly true for some invasive

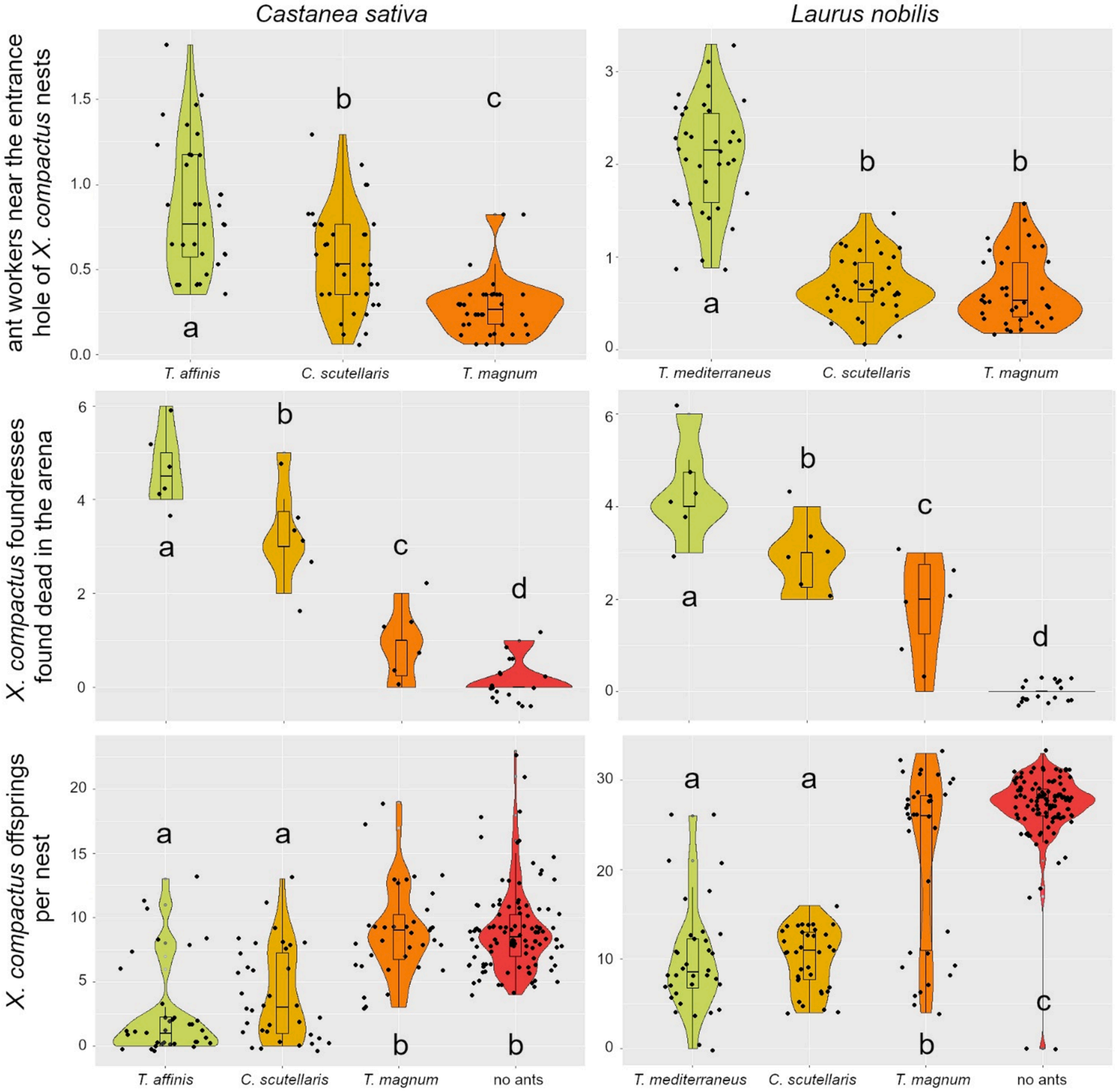

Fig. 2. Attraction of ants to *X. compactus* nest entrances, effect of ants on the mortality of *X. compactus* foundresses, and reproductive success of *X. compactus* nests exposed to ant colonies. Groups with the same latter are not statistically different.

Xylosandrus spp. which biological invasions has the potential to cause a cascade of impacts both at ecological and economic level (Gugliuzzo et al., 2021). Moreover, the peculiar biology and cryptic nature (i.e., completing their lifecycle within galleries inside the wood) of this ecological group of insects typically protects them from many potential predators. However, the relationship between Scolytinae beetles and predators, such as ants, has been rarely investigated, mostly with the coffee berry borer *Hypothenemus hampei* (Ferrari, 1867) rather than Xyleborini (e.g. Larsen and Philpott, 2010; Gonthier et al., 2013).

All the ant species we tested in 1*vs*1 encounters showed a high interest in predating immature stages of *X. compactus* (larvae and pupae), yet aggression against adults was very limited and only rarely damaging for the beetles, with the larger species, *C. scutellaris* and *T. magnum*, performing more attacks. Larvae and pupae are normally hidden deep inside *X. compactus* galleries, which are narrow and guarded by an adult, i.e., the foundress mother. Nonetheless, when beetle nests were exposed to ant colonies, all ant species had a meaningful impact, with ant workers always surrounding the nests' entrances and significantly increasing the mortality of the foundress mother. Albeit weaker in the case of *T. magnum*, this result was associated with a much reduced reproductive success of the beetles. The premature death of the foundress when beetle nests were exposed to ant colonies likely deprived the offspring and the nest of the necessary cares that the mother usually performs (controlling the hygienic conditions and taking care of the mutualistic fungus), greatly limiting their ability to develop into adults. This result appears coherent to what reported by Ogogol et al., (2017), who witnessed a control effect on *X. compactus* populations by *P. megacephala* despite the latter's inability to enter the beetles' nests. However, one of the two smaller ant species used in our tests, *T. mediterraneus* was able to enter the nest galleries, dragging outside larvae, pupae and dismembered adults.

Our data suggest that volatiles may have very little influence over ants' ability to locate *X. compactus* nests. Indeed, results of olfactometer bioassays show that volatiles related to the beetle infestation do not affect the orientation behavior of *C. scutellaris* and *T. magnum* workers. On the other hand, *T. mediterraneus* workers exhibited significant attraction for volatiles released by laurel stem sections infested by *X. compactus* larvae, but not for those emitted by stems infested by pupae. Furthermore, volatiles emitted by stems which galleries were infested by beetle adults were not attractive for workers of *T. mediterraneus* that preferred healthy stem sections. This result could be related to volatile substances potentially involved in the beetle adult aggregation inside galleries that may have affect the orientation of ant workers. Aggregation pheromones are used by many herbivorous insects

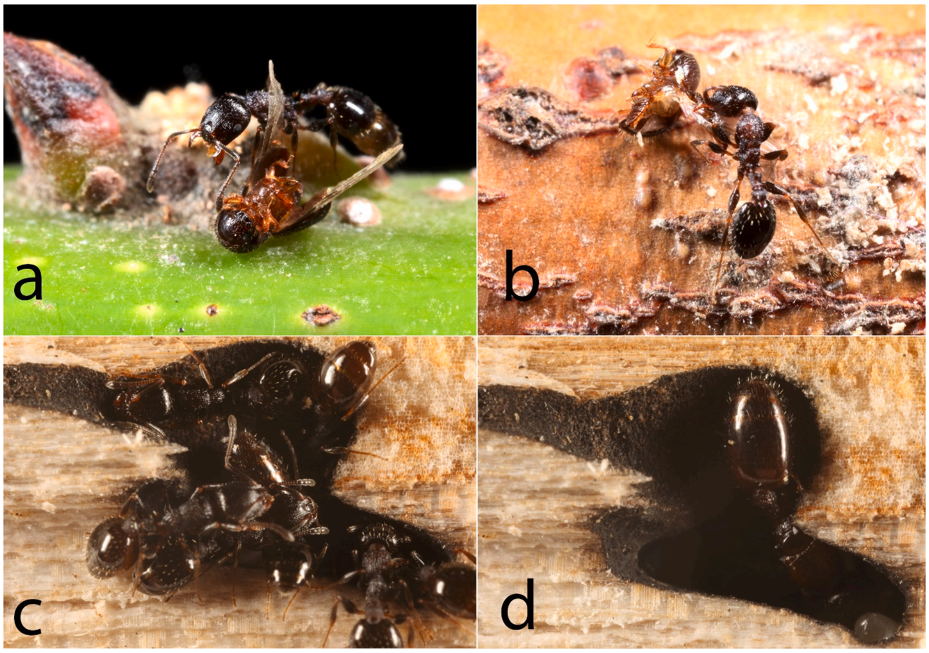

Fig. 3. Interactions between *T. mediterraneus* and *X. compactus*. a,b *T. mediterraneus* workers drag out dead and partly dismembered *X. compactus* adults out of their galleries. c,d *T. mediterraneus* colony occupying a *X. compactus* nest after eliminating the beetles: workers (c) and queen (d) shot after cutting a section of the twig.

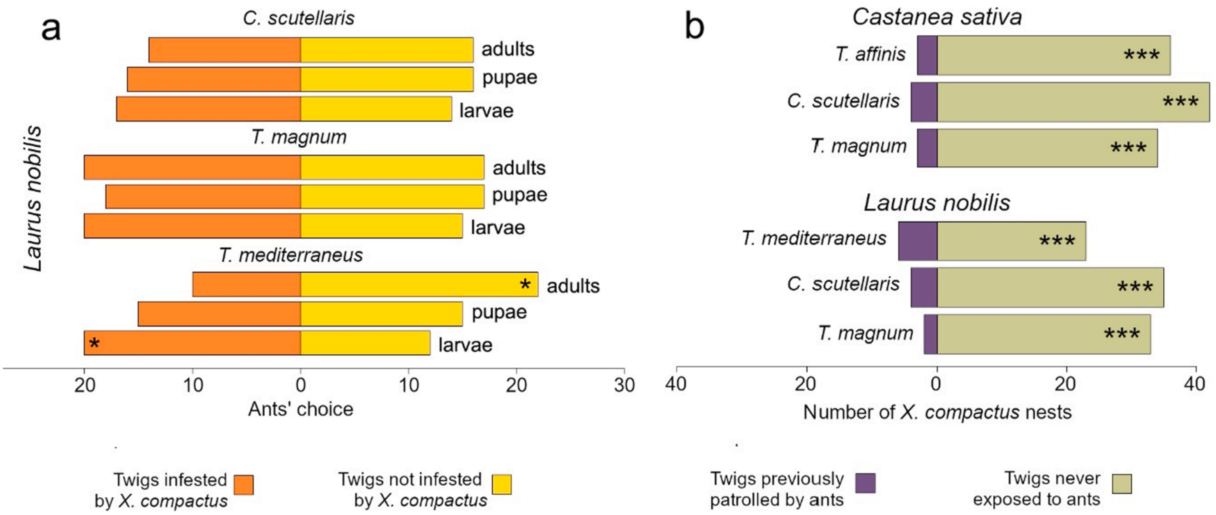

Fig. 4. a) Choice by ant workers among twigs infested by *X. compactus* or not. b) Choice by *Xylosandrus compactus* among twigs previously exposed or unexposed to ants. Asterisks indicate statistical significance of the differences (*: $p < 0.05$; **: $p < 0.005$; ***: $p < 0.0001$).

exhibiting a pheromone-based colonization behavior, including some bark beetle species (Meurisse et al. 2021). On the other hand, these semiochemicals can act as prey-finding kairomones by predators or parasitoids (Scala et al. 2022). However, to the best of our knowledge, there is no evidence of sex or species-specific aggregation pheromone occurrence for most ambrosia beetle species, and despite they could not be ecologically relevant for other species of this group of insects (Kirkendall et al., 1997), it could be worthy investigating this aspect in mass-aggregating *Xylosandrus* species. Moreover, it is possible to hypothesize that other volatile sources, e.g., those produced by mutualistic fungi, related to the wood infestation by these fungus-farming insects (Egonyu and Torto 2018; Ranger et al. 2021), could be involved in the predator–prey interactions. At the same time, our results show that *X. compactus* females would strongly avoid building their nests in twigs that retain the chemical trace of ant activity. Ant trails may be widespread and stable chemical signposts (e.g. Hölldobler and Wilson, 1990; Grasso et al., 1998, 1999) offering the beetles useful cues to avoid unsuitable nesting places, and ant cues may have a strong deterring effect against phytophagous insects (Offenberg et al., 2004; Adandonon et al., 2009; Vayssières et al., 2013).

The results of this study indicate a potential role of native ant species to limit the success of this invasive pest in Europe and in the Mediterranean. It is worth noting that the only ant species so far clearly identified as an effective predator of *X. compactus* was the Afrotropical big-headed ant *P. megacephala* (Ogogol et al., 2017), an invasive species of serious ecological concern in much of the world (Sarnat et al., 2015). In the study by Egonyu et al. (2015) *Plagiolepis* sp. showed to be a potential efficient predator of *X. compactus* in Uganda; however, the ant pictures published in the article seems to be related to ants of the genus *Cardiocondyla*. European *Plagiolepis* species are considered mostly glicliphagous rather than predatory, while *Cardiocondyla* species in Europe do not forage on trees (Seifert, 2018). On the other hand, *Crematogaster* species of the *scutellaris* group and arboreal-nesting *Temnothorax* species are both widespread in the Holarctic region (Prebus, 2017; Ward and Blaimer, 2022). In Italy, *C. scutellaris*, *T. affinis*, and *T. mediterraneus* are commonly observed foraging on *C. sativa* and/or *L. nobilis* (authors unpublished data).

Our data encourage to further investigate the interactions between ants and ambrosia beetles and to extend this investigation to other ant species and genera, especially considering that we obtained comparable results from ants belonging to rather different evolutionary lineages. In the case of *X. compactus*, such investigations should now continue in the field, to verify the potential use of ants as biocontrol agents or the actual ecological consequences of these interactions. In addition, the possible roles of the mutualistic fungi associated to Xyleborini in the relations between the beetles and other arthropods such as ants deserve further investigations.

In conclusion, the pervasive ecological role of ants as generalist predators across terrestrial habitats may have had an evolutionary impact on ambrosia beetles too (Parker and Kronauer, 2021). The results of our study, which is the first to investigate potential natural enemies of *X. compactus* in the Euro-Mediterranean region, encourage to take into account the predatory role of ants in management strategies aimed at controlling this invasive pest.

CRediT authorship contribution statement

Daniele Giannetti: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Enrico Schifani:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Antonio Gugliuzzo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Lucia Zappalà:** Conceptualization, Resources, Writing – review & editing. **Antonio Biondi:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Donato A. Grasso:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Video on *Temnothorax mediterraneus* predation on *Xylosandrus compactus* infested twig.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2022.105032.

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Section I - Ants as biocontrol agents of insect pests

Chapter 4

Interactions between egg parasitoids and predatory ants for the biocontrol of the invasive brown marmorated stink bug *Halyomorpha halys*

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Interactions between egg parasitoids and predatory ants for the biocontrol of the invasive brown marmorated stink bug *Halyomorpha halys*

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Abstract

The brown marmorated stink bug *Halyomorpha halys* is an Asian species that has become a major agricultural pest in North America and Europe. Ants from the genus *Crematogaster* are predators of *H. halys* nymphs in Asia, as well as in the Mediterranean, where known native predators are still few. At the same time, ants usually do not harm *H. halys* eggs, which are the target of the main biological control agents, the scelionid parasitoids of the genus *Trissolcus*. However, ants, as generalist predators and territorial organisms, may kill or displace a variety of other insects, potentially interfering with parasitoids and biological control programmes. We conducted laboratory experiments to investigate the interactions between the Mediterranean ant *Crematogaster scutellaris* and the parasitoids *T. japonicus* and *T. mitsukurii*, evaluating the possibility that the ants could damage the parasitized eggs, attack the parasitoids during emergence or interfere with the egg-laying behaviour of female parasitoids. Our results demonstrate that *C. scutellaris* is not able to damage parasitized eggs and is not aggressive towards adult parasitoids at any stage. The presence of ants can slow down the parasitization rate in *T. mitsukurii* females in the smallest laboratory setups; however, this has not been observed in a more natural setting. We suggest that ants may play a complementary role together with egg-parasitoids in the control of *H. halys* without interfering with each other.

KEYWORDS
Crematogaster scutellaris, intraguild relationships, *Trissolcus japonicus*, *Trissolcus mitsukurii*

1 | INTRODUCTION

The brown marmorated stink bug *Halyomorpha halys* (Stål), native to Eastern Asia and with invasive populations throughout the European, northern and southern American continents, is currently one of the most dangerous pests of fruit and seed crops (Leskey & Nielsen, 2018). Its high invasive capacity is facilitated by human activities and trade (Maistrello et al., 2018) and by high polyphagy (Rice et al., 2014), high mobility of the adults (Lee & Leskey, 2015), and high reproductive potential (Costi et al 2017). In Italy, where it

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Enrico Schifani and Daniele Giannetti contributed equally to this study.

was first officially detected in 2012 (Maistrello et al., 2016), *H. halys* quickly became a key pest of fruit orchards (Maistrello et al., 2017); and in 2019, the estimated damage to fruit production in northern Italy was € 588 million, with yield losses of up to 80%–100% in orchards (CSO Italy, 2020). To counter this invasive pest, the use of broad-spectrum insecticides has increased dramatically, resulting in a major disruption to previous integrated pest management (IPM) programmes with negative consequences on the environment (Maistrello et al., 2017). Long-term and more sustainable management strategies include conservation and classical biological control.

In native Asia, *H. halys* egg masses are attacked by different species of egg parasitoids, among which the Scelionidae *Trissolcus japonicus* (Ashmead) and *T. mitsukurii* (Ashmead) have the highest specificity and parasitization efficiency, ranging between 50% and 90% (Qiu, 2010; Yang et al., 2009; Zhang et al., 2017). In Northern Italy, adventive populations of *T. mitsukurii* and *T. japonicus* were first detected in 2016 (Scaccini et al., 2020) and 2018 (Sabbatini Peverieri et al., 2018), respectively. A large-scale survey conducted throughout northern Italy and Switzerland in 2019, showed that both species had rapidly spread into all types of habitats where *H. halys* is present, with a wide distribution, continuous expansion and high levels of parasitism (Zapponi et al., 2021). Furthermore, in 2020, *T. japonicus* was selected by the Italian Ministry of Environment and the Protection of the Land and Sea as a candidate for classical biocontrol of the invasive pest (MATTM, 2020) and thousands of these parasitoids were released in the northern Italian regions for 3 years, leading to one of the largest biocontrol projects ever attempted in Italy and Europe. Meanwhile, laboratory studies conducted to verify the potential of generalist antagonists showed that ants are among the most efficient predators of *H. halys* (Bulgarini, Badra, et al., 2021; Bulgarini, Castracani et al., 2021; Castracani et al., 2017). Specifically, experiments with the two European ants most frequently encountered in agroecosystems, *Crematogaster scutellaris* (Olivier) and *Lasius niger* (Linnaeus), demonstrated their ability to kill *H. halys* nymphs without damaging eggs or adult stink bugs (Bulgarini, Castracani, et al., 2021; Castracani et al., 2017). Further studies conducted with the Japanese ant *Crematogaster matsumurai* Forel, 1901 and *C. osakensis* Forel, 1900 as well the cosmopolite invasive Argentine ant *Linepithema humile* (Mayr, 1868), had a similar outcome (Kamiyama et al., 2021).

This study aims to investigate the interactions between the native European ant *C. scutellaris* and the exotic egg parasitoids *T. japonicus* and *T. mitsukurii* in terms of the outcome on the efficiency of biological control of *H. halys*.

Ants are ubiquitous across most terrestrial ecosystems of the world, where they often play a significant ecological role as generalist predators (Hölldobler & Wilson, 1990; Lach et al., 2010; Parker & Kronauer, 2021). Their predatory abilities against insect pests can not only make them good key biological control agents (Choate & Drummond, 2011; Offenberg, 2015) but also may negatively affect other important biocontrol agents, including both parasitoids and predators (e.g. Appiah et al., 2014; Jiggins et al., 1993; Mgocheki & Addison, 2009).

We hypothesized that ants may attack *H. halys* egg parasitoids as they do with *H. halys* nymphs (Bulgarini, Castracani, et al., 2021; Castracani et al., 2017). In particular, we investigated two moments in the life of adult parasitoids in which they could be particularly vulnerable: the moment in which the female parasitoid lays her eggs, which requires her to stand still on the stink bug egg mass for an extended time, and the moment of emergence of the newly metamorphosed individuals, as they need time to break an opening in the stink bug egg to free themselves. We also tested whether parasitized eggs might be more susceptible to ant attack than non-parasitized eggs, which are usually not attacked, and whether their attractiveness to ants could vary over time.

2 | MATERIALS AND METHODS

2.1 | Insect rearing and equipment

Adults of *Halyomorpha halys* were collected during the spring and summer of 2020–2021 from urban parks in Modena and Reggio Emilia provinces (Emilia-Romagna, Italy) using the tree-beating technique. Stink bugs were set in BugDorm cages (17.5 × 17.5 × 17.5 cm) and placed in climatic chambers at 26°C and L16: D8. Each cage contained up to 50 adults with a sex ratio of 50:50. The stink bugs were fed twice a week with fresh organic fruits and peanuts. Sheets of filter paper were placed in the cage as egg-laying substrates. Freshly laid egg masses of *H. halys* (<24 h old) with 27–28 eggs were used for the experiments. Rarer egg masses with different numbers of eggs were excluded.

Trissolcus japonicus and *T. mitsukurii* adults were obtained from field-collected *H. halys* egg masses and were reared in BugDorm cages (12 × 12 × 12 cm) in climatic chambers at 23°C and L16: D8, and fed with drops of a honey-water solution (70% organic honey solution). Every 3 days, freshly laid egg masses of *H. halys* (<24 h old) were offered to the parasitoids. The parasitized egg masses were individually transferred to empty vials and stored at 26°C and L16: D8 pending the emergence of the parasitoids. The newly emerged parasitoids of each species were mated (one female and one male) for 1 week in vials (Falcon 50 mL, the lid of which was replaced by a piece of pantyhose fixed with an elastic band) and supplied with drops of the honey-water solution. After the mating period, the females of each species were used in the experiments.

Colony fragments consisting in several hundred workers of *Crematogaster scutellaris* (Olivier, 1792) were collected in the wild from Parma (Italy) and reared in plastic cages under the following conditions: T: 25° ± 1 C, RH: 55 ± 10%, L16: D 8. They were fed with the same honey-water solution used for parasitoids and with *Tenebrio molitor* Linnaeus larvae. Ants endured a 48 h starvation period prior to the experiments.

All tests were conducted in a climatic chamber at 26°C and L16:D8 in the Laboratory of Applied Entomology of the University of Modena and Reggio Emilia.

All video recordings were performed using an HC-V380 Panasonic camera. A binocular microscope Zeiss Stemi 508 was used to verify if ants and/or parasitoids were alive after the experiments.

2.2 | Experimental procedure

We carried out three experiments. Experiment I aimed at verifying whether parasitized eggs and emerging parasitoids can be attacked and damaged by ants. Experiments II and III aimed at evaluating whether ants and adult parasitoids behave aggressively in a simplified context (one-to-one interactions in a Petri dish) and a more complex system (a parasitoid couple, a larger number of ants, and a plant) respectively. In the simplified context of Petri dishes, single workers of *C. scutellaris* retain their basic foraging behaviours, killing and carrying away prey insects (e.g. Giannetti et al., 2022; Schifani, Giannetti, & Grasso, 2023; Schifani, Peri, Giannetti, Alınç, et al., 2023; Schifani, Peri, Giannetti, Colazza, & Grasso, 2023). In all experiments, we counted the number of sting bugs and parasitoids that emerged from the eggs, and the number of surviving parasitoids.

2.3 | Experiment I: Interactions between ants and parasitized eggs or emerging parasitoids

To verify whether parasitized eggs attracted the interest of ants, we prepared egg masses in which parasitization of all eggs by *T. japonicus* or *T. mitsukurii* was established during preliminary observations. Specifically, after introducing a parasitoid female to each egg mass, its activities were video-recorded and the number of markings was checked. The following behaviours have been observed: probing the host, inserting the ovipositor and performing head-pumping movements and body vibrations associated with egg-release, partially extracting the ovipositor and sweeping it over the surface of the host egg with 'figure 8'-shaped movements, as described by Field (1998).

Each egg mass was transferred in the centre of a Petri dish (⌀ = 9 cm), which was followed by the introduction of a single ant worker. The petri dish was then filmed for 40 min to collect behavioural data, after which the ant was removed. Egg masses were exposed to ants after either 0, 2, 4, 6 or 9 days after parasitization, to test the behaviour of ants towards parasitized eggs at different development stages, or during parasitoid emergence, to test ant behaviour towards emerging adults. Six replicates were performed for each developmental stage of each parasitoid species, both for the treatment (presence of the ant) and for the control (no ant).

2.4 | Experiment II: 1 versus 1 interactions in petri dishes (40 min)

Tests were conducted by placing a non-parasitized egg mass in the centre of a Petri dish (⌀ = 9 cm) and introducing a single female parasitoid. As soon as the parasitoid made its first contact with the egg mass, we introduced an ant worker. Once the ant was introduced, we filmed the petri dish for 40 min to collect behavioural data. No ants were introduced into the control replicates, and filming started as soon as the parasitoid made its first contact with the egg mass. At the end of each test, we checked under the microscope whether the ant and the parasitoid were still alive and if any of them had suffered visible injuries. We conducted 10 treatment replicates with ants and 10 control replicates (no ants) for each of the two parasitoid species.

2.5 | Experiment III: Interactions in insect cages (24 h)

Tests were conducted using a 30 × 30 × 30 cm insect cage. At the centre of each cage, we placed the following items: (i) a *Capsicum annuum* L. plant (approximately 15 cm tall); (ii) a Falcon vial containing a female and a male parasitoid of either *T. japonicus* or *T. mitsukurii*; (iii) a plastic jar (⌀ = 4 cm, height = 7 cm) containing a group of 50 ant workers, partially filled with small wood pieces, and with the inner upper edge covered with an ant repellent substance (50% glycerine oil, 50% petroleum jelly) to prevent their escape. To start the experiments, we performed the following steps: (i) on an apical leaf of each plant we clipped a 1 × 3 cm filter paper with a single egg mass previously attached with a glue stick; (ii) we placed a 12 cm wooden stick to connect the plant on one hand and the wood pieces in the plastic jar on the other, allowing the ants to get out of the jar and visit the plant; (iii) we opened the lid of the vial, allowing the two parasitoids to move freely inside the cage. Each experimental test lasted 24 h, after which we removed the egg masses and the parasitoids and checked whether the latter were alive or dead. The egg masses were incubated until they hatched, or parasitoids emerged.

We conducted 24 replicates per parasitoid species (*T. japonicus* or *T. mitsukurii*), equally divided between replicates with ants and control replicates without ants.

2.6 | Behavioural data

The behaviour of ants and parasitoids was analysed by video-recording the experiments and analysing the resulting videos with the software Solomon Coder (https://solomon.andraspeter.com/).

Concerning ants, we recorded the time between their entry into the experimental arena and their first contact with the eggs or parasitoids (contact latency), and the number of times the following six behaviours, directed towards the eggs or the parasitoids as targets, were observed: (i) *antennation* (making contact with the antennae); (ii) *biting with mandibles*; (iii) *licking*; (iv) *walking over* the female parasitoid; (v) *threatening with open mandibles* (assuming a motionless posture with open mandibles); (vi) *threatening with the stinger* by directing it in the direction of the target at close range, as typical of the spatulate stinger of *Crematogaster* ants; (vii) *gaster rising*, consisting in an alarm posture typical of *Crematogaster* ants in which the gaster is raised above in a position perpendicular to the body plane.

Concerning parasitoids, we recorded the number of times the following three behaviours were observed: (i) *oviposition* (*including marking*), which consists of probing the host, inserting the ovipositor, and making head-pumping movements and body vibrations

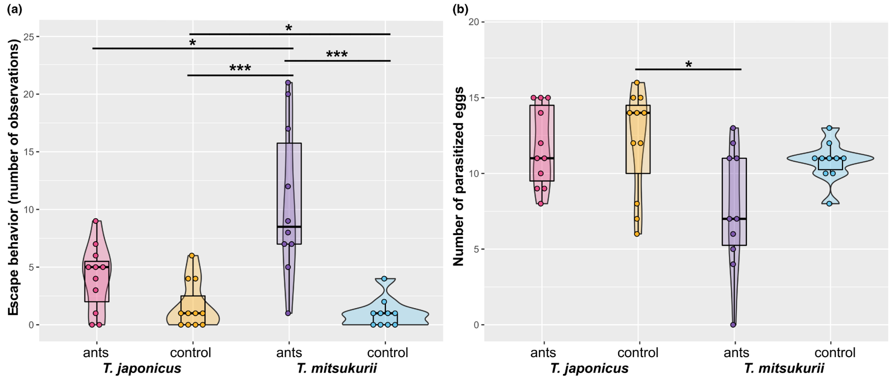

FIGURE 1 Most significant results of the experiments II, in which the effects of *Crematogaster scutellaris* ants on the behaviour of *Trissolcus japonicus* and *T. mitsukurii* females parasitizing *Halyomorpha halys* eggs were observed in the restricted setting of a petri dish for 40 min. (a) The number of times female parasitoids moved away from the egg-masses (escape behaviour); (b) the number of stink bug eggs the parasitoids were able to parasitize. According to pairwise comparisons, significantly different treatments are connected by black horizontal lines, and the above asterisks indicate significance levels (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

associated with the egg-release, partially exerting the ovipositor and sweeping it across the surface of the host egg in ∞-shaped movements as described by Field (1998); (ii) *chase-off*, consisting in running directly towards the ant, sometimes lunging with raised wings, making contact and biting it as described by Field (1998); (iii) *escape*, i.e. moving away from the egg mass; (iv) *resting*, i.e. stopping the oviposition and standing immobile.

2.7 | Statistical analyses

Statistical analyses were conducted using the software R 4.1.2 (R Core Team, 2020). We used Wilcoxon rank-sum tests to analyse differences between two groups, and Kruskal–Wallis tests followed by Dunn's post hoc tests with Benjamini–Hochberg *p*-value adjustment to analyse differences between multiple groups. Statistical tests were not run for behaviours occurring in less than 15% of the trials. For each test, $\alpha = 0.05$.

3 | RESULTS

Data collected in all experiments are provided in Table S1.

3.1 | Experiment I: Interactions between ants and parasitized eggs or emerging parasitoids

Regardless of the *Trissolcus* species tested in the trials, ants never caused any noticeable harm to parasitized eggs, and never attacked emerging parasitoids.

The number of emerged parasitoids did not differ among treatments (distinguishing between *T. japonicus* or *T. mitsukurii*, eggs exposed to ants 0, 2, 4, 6 or 9 days after parasitization, and eggs never exposed to ants before emergence) ($0.09 < p < 1.000$, Dunn's test).

In trials with ants, antennation was always observed and no significant differences were detected between treatments with the two parasitoid species ($p = 0.09 < p < 1.000$, Dunn's test). The escape behaviour was recorded in 58% of the trials with emerging parasitoids and was not significantly different between the two parasitoid species ($p = 0.23$, Wilcoxon rank-sum test). The following behaviours were extremely rare across the 72 trials that were run (<15%): biting (10 trials), licking (1 trial), threatening with open mandibles (6 trials), gaster rising (0 trials) and threatening with the stinger (2 trials).

3.2 | Experiment II: 1 versus 1 interactions in petri dishes (40 min)

Ants were never observed to attack and harm either of the two parasitoid species. Approaching ants often caused *T. mitsukurii* females to temporarily leave the egg masses, slowing their overall parasitization rate. On the contrary, *T. japonicus* females remained on the egg masses even when ants touched them, and their parasitization rate was not affected by the ants' presence.

The escape behaviour of parasitoids was significantly different based on treatment ($p < 0.001$; Kruskal–Wallis test): it was higher for *T. mitsukurii* in presence of ants compared to the other three treatments ($0.035 < p < 0.001$; Dunn's post hoc test), and more frequently observed for *T. japonicus* with ants compared to *T. mitsukurii* without ants ($p = 0.038$; Dunn's post hoc test), while no significant differences were detected in the remaining comparisons (Figure 1). Treatment affected the number of eggs that were parasitized ($p = 0.033$; Kruskal-Wallis test): in presence of ants *T. mitsukurii* parasitized a significantly lower number of *H. halys* eggs compared to *T. japonicus* trials with no ants ($p = 0.023$; Dunn's test), while no significant differences were detected in the remaining comparisons. The parasitoids managed to parasitize 11 eggs on average (39% of all eggs in the egg masses).

Contact latency of ants approaching parasitized eggs was not significantly different between replicates with *T. japonicus* and with *T. mitsukurii* ($p = 0.123$; Wilcoxon rank-sum tests). Walking over was observed in half of the *T. japonicus* trials, threatening with the gaster in one *T. japonicus* trial, while both behaviours were not observed in trials with *T. mitsukurii*. Resting behaviour was only observed once per each parasitoid species. Gaster rising and chase-off behaviours were never observed.

3.3 | Experiment III: Interactions in insect cages (24 h)

Ants had no impact on the parasitization activity and mortality of either *T. mitsukurii* or *T. japonicus*.

There were no statistically significant differences in the number of parasitoids hatched from the eggs in relation to the parasitoid species or the presence of ants ($p = 0.821$; Kruskal–Wallis test) nor any significant difference in the number of parasitoids found alive after the experiments ($p = 0.424$; Kruskal–Wallis test) (Figure 2).

4 | DISCUSSION

In our experiments, ants never directly attacked *Trissolcus* parasitoids, including in the potentially vulnerable moment of their emergence. Furthermore, parasitization did not alter the ant's ability to attack *H. halys* eggs, suggesting that parasitized and non-parasitized eggs are equally unlikely to suffer any damage by this ant. Stink bug eggs are rarely successfully attacked by ants, and the few known examples refer to cases of relatively large ants capable of considerable biting force, while chemical cues (or their absence) may also contribute to avoiding ant attacks (Castracani et al., 2017; Schifani, Giannetti, & Grasso, 2023).

Crematogaster scutellaris still affected the behaviour of *T. mitsukurii* in the confined space of Petri dishes, even if it did not perform any direct attack against the parasitoid. Notably, in the presence of an ant worker, female *T. mitsukurii* significantly more often stopped the egg-laying process and moved away, which diminished the number of stink bug eggs parasitized during the observation time, albeit not significantly. The same did not occur with *T. japonicus*, since the latter mostly ignored the approach of an ant, and even upon contact, it normally avoided abandoning the eggs. However, such interesting behavioural differences did not appear to play a role when ant-parasitoid interactions were observed in the more complex and larger cage environment, where *T. japonicus* and *T. mitsukurii* had similar parasitization success, regardless of the presence of *C. scutellaris* workers. Since *C. scutellaris* ants never harmed the parasitoids in direct encounters and had only a slight disturbance effect when artificially enclosed with *T. mitsukurii* in a very confined space, it is highly unlikely that interactions between *C. scutellaris* and *Trissolcus* parasitoids play a significant role under field conditions. *Crematogaster scutellaris* and the two non-native egg parasitoids *T. japonicus* and *T. mitsukurii* are currently co-occurring and rapidly spreading across the northern Italian regions invaded by *H. halys* (Zapponi et al., 2021). Multiparasitism laboratory experiments with *T. japonicus* and *T. mitsukurii* indicate that the order of arrival on the host's eggs is crucial to ensure the most successful parasitization, and that, competition between the two species did not result in reduced *H. halys* egg mortality (Costi et al., 2022).

As biological control agents, ants are appreciated for their polyphagy, territorial aggressiveness, resistance to starvation, and the possibility to manipulate their behaviour (Choate & Drummond, 2011; Offenberg, 2015). Negative effects are mainly observed when ants have a mutualistic relationship with pest insects, usually, honeydew-producing hemipterans, which they can defend against predatory insects and parasitoids used to control them (e.g. Jiggins et al., 1993; Mgocheki & Addison, 2009). The relationship of ants with parasitoids of ant-mutualistic hemipterans is generally antagonistic but not always relevant to biocontrol (Schifani, Peri, Giannetti, Colazza, & Grasso, 2023), and there are a few exceptions of myrmecophilous parasitoids adapted to exploit ant's presence (Pierce & Mead, 1981; Völkl, 1992). However, as generalist predators, ants may also attack parasitoids that do not interact with their mutualistic networks

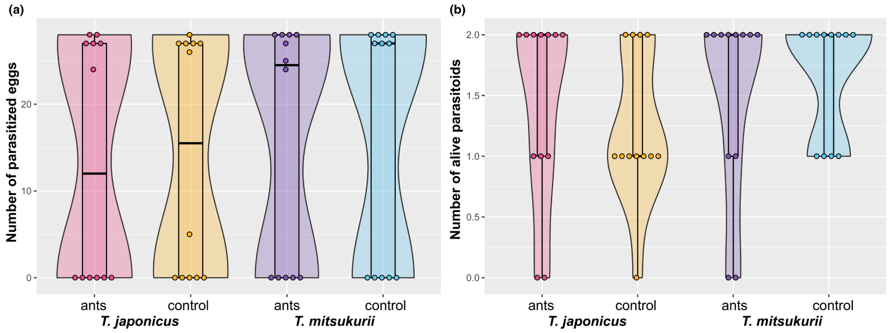

FIGURE 2 Most significant results of the experiments III, in which the effects of *Crematogaster scutellaris* ants on the behaviour of *Trissolcus japonicus* and *T. mitsukurii* females parasitizing *Halyomorpha halys* eggs were studied in the more natural setting of an insect cage with a plant, in which the insects were released for 24 h. (a) The number of stink bug eggs the parasitoids were able to parasitize; (b) the number of alive parasitoids verified at the end of the experiment. In both cases, no statistically significant differences between treatments were detected.

(Appiah et al., 2014). We have observed substantial neutrality between ants and parasitoids in our experiments. *Crematogaster scutellaris* is an ant that may play a useful role in pest management thanks to its common presence in agroecosystems and its predatory abilities against other pests such as the codling moth *Cydia pomonella* (L.), the ambrosia beetle *Xylosandrus compactus* (Eichoff, 1876) or the stink bug *N. viridula* (Giannetti et al., 2022; Schifani, Giannetti, & Grasso, 2023; Schifani, Peri, Giannetti, Alınç, et al., 2023). Notably, both stink bugs and parasitoids are attracted by sugary nectars, whose provision may serve the purpose of manipulating their behaviour or enhancing their efficacy as biocontrol agents (Colazza et al., 2022; McIntosh et al., 2020; Schifani et al., 2020).

By revealing that ants do not interfere with egg parasitization nor they attack egg parasitoids, our study encourages the possibility that ants and parasitoids may be integrated in the control of *H. halys*, with a combined effect on both eggs and nymphs that needs to be evaluated in field assessments (Bulgarini et al., 2022; Campolo et al., 2015; Castracani et al., 2017; Offenberg, 2015; Wright & Diez, 2011).

AUTHOR CONTRIBUTIONS

Enrico Schifani: Conceptualization; methodology; data curation; validation; formal analysis; visualization; resources; writing – original draft; investigation. **Daniele Giannetti:** Conceptualization; methodology; investigation; validation; formal analysis; resources; data curation. **Elena Costi:** Conceptualization; methodology; validation; formal analysis; investigation; resources; data curation. **Giulia Franconi:** Investigation; data curation; resources. **Arianna Campostrini:** Investigation; resources; data curation. **Lara Maistrello:** Conceptualization; validation; project administration; supervision; funding acquisition; resources. **Donato Grasso:** Conceptualization; validation; project administration; supervision; resources; funding acquisition.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data supporting the reported results can be found at https://doi.org/10.5281/zenodo.8082046.

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SUPPORTING INFORMATION

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Section I - Ants as biocontrol agents of insect pests

Chapter 5

Mediterranean ants can increase nymph mortality in the stink bug *Nezara viridula* without interfering with its egg parasitoid *Trissolcus basalis*

Schifani, E., Peri, E., Giannetti, D., Alınç, T., Colazza, S., & Grasso, D.A. (2023). Mediterranean ants can increase nymph mortality in the stink bug *Nezara viridula* without interfering with its egg parasitoid *Trissolcus basalis*. *Entomologia Experimentalis et Applicata*, 171(10), 739-744. https://doi.org/10.1111/eea.13357

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Entomologia Experimentalis et Applicata

ORIGINAL ARTICLE

Mediterranean ants can increase nymph mortality in the stink bug *Nezara viridula* without interfering with its egg parasitoid *Trissolcus basalis*

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Abstract

Ants (Hymenoptera: Formicidae) play a relevant ecological role across terrestrial ecosystems. Recent studies suggest that the presence of ants in crops could lead to a decrease in the populations of insect pests, but how these actions can vary along the different trophic levels is not well known. The southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae), is a cosmopolitan agricultural pest which is regularly found on horticultural agroecosystems closely associated with its main egg parasitoid, *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae). We conducted laboratory experiments to test whether two Mediterranean ant species, the generalist predators *Crematogaster scutellaris* (Olivier) and *Tapinoma magnum* Mayr, attack *N. viridula* eggs or nymphs, and whether they interfere with the parasitization activity of *T. basalis*. The experiment showed that both ant species significantly increased the mortality of *N. viridula* nymphs, whereas they do not attack their eggs and do not interfere with the egg parasitoids. Our results suggest that ants and egg parasitoids may have an integrable role in biocontrol strategies against this pest.

KEYWORDS

agricultural pest, biocontrol agent, biological control, *Crematogaster scutellaris*, crop protection, Formicidae, Hemiptera, Hymenoptera, Pentatomidae, Scelionidae, southern green stink bug, *Tapinoma magnum*

INTRODUCTION

Ants (Hymenoptera: Formicidae) are key ecological actors across terrestrial ecosystems (Hölldobler & Wilson, 1990; Lach et al., 2010; Parker & Kronauer, 2021). Their widespread presence in agroecosystems yields a range of services, e.g., ants produce chemical secretions that may limit certain plant pathogens (Offenberg & Damgaard, 2019), they have positive effects on soil bioturbation and nutrients (Lach et al., 2010; Taylor et al., 2019), and most ants are generalist predators that may suppress several agricultural pests, mostly arthropods but also weeds (Way & Khoo, 1992; Lach et al., 2010; Baraibar et al., 2011; Offenberg, 2015). On the other hand, ants are also known to cause ecosystem disservices to agricultural activities, e.g., they may facilitate mutualistic honeydew-producing hemipteran pests or prey upon insects that are beneficial to biological control programs and may cause damage by consuming seeds of cultivated species (Way, 1963; Baraibar et al., 2011; Offenberg, 2015). Many ants also attack non-prey insects because of territorialism or other forms of aggressiveness (Le Moli et al., 1994; Katayama & Suzuki, 2005; Dejean et al., 2009). Furthermore, they release persistent chemical cues which may attract/recruit nestmates and repel or have a deterrent effect on other insects (Grasso et al., 1998, 1999, 2005; Abandonon et al., 2009; Van Mele et al., 2009; Giannetti et al., 2022). Direct attacks against plants, on the other hand, are essentially restricted to the leafcutter ants that inhabit the American continents (Swanson et al., 2019) and some granivorous species (Rico-Grey & Oliveira, 2007).

Stink bugs (Hemiptera: Pentatomidae) are a large group of insects which includes several agricultural pests, of which ants have proved to be effective predators in some cases (e.g., Kryspin & Todd, 1982; Yang, 1984; Van Den Berg et al., 1995; Jones et al., 2001; Hosetti & Rudresh, 2012; Castracani et al., 2017; Bulgarini et al., 2021; Kamiyama et al., 2021). The southern green stink bug, *Nezara viridula* (L.), is a cosmopolitan pest species whose geographic origin may reside in the Mediterranean region and/or the African continent (Jones, 1988). This highly polyphagous insect is considered one of the most important pentatomid pests worldwide (Conti et al., 2021). The egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) is a key natural enemy of *N. viridula* and it is used in biocontrol programs across the world (Colazza & Bin, 1995; Esquivel et al., 2018). Ant activity may interfere with the action of parasitoids under various circumstances: this is mostly observed for those parasitoids that attack ant-mutualist aphids or coccids (Martinez-Ferrer et al., 2003; Chen et al., 2014), but parasitoids of insect species that are prey for ants can also be repelled (Appiah et al., 2014).

Three tropical ants are known as important predators of *N. viridula*, attacking both eggs and nymphs: the Asian weaver ant, *Oecophylla smaragdina* (Fabricius), and two invasive species, the fire ant *Solenopsis invicta* Buren and the big-headed ant, *Pheidole megacephala* (Fabricius) (Krispyn & Todd, 1982; Yang, 1984; Van Den Berg et al., 1995; Jones et al., 2001). Furthermore, *Monomorium minimum* (Buckley), *Pheidole dentata* (Mayr), and *Tetramorium guineense* (Bernard) are known to be nymph predators (Lockwood & Story, 1986). However, it is unknown whether ants from temperate regions can act as predators of *N. viridula* eggs or nymphs. Moreover, the interactions among ant species, *N. viridula*, and parasitoids like *T. basalis* were never investigated. To fill these gaps, we conducted laboratory experiments investigating the interactions between two Mediterranean ant species, eggs and nymphs of *N. viridula*, and females of the egg parasitoid *T. basalis*. The two ant species we chose as models are widespread in agroecosystems (Campolo et al., 2015; Giannetti et al., 2021; Bazzato et al., 2022; Schifani et al., 2022): *Crematogaster scutellaris* (Olivier) and *Tapinoma magnum* Mayr. The two ants are both very disturbance-tolerant species with large colonies, and their workers actively forage on a wide variety of plants, feeding on honeydew and acting as generalist predators (Campolo et al., 2015; Castracani et al., 2017; Seifert, 2018; Giannetti et al., 2022). At the same time, they are representatives of very different ant lineages with distinct behavioral and morphological adaptations. *Crematogaster scutellaris* is an arboreal-nesting species with monomorphic workers that apply their venom topically with a spatulate stinger, whereas *T. magnum* is a ground-nesting species, characterized by highly polymorphic but on average smaller workers which at short range can spray a toxic secretion produced by their anal glands (Seifert, 2018). We tested whether these ants would increase the mortality of the stink bug nymphs, damage their eggs, or interfere with the parasitoids.

MATERIALS AND METHODS

Plant and insects rearing

Seeds of broad bean plants, *Vicia faba* L. cv. 'Aguadulce Supersimonia' (Fabaceae), were immersed in a slurry of water and soil (1:4 vol/vol) for 24 h, to promote root nodulation. The seeds were then individually seeded in plastic pots (9 × 9 × 13 cm) that were filled with a mixture of agriperlite (Superlite; Gyproc Saint-Gobain, Milan, Italy), vermiculite (Silver; Gyproc Saint-Gobain), and sand (1:1:1 vol/vol/vol). The seeds were germinated, and the plants were grown in a climate-controlled chamber (24 ± 2 °C, 55 ± 10% r.h., L12:D12 h, with light intensity 400 μmol photons m^{-2} s^{-1}). The plants were watered daily and 1 week after germination they were fertilized with an aqueous solution of fertilizer (1.4 g L^{-1}; N-P-K = 5–15-45; Plantfol, Valagro, Italy). Plants of 20–25 cm tall were used for the experiments.

Nezara viridula was reared in wooden cages (50 × 30 × 35 cm), ventilated with mesh-covered holes (5 cm diameter), in an environmental room (24 ± 1 °C, 70 ± 5% r.h., L16:D8). Stink bugs were fed with a diet of seasonal fresh vegetables and sunflower seeds. Food was changed every 2–3 days, and separate cages were used for nymphs and adults. Paper towels were placed inside each adult cage as an ovipositional substrate. Egg masses collected daily were used to maintain the stink bug colony, which was from time to time refreshed with field-collected bugs.

The *T. basalis* colony was established from wasps emerging from naturally laid *N. viridula* egg masses collected from cultivated fields and surrounding uncultivated areas near Palermo (Sicily, Italy). Adult parasitoids were reared in 16-mL glass tubes (density = 50–60 wasps per tube), fed with a solution of honey and water, and kept in an incubator at the same environmental conditions described for the stink bugs. Egg masses of *N. viridula* collected from the colony were exposed to parasitoids for 48 h, then the wasps were removed, and the parasitized eggs were stored for incubation.

Crematogaster scutellaris and *T. magnum* laboratory colonies were established from samples of about 2000 workers from colonies collected near Palermo. Both colonies were temporarily reared in plastic cages (75 × 35 × 20 cm) for the duration of the experiment under the same environmental conditions described for the stink bugs. They were fed with a honey–water solution. Ants used during each experimental trial were not re-used in following tests.

Experimental setup

Experiments were conducted under controlled conditions of 24 ± 1 °C, 66 ± 1.4% r.h., and L16:D8 photoperiod, using 30 × 30 cm insect cages. In the center of each cage, we placed a *V. faba* plant with its plastic pot. On one corner of the cage, we placed a plastic cup (8 cm high, 6.5 cm diameter) filled for 1/3 with broken twig pieces and, at its upper internal margins, covered for 1 cm with an ant-repelling substance (50% glycerin oil, 50% petroleum jelly) to prevent ants from escaping. In each plastic cup, we also inserted a 10-cm-long wooden stick, whose upper end was leaning on the plant. The plastic cup was meant to offer a shelter to the ants during the experiments, allowing them to move towards the plant along the wooden stick, without dispersing in all other directions thanks to the repellent substance. After each experiment, the insect cages and plastic cups were washed with clean water, and the plants, the content of the plastic cups, and the wooden sticks were replaced with new ones. We conducted three tests for each experiment, running 15 replicates per test: one with *C. scutellaris*, one with *T. magnum*, and a control test with no ants.

Experiment 1: effects of ant activity on stink bug nymph mortality

On an apical leaf of each plant, we placed with a clip a 1 × 3 cm filter paper with an artificially made egg mass of 15 eggs attached with a glue stick (Pritt; Henkel, Hemel Hempstead, UK). We then waited for 6–10 h after the eggs hatched and counted the *N. viridula* nymphs (mean ± SD = 12.4 ± 2.8). The experiment then started with the introduction of 50 ants in each plastic cup, and lasted 24 h, during which the cage was kept closed. At the end, we collected the stink bug nymphs, checking how many of them were still alive.

Experiment 2: effects of ant activity on stink bug eggs and parasitoid oviposition and survival

We first introduced on each plant three *N. viridula* virgin females for 24 h to allow them to walk over the plant to contaminate it with chemical footprints that are relevant cues exploited by *T. basalis* in its searching behavior (Colazza et al., 1999). We used a glue stick to attach *N. viridula* egg masses (50–100 eggs each) on 1 × 3 cm filter papers and used a clip to put one of them on the apical leaf of each plant. To start the experiment, we introduced 50 ants into each plastic cup, and three *T. basalis* females (24–48 h old). Each experiment lasted 24 h, during which the cages were kept closed, after which we removed the egg masses and the parasitoids and checked whether the latter were alive or dead. The egg masses were incubated until the eggs hatched, or parasitoids emerged. We discriminated between eggs from which parasitoids emerged and the rest from which stink bugs emerged or that did not hatch.

Statistical analysis

The normality of the data and equality of their variance between treatments were tested by means of Shapiro–Wilk and Levene's tests, respectively. To evaluate the differences between treatments concerning stink bug nymph mortality and the egg parasitization rate, we relied on Kruskal-Wallis tests followed by Dunn's post hoc tests for pairwise comparisons if significant differences between treatments had been detected. As no more than one parasitoid died in each trial, data on parasitoid mortality were binomial and differences between treatments were analyzed using a generalized linear model (GLM). All statistical analyses were conducted with the software R v.4.2.0 and RStudio-2022.02.2–485 (R Core Team, 2022).

RESULTS

Experiment 1: effects of ant activity on stink bug nymph mortality

Stink bug mortality rate differed between treatments (H = 12.24, d.f. = 2, P = 0.002). In particular, the treatment with no ants was different from the other two (no ants vs. *C. scutellaris*: P = 0.023; no ants vs. *T. magnum*: P = 0.003), whereas there were no differences between the treatment with *C. scutellaris* vs. *T. magnum* (P = 0.49). Stink bug mortality was 0 ± 7% in the treatment with no ants, and 17 ± 32% in the treatments with ants (median ± interquartile range; Figure 1A-C).

Experiment 2: effects of ant activity on stink bug eggs and parasitoid oviposition and survival

Stink bug eggs were not removed by ants and their shell was not damaged after the experiments in which they were exposed to ants. There was no effect of treatment on the parasitization rate and the consequent number of stink bugs that hatched (H = 0.27, d.f. = 2, P = 0.87) (Figure 1D, E). Furthermore, there was also no treatment effect on the number of dead parasitoids (0.24 < P < 0.70).

DISCUSSION

Our results show that native Mediterranean ants may act as antagonists of *N. viridula* nymphs, significantly increasing their mortality. Furthermore, we found no evidence of interference between ants and the parasitization activity of *T. basalis* on *N. viridula* eggs. As these ants do not appear to prey upon stink bug eggs, they may be even less likely to interact with parasitoids directly. Although we cannot rule out that increasing the number of ants per plant even more would eventually have posed some problems to the

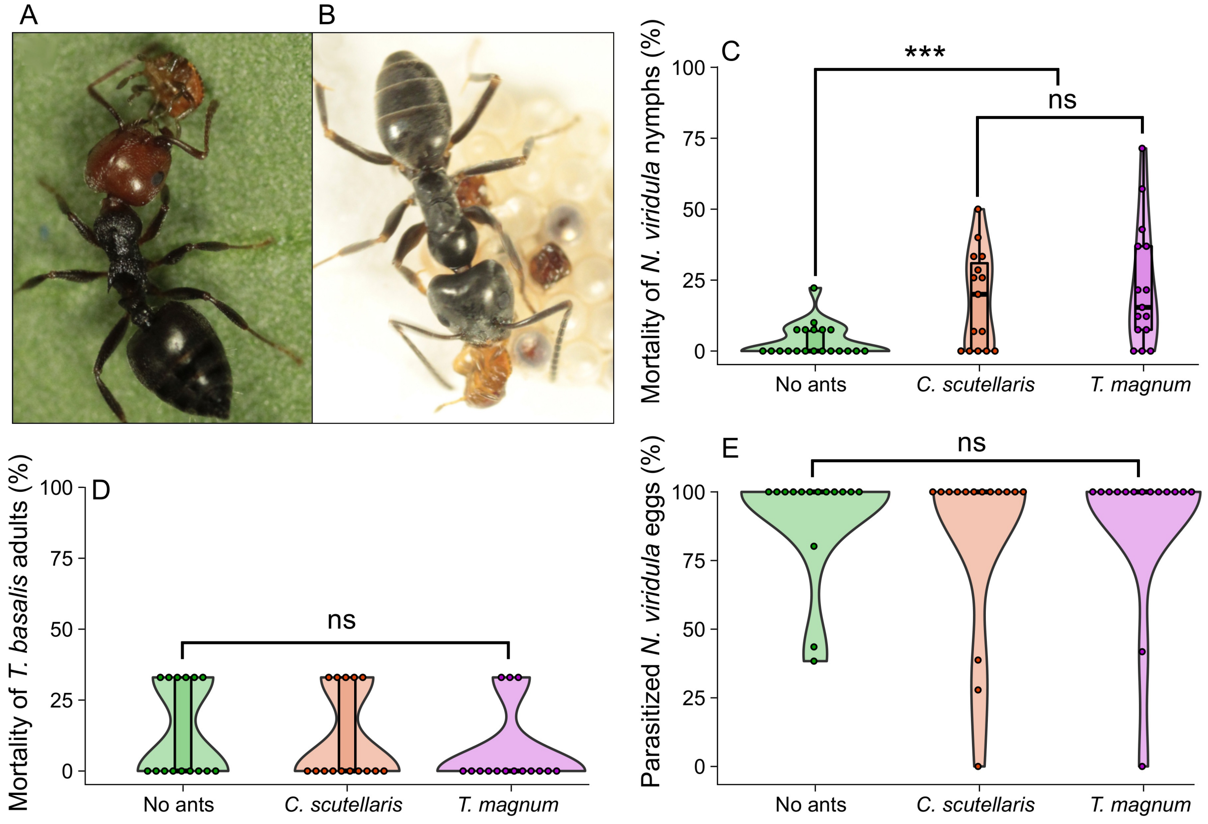

FIGURE 1 Interactions between the ants (A) *Crematogaster scutellaris* and (B) *Tapinoma magnum*, the stink bug *Nezara viridula*, and its egg parasitoid *Trissolcus basalis*. Mortality (%) of (C) stink bug nymphs and (D) the parasitoids. (E) Parasitization rate (%) of stink bug eggs. Asterisks indicate significant treatment effects (Kruskal-Wallis tests followed by Dunn post-hoc tests for pairwise comparisons: $P < 0.001$; ns, $P > 0.05$). Note that the ants in A and B are attacking stink bug nymphs.

parasitoids, and perhaps further increase stink bug mortality, we would not expect such high concentrations to occur under natural conditions.

The fact that we did not record egg predation by the ants, unlike what was observed for *O. smaragdina* (Yang, 1984; Hosetti & Rudresh, 2012), may be due to the relatively smaller size of the ant species we used for the experiments. *Crematogaster scutellaris*, as well as the native European ant *Lasius niger* (L.), are similarly unable to attack the eggs of the brown marmorated stink bug, *Halyomorpha halys* Stål, whereas they do prey upon its nymphs (Castracani et al., 2017; Bulgarini et al., 2022), highlighting a similar pattern among Mediterranean ants interacting with various stink bug species. Larger Mediterranean ant species may be able to prey upon *N. viridula* eggs, but they are seemingly less frequent in agroecosystems than eggs of species such as *C. scutellaris* and *T. magnum* (Mansour et al., 2012; Campolo et al., 2015; Giannetti et al., 2021; Bazzato et al., 2022; Schifani et al., 2022). On the other hand, ants may increase nymphal mortality through different mechanisms, which require further investigation. In addition to suffering from direct attacks (Castracani et al., 2017), disturbance may prompt young nymphs to break aggregations, which leads to a higher risk of death by desiccation and may even expose them to other predators (Lockwood & Story, 1986).

Ants are often reported to either displace or favor parasitoids, often due to specialized myrmecophilic adaptations by either the hosts or the parasitoids themselves (Pierce & Mead, 1981; Völkl, 1992), whereas in our experiments we witnessed a substantial neutrality between the two actors. The lack of direct interference with *T. basalis* and the lack of interest for *N. viridula* eggs by *C. scutellaris* and *T. magnum* opens the possibility of using these ants as complementary tools along with *T. basalis* in the control programs against *N. viridula*. In this perspective, it is notable that the activity of both ants and *T. basalis* can be enhanced or manipulated by using natural and artificial nectars, favoring their presence on target plants, increasing their survivability, and, in the case of ants, even distracting them from tending coccids or aphids (Offenberg, 2001; Rahat et al., 2005; Schifani et al., 2020). Habitat characteristics may significantly contribute to determining whether ants or parasitoids play a more significant role in controlling *N. viridula* and other stink bugs (Wright & Diez, 2011).

Crematogaster scutellaris and *T. magnum* are already known to play an interesting role in the control of other insect pests, including brown marmorated stink bug, *H. halys*, but also horse-chestnut leaf miner, *Cameraria* *ohridella* Deschka & Dimić, Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), codling moth, *Cydia pomonella* (L.), and the ambrosia beetle *Xylosandrus compactus* (Eichhoff) (Radeghieri, 2004; Campolo et al., 2015; Castracani et al., 2017; Giannetti et al., 2022; Schifani et al., 2023). Considering the significant behavioral, morphological, and phylogenetic differences between the ant species known to prey upon *N. viridula* nymphs so far, we expect that several other ants may play a similar role (Offenberg, 2015). However, even superficially similar species may still differ markedly in their attitude towards both stink bugs and their parasitoids (Chen et al., 2014). Further assessments are required to quantify the predatory role of Mediterranean ants on stink bug nymphs in agricultural fields, where generalist species such as *C. scutellaris* and *T. magnum* are expected to co-occur frequently with *N. viridula*.

AUTHOR CONTRIBUTIONS

Enrico Schifani: Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); resources (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Ezio Peri:** Conceptualization (equal); funding acquisition (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); writing – review and editing (lead). **Daniele Giannetti:** Conceptualization (equal); methodology (equal); resources (supporting); writing – review and editing (supporting). **Tuğcan Alınç:** Investigation (supporting); resources (lead); writing – review and editing (supporting). **Stefano Colazza:** Conceptualization (equal); funding acquisition (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); writing – review and editing (lead). **Donato Antonio Grasso:** Conceptualization (lead); funding acquisition (equal); methodology (equal); project administration (lead); supervision (lead); validation (equal); writing – review and editing (supporting).

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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3. SECTION II:

ANTS AS MUTUALIST PARTNERS OF INSECT PESTS

This second section is composed of three chapters. Chapter 6 illustrated an ecological experiment carried out in Sicily, in which ant-exclusion through the application of sticky barriers was used on fava bean plants to study the impact of the local ant community on mutualist and non-mutualist aphid species attacking the plants, and the relationship between the presence of these ants and the presence of aphid natural enemies (parasitoids and predators). According to the results, the three ant species visiting the plants (one exotic and two natives) had no role in deterring aphid parasitoids and predators of the mutualist *Aphis fabae* Scopoli, 1763. Aphids were still more abundant in plants without sticky barriers, but it was unclear whether this was caused by ants or by sticky barriers themselves. In any case, while other ant species are known to protect *A. fabae* from parasitoids and predators, our experiment demonstrates how the identity of the ant species involved matters, and the associations between attending ants and aphids does not automatically imply protection from aphid natural enemies.

Chapter 7 digs deeper into the topic, this time using as a model system the association between the walnut aphid *Panaphis juglandis* (Goeze, 1778), an ant-mutualist, and different ant species that attend it. We carried out field experiments aimed at comparing the behavioral responses of different ants associated with *P. juglandis* to the approach of different ladybeetle species. We detected interesting patterns of behaviors, again emphasizing strong differences between different ants in their behavioral responses and effectiveness at protecting their aphid partners from the ladybeetles, with a gradient from very strong protection to no protection at all.

Finally, in Chapter 8 we expand on a review of management tactics for ant-hemipteran associations that can be employed whenever these associations are demonstrated to produce significant economic damage to cultivated plants. Four main tactics are identified, some of which can be integrated with one another, and their pros and cons are evaluated: *i)* poisoning ant colonies with low-toxicity baits; *ii*) preventing ant access to the plants using physical or chemical barriers; *iii*) diverting ant attention from mutualist hemipterans by offering alternative food sources; *iv*) utilizing parasitoids or predators that are capable of circumventing the defensive abilities of the locally prevailing ant species. Future research directions are also commented upon.

Section II - Ants as mutualist partners of insect pests

Chapter 6

Ant attendance does not necessarily imply protection of aphids from their arthropod natural enemies

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SHORT COMMUNICATION

Ecological Entomology

Ant attendance does not necessarily imply protection of aphids from their arthropod natural enemies

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Abstract

1. Many ants and hemipterans are bound by a mutualistic relationship (trophobiosis) which represents an ecological keystone: ants receive food (honeydew) providing different kinds of protection. Partner protection against arthropod natural enemies is considered to be frequent and to interfere with biological control strategies of hemipteran pests.
2. We carried out an ant-exclusion field experiment in a fava bean (*Vicia faba*) plantation in Italy to monitor the abundance and behaviour of the ants (*Plagiolepis pygmaea*, *Tetramorium semilaeve* and the exotic *Nylanderia jaegerskioeldi*) and their relationship with facultative mutualist (*Aphis fabae*) and non-mutualist (*Megoura viciae*) aphids, the arthropod natural enemies of the aphids, and extrafloral nectaries.
3. Ants concentrated their activity on the attendance of facultative mutualist aphids much more than on the extrafloral nectaries. The ant-exclusion treatment had no effect on the abundance of *M. viciae* and on the parasitization rate of *A. fabae*, while it reduced the abundance of *A. fabae*, aphid predators, and aphid parasitoids.
4. Our results demonstrate that ant attendance does not imply the protection of aphids from arthropod natural enemies and suggest that the identity of the ant species involved is important for the outcome. As relatively few species have been studied in this regard, extending our knowledge to the role of more ant species is desirable to understand the ecology and evolution of ant-aphid mutualisms and to refine integrated control strategies of aphid pests.

KEYWORDS
ant-aphid mutualism, aphid parasitoids, aphid predators, biological control, extrafloral nectaries, honeydew, integrated pest management, trophobiosis

INTRODUCTION

Ants play an important ecological role across terrestrial ecosystems, in both natural and human-managed environments (Hölldobler & Wilson, 1990; Parker & Kronauer, 2021). Trophobiotic mutualism between ants and hemipterans (mostly aphids or coccids) ranges from facultative to obligate and represents a widespread ecological keystone: ants provide their partners with different kinds of protection in exchange for honeydew (Delabie, 2001; Depa, Kaszyca-Taszakowska, Taszakowski, & Kanturski, 2020; Hölldobler & Wilson, 1990; Parker & Kronauer, 2021). Ants may protect mutualist hemipterans from pathogens (Nielsen, Agrawal, & Hajek, 2010), offer them shelter from adverse climatic conditions (Giannetti et al., 2021), and help them colonise new plants (Collins & Leather, 2002). However, their ability to protect aphids against arthropod natural enemies, which can interfere with biological control strategies aimed at containing hemipteran pests, has attracted most attention (Banks & Macaulay, 1967; Jiggins, Majerus, & Gough, 1993). Nonetheless, some aphid parasitoids may benefit from ant presence, developing myrmecophilous adaptations to communicate with ants and receive food via trophallaxis (Völkl, Liepert, Birnbach, Hübner, & Dettner, 1996). Moreover, ants may prey upon their aphid partners under certain circumstances (Offenberg, 2001).

Only relatively few ant species have been studied concerning the protection they may grant to aphids and the consequences for biological control (e.g., *Lasius niger* L., Jiggins et al., 1993). On the other hand, ants themselves often act as predators of phytophagous insects appreciated in biological control, and by attracting ants, aphids may sometimes indirectly protect plant from defoliating herbivores (Styrsky & Eubanks, 2010). The study of these complex ecological networks can be important to develop a better evolutionary perspective but also for integrated pest management (IPM) and biological control in particular.

In this context, we carried out an ant-exclusion experiment on a fava bean (*Vicia faba* L.) plantation in Italy, collecting data on the effects of ant activity on aphids and their arthropod natural enemies, as well as evaluating the possible effects on other arthropods.

MATERIALS AND METHODS

A total of 60 plants of fava beans (*Vicia faba*, "Aguadulce Supersimonia" variety) were obtained from seeds planted by the end of March (2021) into 6 rows of 20 m each, covering an area of (10 × 20 m) within an experimental field in Sicily (38.10652, 13.35030, 50 m asl). This variety possesses extrafloral nectaries (EFNs) whose secretion is attractive for ants. No fertilisers or pesticides were used, and weeds were not removed throughout the experiment. During April 2021, we surveyed the ant and aphid fauna of the area. Specimens were collected by direct sampling and stored in 96% EtOH (vouchers are stored in ES personal collection) and identification was performed under a stereomicroscope (90–180× magnification) (Blackman & Eastop, 2000; LaPolla, Hawkes, & Fisher, 2011; Sanetra, Güsten, & Schulz, 1999; Seifert, 2018). This way we ensured our ability to identify the field the locally occurring species without collecting further specimens. Then, the ant-exclusion treatment was obtained by applying commercially available sticky barriers at the base of the stem (1 cm from the ground) of half of the plants ($n = 30$) (Schifani et al., 2020).

Data were recorded twice per week on each plant from 11:00 am to 14:00 pm (from 6 May to 12 June 2021, for a total of 11 sampling sessions), alternating the order at which plants were inspected on each session. The first sampling session preceded the application of sticky barriers. During 2 min-long observations, we counted the ants visiting the plant, distinguishing those licking or antennating the EFNs, those antennating/licking or transporting aphids, and those attacking other arthropods (biting/stinging/spraying chemicals). Aphid abundance was calculated by selecting the area where aphid density was the highest for each aphid species and counting the number of aphids (excluding mummies) in a corresponding 1.5 cm long section of the plant. The total number of aphid mummies per plant was also counted. To weight the number of mummies based on the size of the aphid colony on the plant, we calculated a ratio by dividing it by its sum with the aphid abundance. Other arthropods were identified at least at the family level by visual observation and divided into functional groups: aphid predators, aphid parasitoids, hyperparasitoids, generalist predators, phytophagous insects. The ratio between the number of aphid natural enemies (aphid predators and parasitoids) and aphid abundance was calculated for each plant. The number of leaves missing visible portions of tissue were counted as a proxy for the potential action of chewing phytophagous insects (Schifani et al., 2020).

We used a Wilcoxon rank sum test to assess whether plants differed in the number of sampling sessions they survived depending on treatment. We used Linear Mixed-Effect models to analyse differences in data collected on arthropods and leaves based on treatment (lme function, nlme R package, Pinheiro et al., 2021). The sampling session was treated as a repeated measure and the plant as a random factor. Arthropod functional groups observed <50 times were excluded. The same model was used to compare the number of ants interacting with aphids with those interacting with EFNs, running the analysis only on Ant+ plants and observations in which ants interacting with aphids were>0. Residual normality was analysed using Quantile-Quantile plots. Statistical analyses were conducted using the software R 4.1.1 and RStudio (R Core Team, 2021). Data are presented as mean ± standard deviation.

RESULTS

Three ant species visited the plants throughout the experiment: *Nylanderia jaegerskioeldi* (Mayr), *Plagiolepis pygmaea* (Latreille), and *Tetramorium semilaeve* André. *Hypoponera eduardi* (Forel), although observed in the area, was never detected on plants. In the control plants, *P. pygmaea* was the most abundant species (7.37 ± 6.62 ants per plant; while *N. jaegerskioeldi* 0.44 ± 1.45, *T. semilaeve* 1.14 ± 4.39), occurring on all of them (while *N. jaegerskioeldi* on 39%, *T. semilaeve* on 29%). On the plants we found two aphid species, *Aphis fabae* Scopoli and *Megoura viciae* Buckton. The first species was more abundant (35.22 ± 38.98 vs. 7.49 ± 19.72) and colonised slightly more plants (100% vs. 93%). Ant-*A. fabae* interactions were observed 2042 times; aphid transportation only once. Ants interacting with EFNs were significantly fewer than those interacting with *A. fabae* (*A. fabae*: 5.56 ± 5.65; EFNs: 0.16 ± 0.86; $F_{1,1001} = 278.57$, $p < 0.001$). We recorded 1142 other arthropods on the plants: 224 aphid parasitoids (Braconidae), 198 aphid predators (Cecidomyiidae, Chrysopidae, Coccinellidae, Syrphidae), 4 hyperparasitoids, 103 phytophagous insects (mostly Cicadellidae) and 821 individuals of *Rhagonycha fulva*

TABLE 1 Differences between ant-exclusion and control group treatments in the arthropods recorded on the plants and on plant survival. Variables with statistically significant differences between the two treatments are highlighted in bold

Variable	Ant-exclusion	Control	*p*-value	Statistics
Number of ants	**1.34 ± 3.09**	**8.95 ± 7.63**	**<0.001**	$\mathbf{F_{1,58} = 48.33}$
Abundance of non-mutualist aphids (*M. viciae*)	8.66 ± 24.71	6.36 ± 13.15	0.412	$F_{1,58} = 0.68$
Abundance of facultative mutualist aphids (*A. fabae*)	**28.35 ± 37.05**	**41.85 ± 39.71**	**0.039**	$\mathbf{F_{1,58} = 4.47}$
Number of *A. fabae* mummies	**4.33 ± 10.37**	**22.33 ± 60.45**	**0.003**	$\mathbf{F_{1,58} = 9.63}$
Number of *Aphis fabae* mummies divided for *A. fabae* abundance	0.20 ± 0.34	0.26 ± 0.33	0.250	$F_{1,58} = 1.35$
Number of aphid predators	**0.26 ± 0.71**	**0.55 ± 1.24**	**0.002**	$\mathbf{F_{1,58} = 10.98}$
Number of aphid parasitoids	**0.32 ± 0.60**	**0.58 ± 0.83**	**0.009**	$\mathbf{F_{1,58} = 7.38}$
Number of aphid enemies divided for *A. fabae* abundance	0.07 ± 0.29	0.04 ± 0.11	0.119	$F_{1,58} = 2.51$
Number of phytophagous insects	0.23 ± 1.15	0.16 ± 1.06	0.409	$F_{1,58} = 0.69$
Number of *R. fulva* individuals	1.37 ± 4.07	1.80 ± 5.03	0.658	$F_{1,58} = 0.20$
Number of damaged leaves	0.69 ± 2.01	0.54 ± 1.65	0.476	$F_{1,58} = 0.51$
Plant survival (number of sampling sessions)	9.03 ± 2.52	9.00 ± 2.30	0.738	W = 472.5

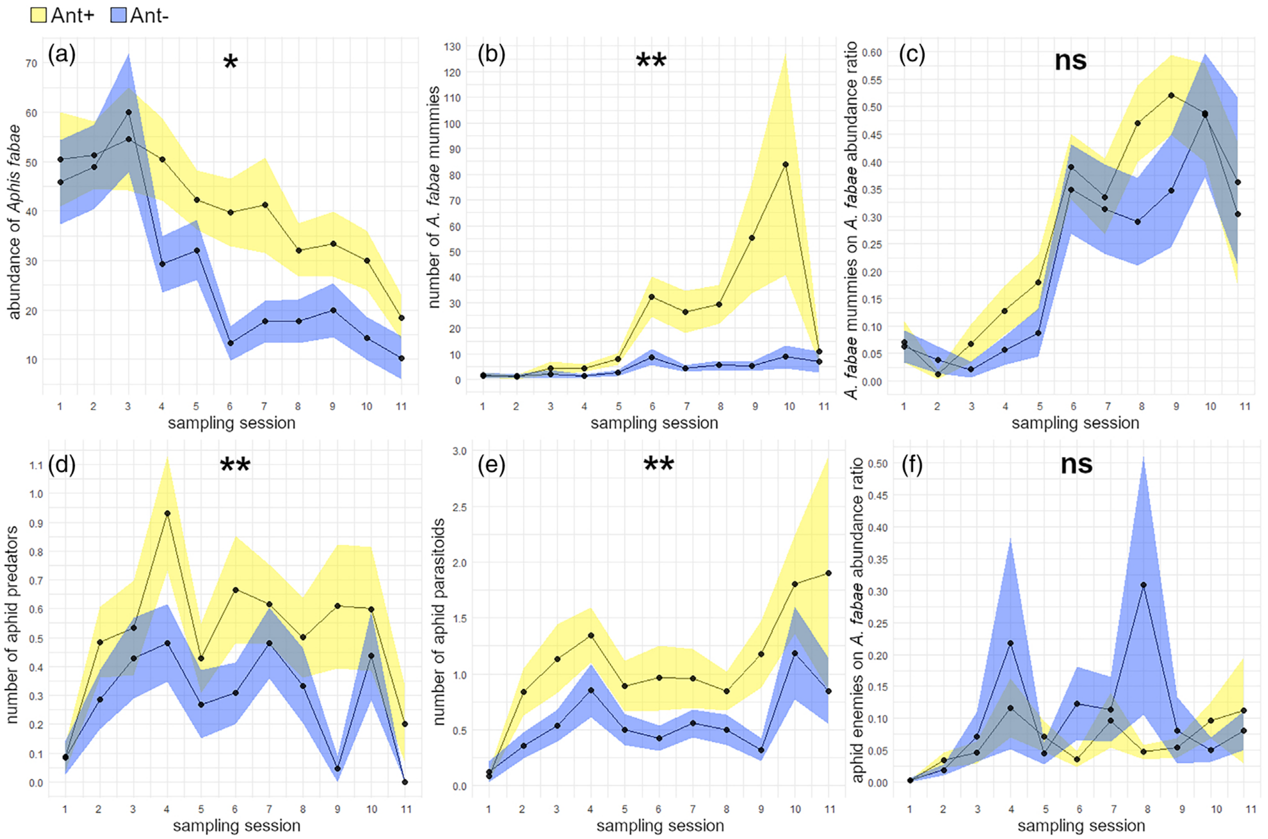

FIGURE 1 Trend of six key variables observed during the 11 sampling sessions of the experiment in plants with (ant+) and without ants (ant−): (a) the abundance of the facultative mutualist aphid *Aphis fabae*; (b) the abundance of *A. fabae* mummies resulting from parasitization; (c) the parasitization ratio (number of mummies divided by its sum with the number of aphids); (d) the abundance of aphid predators; (e) the abundance of aphid parasitoids; (f) the natural enemies per aphid ratio (number of arthropod natural enemies of aphids divided by the number of aphids);. The black dots and black line indicate the mean values, while the coloured envelopes represent the standard error. The statistical significance of the differences between the two treatments is indicated as following: *** if $p \leq 0.001$, ** if $p \leq 0.01$, * if $p \leq 0.05$, ns (non-significant) if $p > 0.05$

(Scopoli) not assigned to previous groups (Table S1). No aggressive interactions between ants and other arthropods were observed.

In ant-excluded plants we recorded fewer facultative mutualist aphids, aphid predators and aphid parasitoids than in the control, while no significant differences were found for the other variables (Table 1; Figure 1). Plant survival was unaffected by treatment (Table 1). Plant numbers decreased gradually from 60 to 23 by the end of the experiment.

DISCUSSION

We document for the first time the relationship between three ant species and the facultative mutualist aphid *A. fabae*. In our experiment, ant attendance did not imply an effective protection against arthropod natural enemies: it was associated both with more abundant *A. fabae* and more numerous aphid predators and parasitoids, and, unlike in other ant-exclusion experiments (Stewart-Jones, Pope, Fitzgerald, & Poppy, 2008), *A. fabae* mummies weighted on the abundance of living individuals were not significantly lower in ant-attended colonies. The increase of *A. fabae* in plants visited by ants was perhaps caused by other types of benefits granted by the attending ants, but it is possible that *A. fabae* simply benefited from the absence of sticky barriers, allowing dispersing wingless individuals to colonise only ant-visited plants.

Despite not being an obligate ant-mutualist, *A. fabae* was observed to obtain significant protection from predators and parasitoids in other experiments (Banks & Macaulay, 1967; Jiggins et al., 1993). While we cannot rule out the presence of ant-adapted parasitoids in our field area (see Völkl et al., 1996 and references therein), at least some of the predators we detected can be repelled by ants as reported by Jiggins et al. (1993), suggesting that the behavioural ecology of the ant species involved in our experiment determined the observed lack of deterrence or attacks against predators and parasitoids. The small size of *P. pygmaea* may limit its ability to repel larger arthropods, while *N. jaegerskioeldi*, an alien species quickly spreading in the Mediterranean region, did not follow the trend of introduced species strongly favouring honeydew-producing hemipterans (Wang, Lu, Peng & Segar, 2021). The degree of dependence of ants on aphids as a trophic resource is expected to influence their commitment to their protection, yet as aphidophilous ants are usually very generalist in their diets, this likely to vary significantly based on available alternatives (Depa, Kaszyca-Taszakowska, Taszakowski, & Kanturski, 2020). Contrary to the results of Engel, Fischer, Wäckers, and Völkl (2001), ant-aphid mutualism did not disadvantage non-mutualist aphids (*M. viciae*), while similarly, ants preferred facultative mutualist aphids over EFNs. Other phytophagous insects were scarce, not allowing us to evaluate potential effects of ants.

Some studies have already reported exceptions to the classical paradigm of ants protecting aphids from arthropod natural enemies (Völkl et al., 1996), but field assessments and examined species are still few. Determining which ants provide significant protection to specific aphid species against certain arthropods is relevant both from and ecological and evolutionary perspective, as well as for pest management strategies. While our knowledge is still limited, farmers should be aware that aphid-tending ants do not necessarily interfere with arthropod biocontrol agents.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Supporting Information

How to cite this article: Schifani, E., Peri, E., Giannetti, D., Colazza, S. & Grasso, D.A. (2023) Ant attendance does not necessarily imply protection of aphids from their arthropod natural enemies. *Ecological Entomology*, 1–5. Available from: https://doi.org/10.1111/een.13226

Section II - Ants as mutualist partners of insect pests

Chapter 7

Fight and rescue or give up and flee? Behavioral responses of different ant species tending the mutualist walnut aphid *Panaphis juglandis* to native and exotic lady beetles

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Schifani, E., Giannetti, D., Castracani, C., Spotti, F.A., Mori, A., & Grasso, D.A. (2023). Fight and rescue or give up and flee? Behavioral responses of different ant species tending the mutualist walnut aphid *Panaphis juglandis* to native and exotic lady beetles. *Bullettin of Entomological Research*, 1-6. https:// doi.org/10.1017/S0007485323000500

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Fight and rescue or give up and flee? Behavioural responses of different ant species tending the mutualist walnut aphid *Panaphis juglandis* to native and exotic lady beetles

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Abstract

Mutualism between ants and honeydew-producing hemipterans is a highly successful evolutionary innovation that attains the status of ecological keystone across many terrestrial ecosystems, involving a multitude of actors through direct or cascading effects. In these relationships, ants often protect their hemipteran partners against their arthropod natural enemies, sometimes interfering with the biological control of pest species. However, the dynamics of these interactions are highly variable based on the specific identity of all the actors involved, and baseline data remain scarce. We performed a field experiment exposing colonies of the walnut aphid *Panaphis juglandis* attended by five European ant species (*Camponotus piceus*, *Ca. vagus*, *Crematogaster scutellaris*, *Dolichoderus quadripunctatus*, *Lasius emarginatus*) to a native and an exotic lady beetle (*Adalia bipunctata* and *Harmonia axyridis*), documenting the behavioural interactions between these insects and the performance of ants in the protection of the aphids. Our results reveal a significant behavioural diversity among the ant species involved, with *D. quadripunctatus* and *L. emarginatus* being the most aggressive and having the best performance as aphid defenders, and *Ca. piceus* being least effective and often fleeing away. *Cr. scutellaris* displayed a rare rescue behaviour attempting to pull away the aphids that the lady beetles grabbed. On the other hand, behavioural responses to *A. bipunctata* and *H. axyridis* were similar. Further investigations are needed to understand the eco-ethological implications of these differences, while a better understanding of ant behavioural diversity may help refine biological control strategies.

Introduction

Mutualistic relationships between ants and honeydew-producing, phytophagous hemipterans represent a highly successful innovation in the evolutionary history of these insects and an ecological keystone in terrestrial habitats (Hölldobler and Wilson, 1990; Parker and Kronauer, 2021). Typically, they are based on the provision of food (the honeydew produced by the hemipterans) in exchange for protection and they are thus defined as trophobiotic (Hölldobler and Wilson, 1990). Ants may defend their partners against pathogens (e.g. Queiroz and Oliveira 2001; Nielsen *et al.*, 2010), adverse climate (Giannetti *et al.*, 2021) or arthropod natural enemies (e.g. Majerus *et al.*, 2007; Dao *et al.*, 2014), and may even help them to colonise new plants (Collins and Leather, 2002). Due to the high diversity of both ants and hemipterans involved in these relationships, several possibilities may co-exist, but little is still known about what exactly ants offer in exchange for honeydew in most cases (Schifani *et al.*, 2023*a*).

Many honeydew-producing hemipterans, including aphids, scale insects and whiteflies, are serious economic pests, and their relationship with ants can have negative impacts on their management. In particular, the major concern is the ability of some ants to defend their partners against arthropod natural enemies such as predators and parasitoids which may interfere with biological control (Franco *et al.*, 2004; Vanek and Potter, 2010; Navarrete *et al.*, 2013; Cocco *et al.*, 2021). However, interactions between these insects are rather complex, and baseline data on the behaviour of most species are missing. Relationships between ants and hemipterans range from facultative to obligate mutualists (Depa *et al.*, 2020), and not all ant species attack the predators and parasitoids of their partners (Schifani *et al.*, 2023*a*). Furthermore, some predators and parasitoids have developed adaptations to circumvent or even take advantage of ant protection (Völkl, 1992; Völkl *et al.*, 1996; Schifani *et al.*, 2023*a*).

To understand the ecology and evolution of these multitrophic networks, the potential negative role of ants as mutualists of pest hemipterans must be analysed without overlooking the other services that ants may provide to the plants they visit, ranging from suppression of plant pathogens (Offenberg and Damgaard, 2019) to predation of non-mutualist phytophagous insects (Styrsky and Eubanks, 2007). Some efficient predatory ants may also be strongly protective of their hemipteran partners (Styrsky and Eubanks, 2007; Offenberg *et al*., 2019), but little is still known about this behaviour for most European ants that may act as pest predators (Campolo *et al*., 2015; Schifani *et al*., 2020).

Lady beetles are key predators of ant-mutualist hemipterans in natural environments and are frequently employed as biological control agents in agroecosystems (Obrycki and Kring, 1998). We carried out a field experiment to study the behavioural interactions between five European ant species associated with the walnut aphid *Panaphis juglandis* and two lady beetle species. These lady beetles are important predators and biological control agents of the walnut aphid (Kök *et al*., 2018; Czechowski *et al*., 2019; Gao *et al*., 2020): the smaller and native *Adalia bipuntata* and the larger allochthonous Asian species *Harmonia axyridis* (Brown *et al*., 2007; Li *et al*., 2021). Our experiment aimed at describing the behaviours of the associated ant species and comparing their differences in relationship with the protective ability they were able to grant to the aphids against the two lady beetle species.

Materials and methods

Data collection was carried out at the Parma University Campus, Italy (44.7684, 10.3140), from 6 to 17 June 2022 (and daily from 11:00 to 15:00), when *P. juglandis* was abundant. A total of 12 walnut trees (*Juglans regia* L.) colonised by *P. juglandis* were selected based on the presence of five different ant species associated: *Camponotus piceus* (Leach, 1825), *Ca. vagus* (Scopoli, 1763), *Crematogaster scutellaris* (Olivier, 1792), *Dolichoderus quadripunctatus* (Linnaeus, 1771) and *Lasius emarginatus* (Olivier, 1792). The identity of these ants was ascertained before the beginning of the experiment by sampling two workers per each species and each tree, identifying them using a ZEISS Stemi 508 stereoscopic microscope and the keys provided by Seifert (2018). Adults of *H. axyridis* were collected in the field, while *A. bipunctata* adults were acquired from Bioplanet (Italy). Both species were temporarily reared in plastic cages under laboratory conditions (*T*: 25 ± 1°C, R.H.: 55 ± 10%) and fed with field-collected *P. juglandis* aphids.

In each experimental trial, we carefully introduced one adult lady beetle of either species to the dorsal surface of a leaf containing at least 15 *P. juglandis* aphids attended by ant workers. The introduction of the lady beetle was conducted slowly to avoid causing any reaction in the ants or aphids by creating vibrations on the plant – experimental trials in which the introduction itself accidentally caused visible vibrations and reactions by aphids or ants were aborted and another leaf was taken. In all cases, only one ant species was present on each leaf, and the leaves were located at a height of about 1.5 m from the ground. Once the lady beetle was introduced, we filmed the insects on the leaf until at least one of the following events occurred: (i) the lady beetle left the dorsal surface of the leaf; (ii) all aphids were either killed by the lady beetle or left the dorsal surface of the leaf; (iii) five minutes have passed since the introduction of the ladybug. Videos were analysed with the software Solomon Coder (solomon.andraspeter.com) to collect data on the interactions of ants, lady beetles and aphids.

We recorded three parameters as continuous variables:

- ➢ Ant workers on the leaf. The number of ant workers per leaf at the beginning of each experimental trial.
- ➢ Lady beetle on the leaf (time). The cumulative time (seconds) during which the lady beetle stayed on the leaf since the first encounter with ants.
- ➢ Lady beetle with the aphids (time). The cumulative time (seconds) during which the lady beetle stayed in proximity (<3 mm) of the aphids.

Moreover, ten behaviours were recorded as binary variables (presence/absence):

- ➢ Lady beetle biting aphids. The lady beetle bites and/or eats at least one of the aphids.
- ➢ Aphids flee from the leaf. At least one of the aphids stops feeding and walks away. In all our observations, this behaviour was always involving multiple aphids simultaneously.
- ➢ Ants flee from the leaf. At least one of the ants abandons the leaf by walking away to other parts of the plant.
- ➢ Ants opening mandibles (threat). At least one of the ants opens its mandibles towards the ladybug, without a successful bite following; genuine but unsuccessful biting attempts are not distinguished from bite threats.
- ➢ Ants biting the ladybug. At least one of the ants successfully bites the ladybug, grasping any part of its body or appendages.
- ➢ Ants bending their gaster. At least one of the ants directs its gaster towards the lady beetle at a short distance. This behaviour corresponds to threatening the release or releasing toxic chemicals against an enemy, the two being normally indistinguishable in the field. There are slight differences in the movements performed by the examined species: in *Camponotus* spp. and *L. emarginatus*, the gaster passes under the body and the head; in *Cr. scutellaris* the gaster may be directed at almost any possible angle and the spatulate stinger connects with the target to release its venom topically; in *D. quadripunctatus*, the gaster is oriented laterally, diverging from the body axis.
- ➢ Ants chasing the ladybug. While the lady beetle is far from the aphids (>5 mm distance), at least one of the ants stops attending to the aphid colony to threaten or attack the lady beetle with one of the abovementioned behaviours (ants open mandibles (threat), ants biting the ladybug or ants bending their gaster).
- ➢ Ants falling off the leaf. At least one ant falls off the leaf after approaching the ladybug. Ants were never observed falling off the leaf under any other circumstances.
- ➢ Ants grooming. At least one ant cleans its mouthparts after biting the ladybug.
- ➢ Ants rescuing aphids. After the lady beetle grabs an aphid with its mouth (see above lady beetle biting aphids), at least one ant starts pulling the aphid in the opposite direction. We recorded whether the aphid was freed or not from the lady beetle because of this behaviour.

We conducted 12 experimental trials for each combination of ant and lady beetle species for a total of 120 trials (12 replicates × 2 lady beetle species × 5 ant species). Data were analysed using the software R and RStudio (R Core Team, 2022; RStudio Team, 2022). We preliminarily explored the data through generalised linear models considering the role of the lady beetle species (factor with two levels), the ant species (factor with five levels) and their interaction on each of the continuous or binomial variables recorded. The interaction term was never statistically significant, which led us to analyse the role of the ant or lady beetle species through separate statistical tests. For continuous variables (ant workers on the leaf, lady beetle on the leaf and lady beetle with the aphids), we analysed differences between ant or lady beetle species using Kruskal–Wallis tests, which were followed by Conover's tests of multiple comparisons from the PMCMRplus R package whenever significant differences were detected. The Bonferroni correction was applied to Conover multiple comparison tests. Binomial variables (lady beetle biting aphids, aphids flee from the leaf, ants flee from the leaf, ants opening mandibles (threat), ants biting the ladybug, ants bending their gaster, ants chasing the ladybug, ants falling off the leaf, ants grooming, ants rescuing aphids) were analysed by using χ^2 tests, which were followed by the analysis of standardised residuals if significant differences were detected.

Results

Concerning the continuous variables, there were no statistical differences between experiments conducted with the two lady beetle species (ant workers per leaf: $H_{(1)} = 0.06$, $P = 0.80$; lady beetle on the leaf (time): $H_{(1)} = 3.13$, $P = 0.07$; lady beetle near aphids (time): $H_{(1)} = 1.33$, $P = 0.25$), while differences between ant species were always significant (ant workers per leaf: $H_{(4)} = 101.25$, $P < 0.001$; lady beetle on the leaf (time): $H_{(4)} = 17.80$, $P = 0.001$; lady beetle near aphids (time): $H_{(4)} = 25.50$, $P < 0.001$). Regarding ant workers per leaf, pairwise comparisons revealed that all ant species differed significantly from each other ($0.014 < P < 0.001$), except for *Ca. piceus* and *Ca. vagus* which shared the lowest numbers of workers ($P = 0.998$), while *D. quadripunctatus* had the highest one (fig. 1A). The lady beetle on the leaf

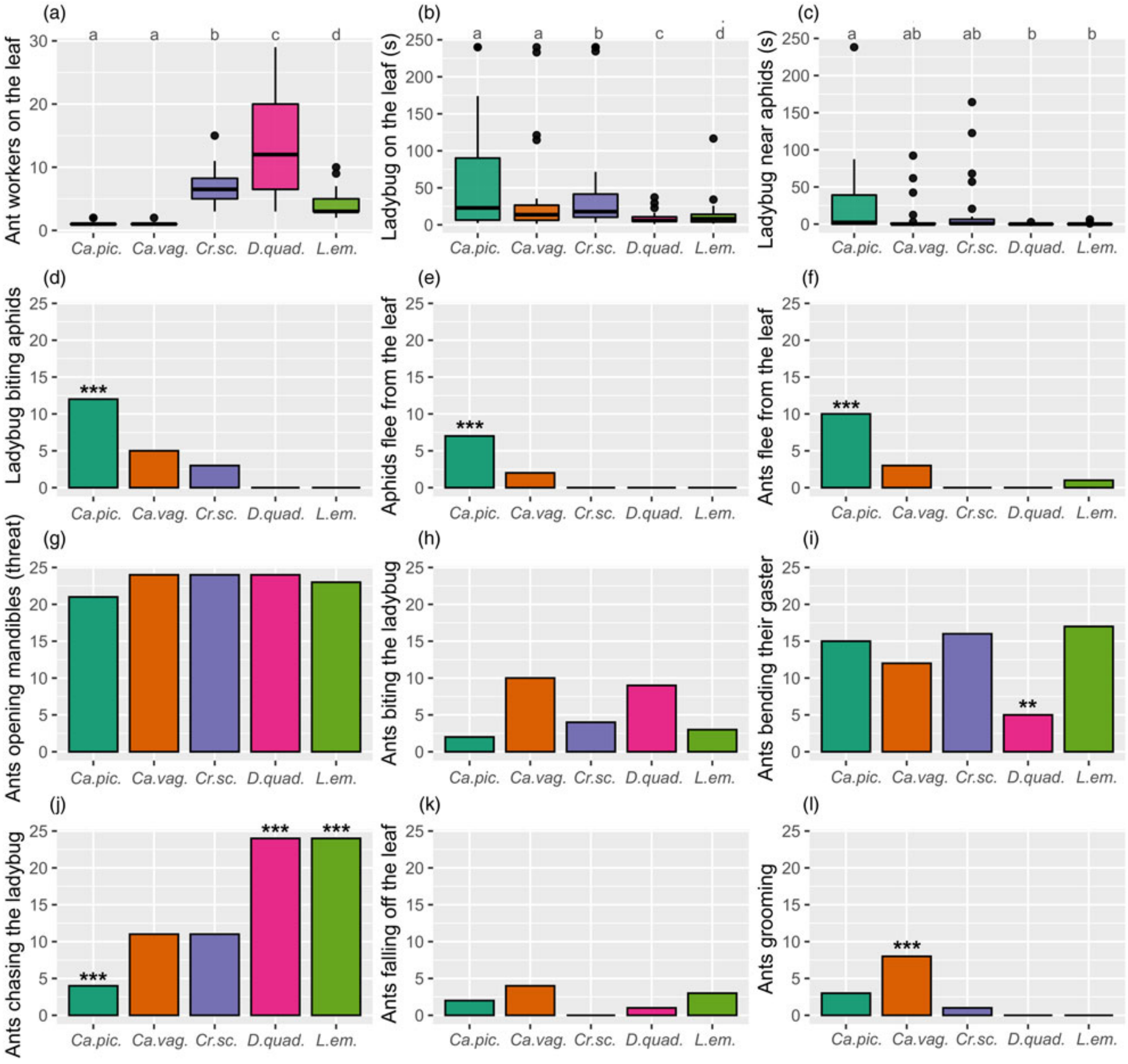

Figure 1. Behavioural data collected during the experiments, divided according to the identity of the ant species involved in the trials. Concerning continuous numerical data (A–C), groups marked with the same lowercase letter were not significantly different according to pairwise comparisons. In the case of binomial data (D–L), groups significantly different than the expected are highlighted using asterisks according to the significance level (*$P \leq 0.05$; **$P \leq 0.005$; ***$P \leq 0.001$).

Table 1. Results of χ^2 analyses of differences between lady beetles and among ant species for nine binomial behavioural variables recorded in this study

Variable	Between lady beetle species	Among ant species
Lady beetle biting aphids	$\chi^2_1 = 0.54$, $P = 0.46$	$\mathbf{\chi^2_4 = 29.40}$, $\mathbf{P < 0.001}$
Aphids flee from the leaf	$\chi^2_1 = 0.48$, $P = 0.48$	$\mathbf{\chi^2_4 = 22.41}$, $\mathbf{P < 0.001}$
Ants flee from the leaf	$\chi^2_1 = 0.73$, $P = 0.39$	$\mathbf{\chi^2_4 = 28.60}$, $\mathbf{P < 0.001}$
Ants opening mandibles (threat)	$\chi^2_1 = 0.26$, $P = 0.61$	$\chi^2_4 = 8.79$, $P = 0.07$
Ants biting the ladybug	$\chi^2_1 = 0.73$, $P = 0.39$	$\mathbf{\chi^2_4 = 12.3}$, $\mathbf{P = 0.015}$
Ants bending their gaster	$\chi^2_1 = 0.54$, $P = 0.46$	$\mathbf{\chi^2_4 = 15.80}$, $\mathbf{P = 0.003}$
Ants chasing the ladybug	$\chi^2_1 = 0.03$, $P = 0.85$	$\mathbf{\chi^2_4 = 55.40}$, $\mathbf{P < 0.001}$
Ants falling off the leaf	$\chi^2_1 = 0.11$, $P = 0.74$	$\chi^2_4 = 4.40$, $P = 0.35$
Ant grooming	$\chi^2_1 = 0.00$, $P = 1.00$	$\mathbf{\chi^2_4 = 20.09}$, $\mathbf{P < 0.001}$

Significant differences are highlighted in bold.

(time) was significantly different between *D. quadripunctatus* and *Ca. piceus* ($P = 0.021$), *D. quadripunctatus* and *Cr. scutellaris* ($P = 0.05$) and between *Cr. scutellaris* and *L. emarginatus* ($P = 0.04$) (fig. 1B). The lady beetle near aphids (time) differed between *Ca. piceus* and *D. quadripunctatus* ($P < 0.001$) and *Ca. piceus* and *L. emarginatus* ($P = 0.005$) (fig. 1C).

For any of the other recorded binary variables, there were no statistically significant differences between experiments conducted with the two lady beetle species, while all of them differed significantly between trials conducted with different ant species except for the ants opening mandibles (threat) behaviour (table 1). The analysis of standardised residuals of χ^2 tests revealed that lady beetle biting aphids, aphids flee from the leaf and ants flee from the leaf behaviours were all observed more frequently than the expected with *Ca. piceus* ($P < 0.001$, fig. 1D–F). The frequency of ants bending their gaster was lower than expected in *D. quadripunctatus* ($P = 0.002$, fig. 1I). Ants chasing the lady beetle were observed less frequently than expected in *Ca. piceus*, and more frequently than the expected in *D. quadripunctatus* and *L. emarginatus* ($P < 0.001$; fig. 1J). Finally, ants grooming was observed more frequently than expected in *Ca. vagus* ($P < 0.001$; fig. 1L).

The ants rescuing aphids behaviour was the only one not to be statistically analysed as it was exhibited only twice (fig. 2). In both cases, this behaviour was performed by *Cr. scutellaris*, once interacting with *A. bipunctata*, and once with *H. axyridis*. In the first case, the ant successfully managed to free the aphid from the ladybug, while in the second case, both the lady beetle and the ant kept pulling the aphid in opposite directions beyond the time duration of the video.

Discussion

The high diversity of interactions between ants, their hemipteran partners and the arthropod natural enemies of the hemipterans is still undocumented in most cases, but of high interest in evolutionary terms as well as in an applied perspective for pest management and biological control (Oliver *et al.*, 2008; Depa *et al.*, 2020; Parker and Kronauer, 2021; Castracani *et al.*, 2023; Schifani *et al.*, 2023*a*). Our results highlight how a large proportion of this variation may depend on the identity of the ant species (Völkl *et al.*, 1996; Schifani *et al.*, 2023*a*).

On one hand, *D. quadripunctatus* and *L. emarginatus* were the most effective species in the protection of aphids. Workers of *D. quadripunctatus* were on average more numerous than those of *L. emarginatus* and used fewer chemical attacks, but the behaviour of both species similarly caused the aphids to stay safe. Both aggressively attacked the lady beetles even if they were not close to the aphids, causing them to abandon the leaves rapidly. These results suggest that *D. quadripunctatus* can be a pugnacious species (Schifani *et al.*, 2022), dismissing the idea that it does not defend the associated *P. juglandis* colonies from lady beetles as stated by Czechowski *et al.* (2019).

On the other hand, the attacks of *Ca. piceus* were mostly ineffective at repelling the lady beetles, and its workers (which were

Figure 2. Aphid rescue behaviour performed by *Cr. scutellaris* in filmed interactions with *A. bipunctata* (left) and *H. axyridis* (right). In both cases, an ant worker is shown attempting to save a *P. juglandis* aphid grabbed by the ladybug.

normally few in numbers) rarely attacked the lady beetles if these were not close to the aphids, and often completely abandoned the leaf soon after the lady beetle attacks on the aphids started. This resulted in the lady beetles spending considerably more time on the leaves, and frequently killing aphids, often to the point of causing the whole aphid colony to flee.

An intermediate performance in terms of aphid protection was recorded for *Ca. vagus* and *Cr. scutellaris*. *Ca. vagus* workers occurred in small numbers like *Ca. piceus* but are much larger than any other ant species observed in this study (Seifert, 2018). Stronger biting force because of larger size may have determined their more effective protective service compared to *Ca. piceus*, and the higher frequency of self-cleaning behaviour after bites. *Cr. scutellaris*, an aggressive and dominant species of the canopy (Castracani *et al*., 2017; Seifert, 2018; Giannetti *et al*., 2019, 2022; Schifani *et al*., 2022, 2023*b*, 2023*c*), was the only species to perform the aphid rescue behaviour. Considering that *Cr. scutellaris* queens sometimes store in special chambers of their nests living *P. juglandis* aphids during the earlier stages of colony foundation (Giannetti *et al*., 2021), our observations on the aphid rescue behaviour reinforce the idea of a special relationship between the two species. Both *Ca. vagus* and *Cr. scutellaris* often did not attack lady beetles that were not close to the aphids, and in their presence, lady beetles managed to kill some aphids. However, only in the case of *Ca. vagus* whole aphid colonies were observed to flee.

It was interesting to observe how ants had similar interactions with the two lady beetle species. Ant–lady beetle interactions can sometimes deeply differ based on the specific characteristic of the lady beetle species involved, with some specialised species being completely immune to ant attacks or even taking advantage of ant presence (Liere and Perfecto, 2008). Apart from their geographic origin, *A. bipunctata* and *H. axyridis* are not known to possess any highly specific adaptation to cope with ants, but differ in size, with the latter being considerably larger than the former. However, the larger ants examined in our experiment, *Ca. piceus* and *Ca. vagus*, performed as good or worse than the remaining smaller species, suggesting that size is not a key determinant in the outcomes of ant–lady beetle encounters.

Mutualistic aphids are attacked by several different predators and parasitoids, which often co-occur and may compete for the same trophic resource (Schifani *et al*., 2023*a*). While the behavioural response of ants may also vary significantly at the individual level (Novgorodova, 2015), the differences among ant species may be crucial in favouring some arthropod natural enemies over others for pest control (Völkl *et al*., 1996), and a better understanding of these dynamics may lead to improved biological control of several pest hemipteran species. In our experiment, the worse aphid protector, *Ca. piceus*, is a species with small- to medium-sized colonies that is generally described as timid, while the remaining, more effective species are characterised by either very large worker size (*Ca. vagus*) or very large colony size (*Cr. scutellaris*, *D. quadripunctatus*, *L. emarginatus*) (Seifert, 2018). Interestingly, in comparison to the other three, *D. quadripunctatus* is not traditionally described as an aggressive species. While general aggressiveness has been indicated as important, no conclusive evidence has yet emerged over natural history traits that can be used to predict the role of an ant species as a good or bad defender of its hemipteran partners, making it still important to extend the baseline knowledge to the behaviour of more species (Buckley and Gullan, 1991; Novgorodova and Gavrilyuk, 2012; Wang *et al*., 2021; Schifani *et al*., 2023*a*).

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Section II - Ants as mutualist partners of insect pests

Chapter 8

Towards sustainable management of ant-hemipteran mutualism in agricultural setting

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Toward sustainable management of ant-hemipteran mutualism in agricultural settings: a comparison of different approaches

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ABSTRACT

Mutualistic associations between ants and honeydew-producing hemipterans have a great ecological and evolutionary significance across terrestrial habitats but can also cause pest outbreaks in agroecosystems. At the same time, ants are often effective predators of several agricultural pests, can improve soil quality, and can control some plant pathogens. Neither ant attendance of hemipteran pests alone, nor a positive correlation between the abundance of ants and hemipterans automatically imply that ants are a worthy target or a key element in hemipteran management strategies. The main tactics in the management of ant-hemipteran associations in agroecosystems include the use of sticky or insecticidal barriers, low-toxicity baits, alternative sugary sources, or ant-adapted biocontrol agents. Barriers can quickly seal ground-nesting ants from the canopy of perennial plants but are unselective towards other arthropods and costly in terms of maintenance. Low-toxicity baits have been particularly tested against invasive and supercolonial ant species and yet are worth considering only when the complete elimination of ant colonies can be desirable. More recently developed and not yet widely available methods based on the provision of alternative sugary sources to manipulate ant behavior can allow to retain or even enhance the contribution of ants to the control of other phytophagous insects or plant pathogens while effective disrupting their mutualism with hemipteran pests. Finally, many parasitoids and predators possess specific adaptations to bypass attending ants, but the existence of species-specific factors complicatesthese networks. Further basic research and longer-term studies are needed to refine and improve the development of sustainable management strategies.

1. Introduction

1.1. *The ecological success of ant-hemipteran mutualisms*

Ants are among the most successful groups of insects across the world, establishing a myriad of interactions within complex multi-trophic networks that involve other animals, plants, and fungi (Hölldobler and Wilson, 1990; Rico-Gray and Oliveira, 2008; Stadler and Dixon, 2008; Lach et al., 2010; Parker and Kronauer, 2021). Trophobiotic interactions between ants and honeydew-producing hemipterans of the suborder Sternorrhyncha significantly impacted the evolutionary radiations of some of today's main ant, aphid, scale, psyllid, and whitefly lineages, and became an ecological keystone in many ecosystems (Hölldobler and Wilson, 1990; Lach et al., 2010; Parker and Kronauer, 2021). The attractiveness of the honeydew produced by hemipterans usually represents the most important reason ants visit plants apart from the eventual presence of extrafloral nectaries (Blüthgen et al., 2004; Engel et al., 2001; Campos and Camacho, 2014; Grasso et al., 2015). Ant-hemipteran mutualistic associations have offered a remarkable model for evolutionary biologists. In exchange for honeydew, ants may help their partners in several ways: *i)* displacing or killing their natural enemies, including parasitoids and predators (Jiggins et al., 1993; Kaplan and Eubanks, 2002; Martinez-Ferrer et al., 2003; Majerus et al., 2007; Dao et al., 2014), and reducing their need to invest in protective microbiota (Henry et al., 2015; Mandrioli et al., 2016); *ii*) reducing the abundance of competing non-mutualist honeydew-producing hemipterans (Engel et al., 2001; Miñarro et al., 2010); *iii*) controlling the hygienic conditions of their colonies by cleaning excess honeydew and releasing anti-microbial and anti-fungi substances (Lit et al., 1999; Morales, 2000; Queiroz and Oliveira, 2001; Matsuura and Yashiro, 2006; Nielsen et al., 2010); *iv*) actively dispersing them to new plants (Das, 1959; Collins and Leather, 2002; Giannetti et al.,

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2021); *v*) offering them protection from adverse meteorological conditions and offering them shelter inside their nests during adverse seasons (Maschwitz and Hänel 1985; Giannetti et al., 2021). Those ant and hemipteran species that participate in these mutualistic relationships developed a variety of morphological and behavioral adaptations based on their level of dependence, spanning from facultative to strictly obligate mutualisms depending on the ant or hemipteran species examined (Delabie, 2001; Stadler and Dixon, 2005; Depa et al., 2020; Parker and Kronauer, 2021). The emergence of mutualism between ants and honeydew-producing hemipterans also caused an evolutionary arms race with the natural enemies of the latter, resulting in many different adaptations to reduce their vulnerability to ants (Stadler and Dixon, 2008). For instance, ladybug larvae of several groups possess specific defensive structure to deter ant attacks (Majerus et al., 2007; Schwartzberg et al., 2010). Myrmecophilous aphid parasitoids even benefit or depend on the presence of certain ants attending their host species (Völkl, 1992, 1994; Völkl and Mackauer, 1993; Völkl et al., 1996), and so do certain coccidophagous lady beetles (Liere and Perfecto, 2014). In any case, the presence of mutualist hemipterans on plants represents a key attracting factor for many ant species to visit the plants (Styrsky and Eubanks, 2007).

1.2. Ant-hemipteran associations as a threat to crop protection

Ant-hemipteran mutualism is by far the most frequently reported problematic interaction involving ants in agroecosystems (Way and Khoo, 1992; Anjos et al., 2022) and, unlike plant foraging or seed predation, is not restricted to few specialized ant lineages (leafcutter ants and harvester ants, see Della Lucia et al., 2014; Uhey and Hofstetter, 2022). While many honeydew-producing hemipterans that are important agricultural pests are not ant-mutualists, several species of ant-mutualist hemipterans rank among the top agricultural pests. For instance, out of 16 studies documenting the effect of ants on honeydew-producing citrus pests that are not obligate mutualists, 10 reported a higher abundance of the pest species in the presence of ants (Anjos et al., 2021). On fava bean plantations, Banks and Macaulay (1967) recorded up to a 50% decrease in the number of seeds produced when facultatively mutualist aphids were attended by ants. Examples of

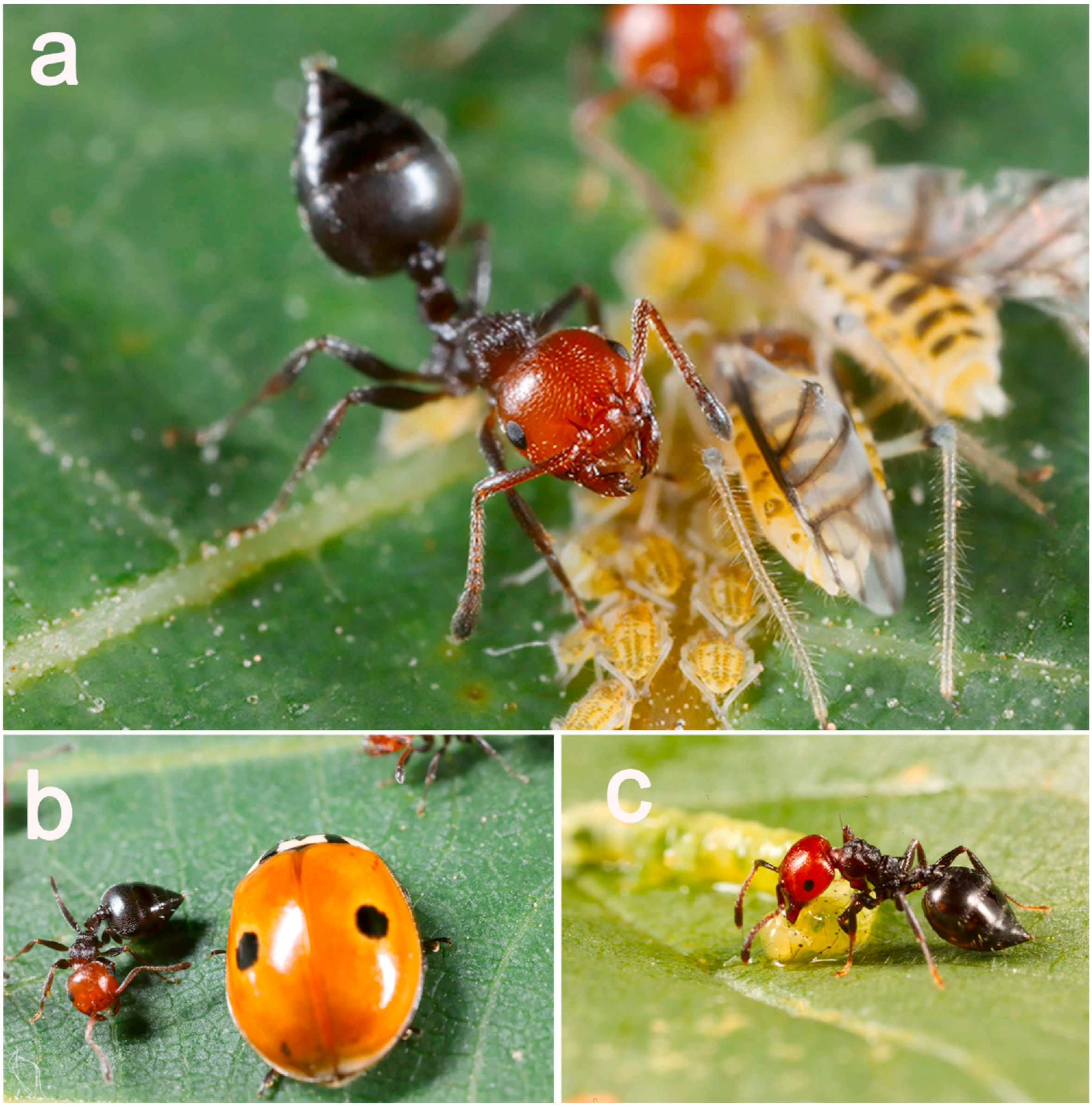

Fig. 1. *Crematogaster scutellaris* ant workers attending an aphid colony (a), defending the aphid colony against the ladybeetle *Adalia bipunctata* (b), and preying upon a caterpillar (c).

hemipteran pests attended by ants include economically important species of both aphids (e.g., the black bean aphid *Aphis fabae* Scopoli and the cotton aphid *A. gossypii* Glover – see Banks and Macaulay, 1967; Kaplan and Eubanks, 2002; Powell and Silverman, 2010; Mirzamohammadi et al., 2019), mealybugs (e.g., the vine mealybug *Planococcus ficus* Signoret, the citrus mealybug *P. citri* Risso, or the cotton mealybug *Phenacoccus solenopsis* Tinsley – see Daane et al., 2007; Marras et al., 2008; Mgocheki and Addison, 2009; Zhou et al., 2012; Cocco et al., 2021; Delabie et al., 2021), and whiteflies (e.g., the wooly whitefly *Aleurothrixus floccosus* Maskell – see Anjos et al., 2021). Notably, all these hemipteran species are facultative mutualist, not strictly requiring ant attendance to survive, yet often visited by a variety of different ant species. Interference with biological control agents is by far the most frequently documented cause of hemipteran outbreaks in the presence of ants (e.g., Kaplan and Eubanks, 2002; Mgocheki and Addison, 2009; Cheng et al., 2015; McCalla et al., 2023; Plata et al., 2023; Fig. 1a and b), while other mechanisms (such as protection from fungal outbreaks on uncollected honeydew or increased survival of first-instar hemipterans) are more rarely investigated but may play a significant role (Queiroz and Oliveira, 2001; Daane et al., 2007). Moreover, it is important to note that not all ant species associated with hemipteran pests have a significant protective role against their natural enemies (e.g., de Jesus et al., 2016; Schifani et al., 2023a; 2023e). In this context, aggressive ant species (Buckley and Gullan, 1991; Novgorodova and Gavrilyuk, 2012), and more importantly invasive alien species are more likely to efficiently defend hemipterans against parasitoids and predators (Wang et al., 2021).

1.3. *Provision of ecosystem services by ants in agricultural settings*

A large part of the interactions of ants with phytophagous insects are positive for agriculture, resulting in the killing or displacement of these insects due to predation or territorial aggression, with an overall positive effect on pest control (Choate and Drummond, 2011; Anjos et al., 2022; Fig. 1c). Ants are known as the first reported example of the use of a biological control agent in agriculture with the Asian weaver ant *Oecophylla smaragdina* (Fabricius) used against citrus pests in China since 304 AD (Huang and Yang, 1987; Van Mele, 2008; Offenberg, 2015). The active use of many species as biocontrol agents across several countries continues today (Peng and Christian, 2010; Offenberg, 2015). However, ant services as natural enemies of pest insects are significant even before the employment of active strategies and are globally reported, and in many systems may lead to neat crop yield increases (Anjos et al., 2022). Most literature focused on the tropics, with weaver ants in Asian fruit orchards (Offenberg, 2015) or *Azteca* ants in Mesoamerican coffee agroforests serving as primary examples, but increasing efforts focused on agroecosystems of temperate regions (Campolo et al., 2015; Schifani et al., 2020; Jensen et al., 2023). The predatory services that ants provide against particularly problematic phytophagous insects may sometimes offer to the plant a much greater benefit compared to the costs caused by sustaining ant-mutualist hemipterans at the same time (Styrsky and Eubanks, 2007, 2010). Apart from aggressive interactions with phytophagous arthropods, by simply visiting plants, ants can also contribute to suppress plant pathogen incidence by passively releasing many effective antibiotics on the surfaces they walk over (Offenberg and Damgaard, 2019; Offenberg et al., 2022). Even though less frequently documented, these interactions appear abundant and of probably high ecological impact (Offenberg and Damgaard, 2019; Giannetti et al., 2019; Offenberg et al., 2022).

On the ground, ants also act as very important ecosystem engineers and agents for soil bioturbation and enrichment, with most species excavating their nests in the soil and the nests becoming a hotspot of nutrients due to the amount of food ants concentrate (Altfeld and Stiling, 2009; Lach et al., 2010; Solida et al., 2011; Taylor et al., 2019). Furthermore, ants can also be important seed predators playing a role in the control of weeds, although the same ability may result in losses of cultivated seeds in other contexts (Baraibar et al., 2011).

2. Management tactics for ant-hemipteran associations

A number of tactics have been developed to deal with the problematic role of ants as partners of honeydew-producing hemipteran pests (Fig. 2), and their pros and cons are here reviewed and discussed. The application of any strategy should however be ideally preceded by an assessment of the real contribution of the local ants to a possible problem related to these hemipterans, keeping in mind that attendance by ants does not necessarily equals the provision of important services from the ants to their partners (Schifani et al., 2023a,e). While such assessments are infrequent or sometimes limited to correlational evidence, it is important to note that any strategy to reduce ant-hemipteran mutualism bears some costs, in some cases implying a meaningful loss of ecosystem services otherwise provided by ants. Sometimes, the same ant species that provide important pest control services may also cause disservices related to the control of ant-mutualist hemipteran pests, although their extent can greatly vary (Anjos et al., 2022). For instance, *Oecophylla* tropical weaver ants, while being widely recognized as excellent control agents for countless phytophagous pests, can sometimes still have some negative effect on the biological control of mealybugs (Offenberg, 2015; Forbes and Northfield, 2017), in certain case worth the application of appropriate management tactics (Correa et al., 2023). Elsewhere, ants of the Mediterranean *Tapinoma nigerrimum*-complex are often associated with mealybugs in citrus orchards and vineyards, and laboratory experiments suggest that they may significantly lower the success of parasitoids and predators of the citrus and vine mealybugs, respectively (Mansour et al., 2012). However, field data demonstrate that they can also significantly lower the survival rate of the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Campolo et al., 2015), while laboratory experiments suggest that they can act as an enemy of the ambrosia beetle *Xylosandrus compactus* (Eichhoff), the codling moth *Cydia pomonella* (L.), and the green stink bug *Nezara viridula* (L.) (Giannetti et al., 2022; Schifani et al., 2023b,c,d). In Europe, *Formica rufa*-group ants have historically attracted significant attention as biocontrol agents of pests in forestry systems, such as the pine processionary moth *Thaumetopoea pityocampa* (Denis & Schiffermüller) and the western larch case-bearer *Coleophora laricella* (Hübner) (e.g., Pavan, 1951, 1961; Adlung, 1966). However, more recent studies dealt with their application in north-European apple plantations, where *F. rufa*-group ants can cause a significant reduction of the winter moth *Operophtera brumata* (L.) and the apple scab *Venturia inaequalis* (Cooke), but may also significantly increased aphid infections by *Aphis pomi* de Geer if not properly managed (Offenberg et al., 2019; Jensen et al., 2023). A common European ant, *Lasius niger* (L.), can have a negative impact over aphid natural enemies (Jiggins et al., 1993; Völkl and Mackauer, 1993) but also be a predator of the brown marmorated stink bug *Halyomorpha halys* (Stål) (Bulgarini et al., 2021). Finally, the red imported fire ant *Solenopsis invicta* Buren, an invasive alien species of global relevance and a threat to agriculture and biodiversity (Menchetti et al., 2023), is at the same time a major predator of phytophagous insects and a promoter of mutualist hemipteran outbreaks in the US (Kaplan and Eubanks, 2002; Hood et al., 2003; Coppler et al., 2007; Rashid et al., 2013). In this context, a careful evaluation of the cost-efficiency of different alternative tactics is important.

2.1. *Preventing ants to access plants by applying barriers*

The use of barriers to prevent ants from climbing on plants probably represents the earliest attempt to deviate from the much damaging unselective wide use of pesticides in the control of ant-hemipteran associations in agroecosystems (Davis and Van Schagen, 1993). The barriers may be either insecticidal or sticky, and their use is normally limited to perennial crops of plants whose trunk is suitable for their applications and in which the prevailing ants are ground-nesting and need to climb

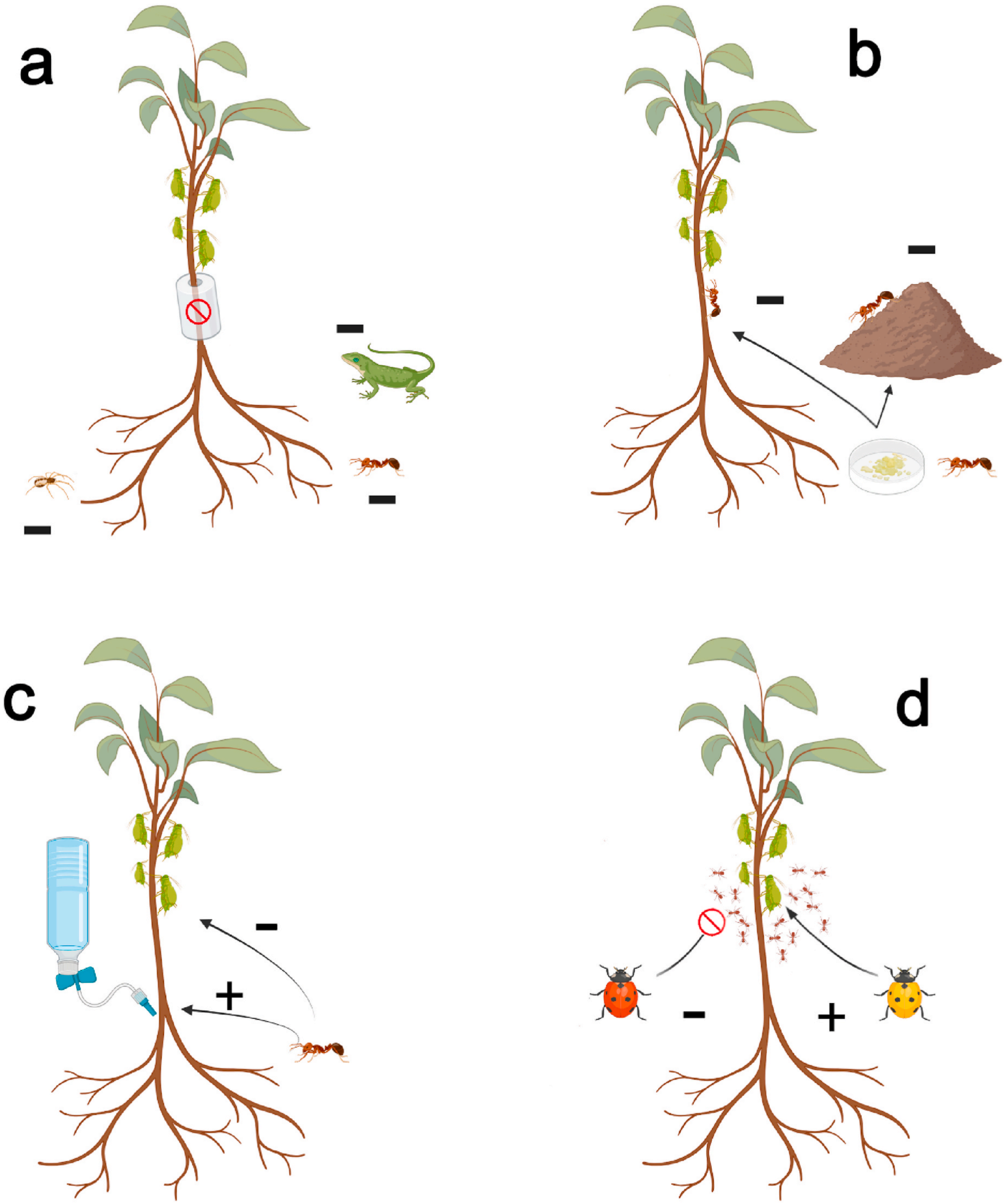

Fig. 2. The four main approaches for the management of ant-hemipteran mutualistic relationships in agroecosystems are exemplified: (a) exclusion of ants from the plants with the application of physical or toxic barriers, which can also limit the access to climbing predators; (b) use of selective toxic baits that eliminate ants and their colonies; (c) behavioral manipulation of ants through alternative sugary sources that reduce their dependance on hemipteran colonies; (d) use of selected natural enemies species with the ability to bypass the locally dominant ants as opposed to those that ants are effectively able to repel. Minus and plus symbols indicate the expected abundance increase (+) or decrease (−) of ants and natural enemies based on each of the four tactics. Image created with biorender.com and modified.

on the plant to access the hemipteran colonies (Juan-Blasco et al., 2011). With a relatively simple functioning, the correct application of barriers immediately stops ants from accessing the plants they are applied to, making them a very rapid method of intervention. Sticky barriers are also often used in research experiments on ant exclusion (e.g., Piñol et al., 2010, 2012; Nagy et al., 2015; Schifani et al., 2020, 2023a). However, their use can also bear some noteworthy difficulties and costs. Sticky barriers usually need to be replaced at least monthly if not more frequently, since rain and dust, as well as dead arthropods attached on the glue can greatly affect their effectiveness (Schifani et al., 2020; McCalla et al., 2023). Their application is particularly difficult on plants with very irregular surfaces, preventing their complete adherence, since the minimal gaps can often be exploited by ants. For the same reason, their effective use requires a continuous grove micromanagement to prevent ants from circumventing the barriers, including the removal of debris and weeds (Juan-Blasco et al., 2011; McCalla et al., 2023). All these factors contribute to making large-scale application of sticky barriers relatively costly, but are partly overcome by insecticidal barriers, whose effect tends to last for much longer even if being still to a certain degree susceptible to irrigation and temperature (Juan-Blasco et al., 2011).

As barriers have the intended effect of stopping or killing any ant that tries to climb on a plant, they obviously also stop any beneficial effect that ants can have on the targeted plants. Nonetheless, their effect on ant colonies is normally not as heavy, and ant presence on the ground is maintained. Longer-term studies evaluating a potential effect on ant species composition due to the exclusion of ants from hemipteran colonies on the plants are lacking. However, it also important to note that barriers are unselective, stopping or killing all arthropods trying to access the plants by climbing, including natural enemies and other non-target species (Juan-Blasco et al., 2011; McCalla et al., 2023). Some of the more powerful commercially available sticky barriers may easily kill even small vertebrates such as young *Podarcis* lizards and may be even attractive to some dipterans (authors observations), while insecticide paints able to create toxic barriers for months can obviously affect a wide range of non-target organisms (McCalla et al., 2023).

2.2. *Using toxic baits to poison ant colonies*

Low-toxicity baits using different chemical compounds and concentrations have been especially used to suppress the populations of the invasive Argentine ant *Linepithema humile* (Mayr) in both citrus orchards and vineyards to disrupt its strong mutualistic relationship with hemipteran pests (Klotz et al., 2003; Tollerup et al., 2004; Greenberg et al., 2006; Daane et al., 2008; Nyamukondiwa and Addison, 2011, 2014; Buczkowski et al., 2014; McCalla et al., 2023), while uses against other ant species for the same purpose are rarely reported (Nyamukondiwa and Addison 2011, 2014; Nondillo et al., 2016). In its invasive range, *L. humile* is known to form dense supercolonial populations while displacing almost all native ant species and monopolizing sugary food resources, therefore ant-attractive toxic baits *de facto* target almost exclusively *L. humile* and should have minimal effects on other ants or arthropods in heavily invaded areas (Silverman and Brightwell, 2008; McCalla et al., 2023). These baits are based on the principle of delayed toxicity, as it happens with the baits used to eradicate certain ants in other contexts: this increases the chances that worker ants pass to each other and to the queens the toxic compound through the mechanism of trophallaxis (Rust et al., 2004). The supercolonial population structure of *L. humile* makes conspecific ants behave as nestmates and therefore easily exchange food through trophallaxis, enhancing the effectiveness of delayed toxicity (Nyamukondiwa and Addison, 2014). *Linepithema humile* has been shown to have a marked effect on the increase of mutualistic hemipterans such as *Aonidiella aurantii* (Maskell), *Coccus hesperidium* L., *Diaphorina citri* Kuwayama, or *P. citri*, and its successful almost complete eradication with low-toxicity baits has been observed to very cost-efficiently cause a collapse in the populations of these pests (McCalla et al., 2023).

The use of toxic baits obviously allowed to greatly narrow the range of impacted organisms as compared to earlier methods that consisted in the application of toxic compounds over part of the plants (Klotz et al., 2003; McCalla et al., 2023). However, their target specificity and effectiveness can greatly decrease in more diverse ant communities of non-invaded areas, where ant species may greatly differ in their food and bait preferences and most species are likely to organize in multiple, competing rival colonies rather than in supercolonies (Nyamukondiwa and Addison, 2014). While their effect is less immediate than that of barriers (Juan-Blasco et al., 2011), the expected result is the almost complete eradication of the targeted ant colonies from the treated area (McCalla et al., 2023), which implies the unselective complete loss of any ecosystem function ants can provide both on the ground and on the plants. While toxic baits can be temporarily used to promote ecological restoration in natural areas when aimed at the eradication of invasive ant species (e.g., Hoffmann, 2010), the ecological effects (even in a multitrophic cascade context) and feasibility of a sustained use aimed at the elimination of any trophobiotic ant species have never been studied.

2.3. *Manipulating ant behavior with alternative food sources*

The honeydew produced by hemipterans represents a key source of energy for many ant species but can be replaced. One of the hypotheses on the evolutionary origin and significance of ant-attracting extrafloral nectaries is that these can attract the services ants provide on the plants without paying the cost implied by hosting hemipteran colonies (Engel et al., 2001; Blüthgen et al., 2004; Campos and Camacho, 2014). To manage ants, it is possible to offer sugary solutions that outcompete honeydew-producing hemipterans, redirecting the attention of the ants and leaving the hemipterans without the defense of their partners (Nagy et al., 2013, 2015; Wäckers et al., 2017; Parrilli et al., 2021; Pérez-Rodríguez et al., 2021; Borbély and Nagy, 2022; Correa et al., 2023; Jensen et al., 2023). In front of an abundant supply of sugary resources, some ants may even shift their relationship with their mutualist partners from mutualism to antagonism, starting to significantly prey on them (Offenberg, 2001). More in general, different concentrations and compositions have been tested so far, normally consisting in sucrose solutions, causing a marked decrease of hemipteran populations usually associated with a sensible reduction of ant attendance and an increase in the abundance of natural enemies across different agroecosystems and targeting ants from different genera (Nagy et al., 2013, 2015; Wäckers et al., 2017; Parrilli et al., 2021; Pérez-Rodríguez et al., 2021; Borbély and Nagy, 2022; Correa et al., 2023; Jensen et al., 2023). The dispensers, sometimes also known as artificial nectaries to highlight the biomimetic inspiration based on extrafloral nectaries (Nagy et al., 2015; Schifani et al., 2020), can be placed on different parts of the plants or on the ground, effectively redirecting most ant activity. It is worth noting that in this sense the provision of sugary liquids can also be used to augment the presence of ants on the plants even in systems without problematic ant-mutualist hemipterans, enhancing their beneficial effects against other phytophagous insects or plant pathogens (Schifani et al., 2020). More in general, as recently demonstrated by Correa et al. (2023) and Jensen et al. (2023) with *Oecophylla*- and *Formica rufa*-group ants, respectively, the sugary provision has the potential to inhibit ant-hemipteran relationship while at least fully preserving the predatory and anti-pathogen activity of ants on cultivated plants.

As a relatively recent innovation, to the best of our knowledge, dispensers of sugary liquids for ants are unfortunately not yet commercially available. The economic cost of a prolonged application of such system has not been calculated, although it would perhaps be comparable to that of toxic baits, and therefore lower than sticky barriers. On the other hand, long term studies are lacking in this case too. Some challenges may also arise applying this method to supercolonial ant species with a diet heavily based on carbohydrates, which may be more difficult to satiate and may be more capable of increasing their colony size in response to the provision of sugary liquids due to a lesser dependance on protein sources. Finally, other potential developments of these tools could be based on further exploration of the composition of extrafloral nectaries and of their manipulative effect on ant behavior and predatory activity (Grasso et al., 2015; Nepi et al., 2018). It will also be important to explore the option of using plants with extrafloral nectaries, capable of altering the ant behavior also in neighboring plants (Staab et al., 2023).

2.4. *Bypassing ant protection by using the most appropriate biocontrol agents*

Ants are far from perfect guardians of their hemipteran partners, and many hemipteran predators and parasitoids have evolved specific adaptations to bypass them. Adjusting biocontrol techniques by selecting the best biocontrol agents based on the identity of the locally dominant ants is a promising opportunity: it can allow to deal with the hemipteran pests without negatively affecting ants in a direct way, with costs comparable to standard biological control tactics. Either in the form of active use, or in terms of conservation biological control, the role of ant-adapted natural enemies in the control of ant-mutualist hemipterans is already a naturally established reality. However, it is at the same time still little studied and requires a good understanding of the complex variety of ant and hemipteran species and their interactions.

Such complex networks require experimental testing to be understood and to define the appropriate natural enemies for each context. For example, *Nylanderia jaegerskioeldi* (Mayr), *Plagiolepis pygmaea* (Mayr), and *Tetramorium semilaeve* André ants associated with *A. fabae* aphids had no repellent effect on the locally occurring parasitoids and predators (Schifani et al., 2023a), but *L. niger* ants associated with the same aphid species can effectively repel lady beetles and parasitoids according to other studies (Jiggins et al., 1993; Völkl and Mackauer, 1993). However, while *L. niger* significantly reduces the parasitization success of the parasitoid *Lysiphlebus testaceipes* Cresson and can frequently kill *Trioxys angelicae* (Haliday), it had no impact on the parasitization of another parasitoid, *Lysiphlebus cardui* (Marshall) (Völkl and Mackauer, 1993). Therefore, on an *A. fabae*-ant system, an ideal biocontrol program should consider the specific identity of the ants associated with the aphids and the biocontrol agents used for their control. In a field experiment, most ant species associated with the walnut aphid *Panaphis juglandis* (Goeze) were able to quickly chase away the lady beetles *Adalia bipunctata* (L.) and *Harmonia axyridis* (Pallas) (Schifani et al., 2023e), and *Crematogaster scutellaris* (Latreille) ants even allowed *P. juglandis* aphids to overwinter in their nests (Giannetti et al., 2021). However, *Camponotus* ants had a much-reduced protective role against the same lady beetles, often fleeing and leaving the *P. juglandis* colonies unprotected (Schifani et al., 2023e). A further stark example of the complex variation of these networks is that of the root aphid parasitoid *Paralipsis enervis* (Nees), which possess myrmecophilous adaptations that enable it to receive food through trophallaxis from *L. niger* (Völkl et al., 1996). However, *Tetramorium caespitum*-complex species or *Myrmica laevinodis* Nylander did not offer food to *P. enervis* and instead decreased its survival time from five days to just one (Völkl et al., 1996). Even more interestingly, *P. enervis* was regularly killed within just 10 min when it encountered *Lasius flavus* (Fabricius), a congeneric species to *L. niger* that acted in a completely different manner (Völkl et al., 1996). Obviously, not all ant-mutualist hemipterans may be suitable targets for ant-adapted parasitoids or predators, and perhaps not all mutualist ants may be bypassed by them.

3. Conclusions

Sustainable management of ant-hemipteran associations would ideally require the understanding of these associations to be much more refined than it currently is. The first step before taking any management action should be to assess whether ant attendance of hemipteran pests truly poses any significant cost, and whether the same ants are providing significant useful services at the same time. Answering each of these questions is not a trivial task given the complexity of the ecological networks that involve ants even in the simplified context of agricultural settings. However, wasting resources on controlling non-problematic ant species may unnecessarily divert resources from the direct control of the hemipterans, while ignoring other services provided by ants may result in unexpected outbreaks of other pests after ant control, including other honeydew-producing hemipterans (Styrsky and Eubanks, 2007, 2010; Schifani et al., 2023a). To this regard, it is important to remind that a higher number of honeydew-producing hemipterans is likely to attract a higher number of ant workers, and such correlation alone is not sufficient prove that ants are actively favoring a growth in numbers of their hemipteran partners. The structure and diversity of ant communities themselves can be relevant for the overall effect of ants on mutualist hemipterans: some dominant species play a positive role in reducing ant-tended hemipterans, while invasive alien species that frequently lead to hemipteran outbreaks tend to eliminate other ants from the invaded areas (Wang et al., 2021; Costa-Silva et al., 2023).

The application of barriers can be rapid and effective at excluding ground-nesting ants from the canopy of many perennial plants, but also costly in terms of maintenance across entire seasons and totally unselective, targeting any arthropod that attempts to climb up. Low-toxicity baits can be relatively selective in their targeting and require less maintenance, performing particularly well for the control or eradication of invasive supercolonial ant species. However, their sustained use implies renouncing all the ecosystem services that ants can offer both to the plants and to the soil, which may be unjustified (Styrsky and Eubanks 2007; Offenberg and Damgaard 2019; Anjos et al., 2022). The more recent use of alternative sugary force to manipulate ant behavior and distract their activity still requires further testing and development before becoming widely accessible but can allow to fully retain or even enhance the many positive services ants may provide while completely disrupting ant-hemipteran mutualism. Finally, relying on the role of biocontrol agents able to bypass the locally prevailing mutualist ant species would similarly have a limited impact on ant-provided services while potentially requiring minimal intervention. Still, it requires widening the knowledge of ant-hemipteran-natural enemy interactions to more species to become easily applicable to more contexts.

Apart from further research on the characteristics and behaviors of ants involved in associations with hemipteran pests, other interesting directions concern the development of more advanced management techniques. A recently discovered opportunity of ant-hemipteran associations is using ant activity as a proxy to monitor the infestation level of ant-associated hemipterans in ways that can be hard to achieve with other methods. Castracani et al. (2022) demonstrated that monitoring the activity of ants associated with vine mealybugs allows to detect the presence of the mealybugs on individual plants significantly earlier in the season and at much lower densities compared to what is achieved by traditional methods that rely on visually searching mealybugs. Ants tend to build permanent foraging trails to stable resources such as associated hemipteran colonies, so that exploiting ant activity to monitor the abundance of hemipteran pests is likely possible and potentially useful in other contexts.

In conclusion, despite decades of research, ant-hemipteran associations remain a fertile ground for both basic and applied research. Across the different existing management tactics, there is a general lack of data for what concerns the effects of their long-term application. A promising direction for further management option is to continue taking inspiration from natural systems, for example by further developing the ability of manipulating ants with alternative sugary sources like plants do through extrafloral nectaries. At the same time, more habitat-wide agroecological measures on crop structure also deserve more attention (Choate and Drummond, 2011). For instance, changing the vegetation structure at the base of an orchard or on top of a cropping system dramatically affects shade, which is a crucial resource for ants, and can therefore promote a complete turnover in ant communities (Samways 1983; Choate and Drummond 2011), potentially leading to the partial or complete replacement of problematic species with others. Some of the different tactics revised in this paper can be integrated with one another (e.g., the use of alternative food sources and selected biocontrol agents), and their ultimate success is expected to be influenced by the broader management strategies adopted and ecological characteristics of the affected areas.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4. SECTION III:

FIRST DATA ON ANT DIVERSITY AND DISTRIBUTION IN ITALIAN AGROECOSYSTEMS

This last section is composed of three chapters. In Chapter 9 and Chapter 10, we present some first systematic data on the still largely unexplored ant fauna of common agroecosystems of Italy. Chapter 9 illustrates data gathered across ten vineyards in a key vinicultural region of Northern Italy, through pitfall traps placed in the soil and on the leaves of the plants. The abundance of ants found in these habitats was compared to the abundance of other arthropod groups, while recording a total of 22 ant species, 13 of which were found to climb on the plants. Ant abundance was overall much higher than that of any other arthropod group, but most ant individuals belonged to an alien species.

Chapter 10 compares two pear orchards of Northern Italy and Sicily with a specific focus on those ant species that climb or nest on the trees and on the role of tree size in shaping ant communities. A total of 20 species was found, with a clear trend of increasing tree size favoring richer ant communities and arboreal-nesting species being mostly confined to the larger trees. The ant communities in Sicily and Northern Italy were relatively similar in terms of major lineages and species groups, but very different in terms of actual species, based on different biogeographic characteristics.

Finally, Chapter 11 presents the inclusion of all the 31 ant species mentioned in Chapters 1-10 and inhabiting Italian agroecosystems into the European barcoding library through the “AntGem” project leaded by the Institute of Evolutionary Biology of Barcelona. At least two sequences per each species were included, using samples from regions far apart whenever possible. The aims of the project include facilitating the identification of ant biodiversity and mapping intra- specific phylogeographic patterns across Europe.

Section III - First data on ant diversity and distribution in Italian agroecosystems

Chapter 9

Assessing ant diversity in agroecosystems: the case of Italian vineyards of the Adige Valley

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ASSESSING ANT DIVERSITY IN AGROECOSYSTEMS: THE CASE OF ITALIAN VINEYARDS OF THE ADIGE VALLEY

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Giannetti D., Schifani E., Castracani C., Ghizzoni M., Delaiti M., Penner F., Spotti F.A., Mori A., Ioriatti C., Grasso D.A. - Assessing ant diversity in agroecosystems: the case of Italian vineyards of the Adige Valley

Agroecosystems have gained a dominant position on worldwide land-usage, and therefore preserving their biodiversity is crucial for environmental sustainability. Ants are one of the most widespread groups of terrestrial arthropods, and, thanks to their significant diversification, they are considered as a good proxy group for biodiversity monitoring, also in agroecosystems. Vineyards are economically valuable cultures widespread worldwide, and hosting many ant species, that provide meaningful ecosystem services and disservices. Despite the important role that ants play in these agroecosystems, ant biodiversity in vineyards is still poorly studied, especially in Italy. In this context, we present a first detailed quantitative and qualitative assessment of the ant fauna of Italian vineyards from the Adige Valley based on pitfall traps data, and discuss the results in comparison with the few other similar assessments from Europe and other continents. We document an assemblage of 22 species (7-16 per orchard), mostly dominated by three disturbance-tolerant species (including an introduced species). Vineyards' ant faunas appear to be rather heterogeneous worldwide, mainly following local ecological and biogeographical constraints, and the role that most ant species play in these agroecosystems is presently unknown.

KEY WORDS: vines; biodiversity monitoring; myrmecofauna; Prealps.

INTRODUCTION

Since agriculture has become a dominant category of land usage worldwide, crop management practices have become a decisive factor to preserve the environment (TILMAN *et al.*, 2001; GREEN *et al.*, 2005; TSCHARNTKE *et al.*, 2005; FIRBANK *et al.*, 2008). Overlooked for decades, insect and arthropod decline and its severe potential outcomes on ecosystems functioning recently attained much attention showing the need for a deeper commitment in the development of effective monitoring systems in contexts with different anthropic impacts (BURGIO & SOMMAGGIO, 2007; CAMPANARO *et al.*, 2011; BURGIO *et al.*, 2015; DIRZO *et al.*, 2014; HALLMANN *et al.*, 2017; LEATHER, 2017; PIZZOLOTTO *et al.*, 2018; HOMBURG *et al.*, 2019). While agricultural transformations may play a key in this process, diversity and distribution of the arthropodofauna in cultivated areas is still insufficiently documented.

Wine grapes (*Vitis vinifera* L.) are a widespread cultivated species of important economic value, whose cultivated surface is likely to increase in the future due to climate change (HANNAH *et al*., 2013; MORIONDO *et al.*, 2013). European vineyards alone cover 3.2 million ha representing 45% of the world's total areas under vines and 1.8% of the total utilized agricultural area. Over 20% of them is located in Italy (688,000 ha), representing about 5% of the total utilized agricultural surface (SAU) of the country (EUROSTAT, 2017; ISTAT, 2019). Under conventional management practices, establishment of viticulture is often associated with notable negative impacts on soil and local biodiversity, and thus may represent a serious conservation threat in certain contexts (ALTIERI & NICHOLLS, 2002; FAIRBANKS *et al.*, 2004; HILTY & MERENLENDER, 2004; COULOUMA *et al.*, 2006; HILTY *et al.*, 2006; HILDENBRANDT *et al.*, 2008; COLL *et al.*, 2011; LAWRENCE *et al.*, 2011; ROSADO *et al.*, 2013). However, implementing correct agro-ecological practices can be an effective way to address some of these issues (VIERS *et al.*, 2012): for example, organic viticulture may allow richer communities of organisms to thrive, both within the vineyards themselves and in neighboring forested areas (e.g. GAIGHER & SAMWAYS, 2010; COLL *et al.*, 2012; KEHINDE & SAMWAYS, 2014; CAPRIO *et al.*, 2015; MASONI *et al.*, 2017; DAANE *et al.*, 2018).

Due to their high diversity and strong ecological impacts, ants are considered an important group for biodiversity monitoring in both natural and anthropic impacted ecosystems, including agroecosystems (e.g. PECK *et al.*, 1998; DE BRUYN *et al.*, 1999; AGOSTI *et al.*, 2000; LACH *et al.*, 2010; GIBB *et al.*, 2017), where they provide impactful services and disservices. For example, they may control other arthropods, fungi or even weeds (e.g. RISCH & CARROL, 1982; BARAIBAR *et al.*, 2011; OFFENBERG & DAMGAARD, 2019) and favor foliar uptake of nitrogen (e.g. PINKALSKI *et al.*, 2018), but may also favor mutualistic pest species (e.g. PEKAS *et al.*, 2010; CALABUIG *et al.*, 2013; DAO *et al.*, 2014). As a result, ants can be employed as biocontrol agents in

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some cases (e.g. WAY & KHOO, 1992; PENG *et al.*, 2010; CHOATE & DRUMMOND, 2011; OFFENBERG, 2015; CASTRACANI *et al.*, 2017; SCHIFANI *et al.*, 2020), but they can be target of control strategies in other situations (e.g. TOLLERUP *et al.*, 2004; GREENBERG *et al.*, 2013). The balance between negative and positive effects of the ant presence in agroecosystems is variable, and it depends on many factors (e.g. STYRSKY & EUBANKS, 2006). In the last two decades, several studies began to investigate the role of ants in Italian agroecosystems and their possible use as bioindicators (e.g. CASTRACANI & MORI, 2006; OTTONETTI *et al.*, 2008; LA PERGOLA *et al.*, 2008; SANTINI *et al.*, 2011; MASONI *et al.*, 2017; CAMPOLO *et al.*, 2015; CASTRACANI *et al.*, 2015; SCHIFANI *et al.*, 2020). However, most contexts of the highly diversified Mediterranean agriculture remain currently unexplored in this sense.

Accounts of the ant fauna inhabiting vineyards are available through scattered checklists from very different geographic regions. For example, ant check-lists in Australian and South American vineyards were provided by CHONG *et al.* (2011) and ROSADO *et al.* (2012; 2013), while in Europe some assessments were provided by BELTRÀ *et al.* (2017) in Spain, GONÇALVES *et al.* (2017) in Portugal and MASONI *et al.* (2017) in central Italy (Tuscany region). However, European vineyards are found under several different climatic conditions. Italy offers a great variety of climatic conditions in this sense: on one hand, vineyards can be found under hot temperate and subtropical temperate climate in Sicily, while they are affected by a sub-continental climate in the Prealpine river valleys (FRATIANNI & ACQUAOTTA, 2017). While MASONI *et al.* (2017) offered a first assessment from an Italian area characterized by a sub coastal temperate climate, we decided to investigate vineyards' ants at the northernmost latitudes of Italian viticulture, considering that ants colonizing vineyards under a sub-continental climate have never been documented elsewhere in Europe. Therefore, we conducted a first qualitative and quantitative assessment of ant diversity in vineyards from the Prealpine Adige Valley in Italy in order to provide a baseline overview and compare the results to the accounts from other geographic regions.

MATERIALS AND METHODS

A total of 10 vineyards from the Adige Valley in northern Italy (region: Trentino-Alto Adige; cities: Rovereto and Trento), treated under conventional agriculture, were selected for this study (see Table 1).

The vines (Pinot grigio variety) were grown with a straight, single trunk and then trained onto a pergola system (Fig. I). The vineyards ground was permanently grass covered between the rows while chemical weed control was applied on a 50 cm strip under the vines. Grass was periodically mowed and mulched on place. Pest control was performed with repeated applications of fungicides and one or two insecticide treatments.

Our monitoring program was conducted from June to September 2016 (which is a good coverage of ants' activity season in the study area), focusing on two rows of each vineyard (each consisting of 16 vines). To obtain data on the arthropodofauna, we relied on pitfall traps (50-ml polypropylene Falcon vials) filled with 30 ml of propylene glycol. In each row, 12 traps were employed at a time: 4 placed on the vines' branches (B traps), 4 in the soil between two vines (S2 traps) and 4 at 1 m from the vines, between the rows (S1 traps) (Figs. II, III). Traps were replaced every 15 days, resulting in 7 sampling dates (from 07.06.2016 to 07.09.2016). Therefore, a total of 1680 traps were used (12 traps x 2 rows x 10 vineyards x 7 sampling dates).

Table 1- List of investigated vineyards.

Site name	**Latitude and longitude**	**Altitude (m)**
A: La Favorita	45.862860, 11.002325	175
B: De Bellat-Pulito	45.845952, 11.007980	155
C: Serravalle Campanella Alto	45.801827, 11.027747	210
D: Serravalle Campanella Basso	45.796905, 11.020143	135
E: Avio Depuratore	45.732027, 10.946634	130
F: Carnal Avio	45.739156, 10.941774	210
G: Avio Campei Alto	45.753609, 10.984719	175
H: Avio Campei Basso	45.752127, 10.984354	140
I: Marine	46.036315, 11.113790	215
J: Maso Grande Ravina	46.032534, 11.107437	260

Systematic identification was achieved using general dichotomous keys for arthopods and for soil microarthopods (CHINERY, 1986; AA VV, 2005). Specimens were recognized at different systematic levels depending on their taxon, but at least at order level. Ants were sorted and identified to species level and identification was achieved using the information provided by WAGNER *et al.* (2017) and SEIFERT (2018; 2020). Ants from the cryptic *Tetramorium caespitum* complex were initially not identified during 2016 as the taxonomy of this complex was still unclear (SCHLICK-STEINER *et al.*, 2006). After WAGNER *et al.* (2017) eventually provided taxonomic keys, only a part of the initial collection still remained in our possession. Since all available specimens were identified as *T. immigrans* (see Results), we refer as *T.* cf. *immigrans* to all the collected specimens from this group.

Species accumulation curves were computed using R 4.0.3 and the `specaccum()` function of the vegan package (OKSANEN *et al.*, 2017; R CORE TEAM, 2021).

Fig. I - Vines grown with a single straight trunk and trained onto a pergola system in one of the investigated vineyards.

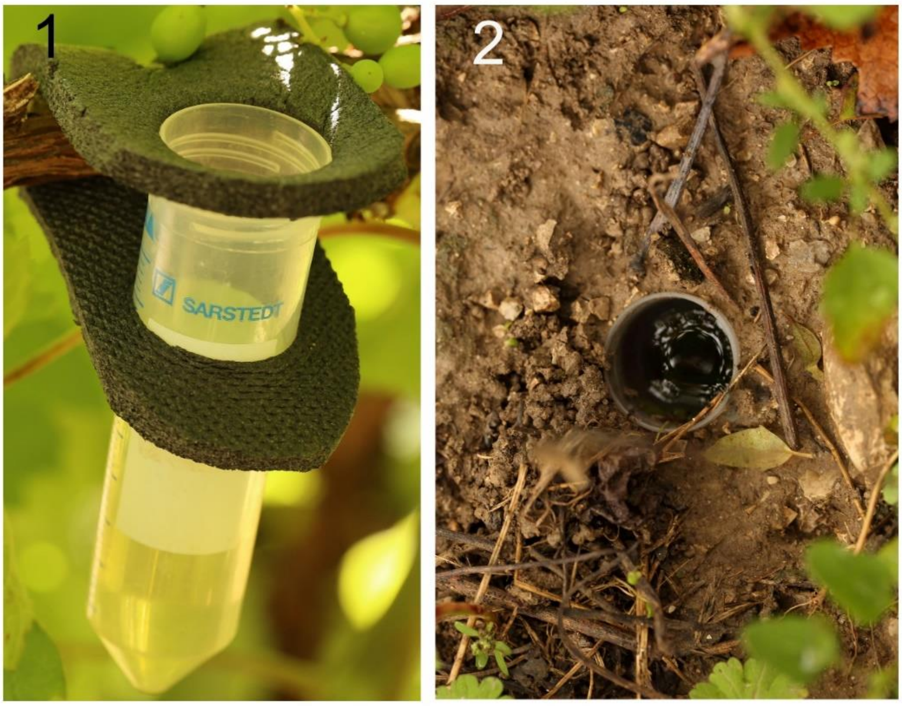

Fig. II - Pitfall traps placed on vines' branches (1) and in the soil (2).

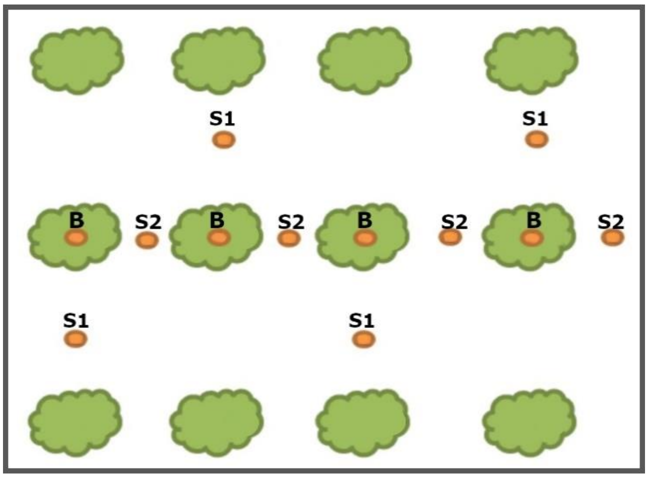

Fig. III - Traps placement in the vineyards' rows: B traps on the vines' branches, S1 traps between the rows and S2 traps between the vines.

Table 2 - Arthropod groups collected during the survey.

Class	Order	Tot. Ind. (n = 20,284)	% Ind.	Tot. Traps (n = 1,680)	% Traps
Arachnida	Acarina	135	0.7	85	5.5
	Araneae	1,210	6.5	526	31.3
	Opiliones	171	0.9	111	6.6
Crustacea	Isopoda	264	1.4	141	8.4
Hexapoda	Collembola	471	2.5	132	7.8
	Coleoptera, Adephaga	2,521	13.4	688	41.0
	Coleoptera, Polyphaga	1,137	6.1	395	23.5
	Coleoptera, larvae	401	2.1	243	14.5
	Dermaptera	168	0.9	115	6.8
	Diptera	1,036	5.5	473	28.1
	Hemiptera	274	1.5	202	12.0
	Hymenoptera, Formicidae	**10,501**	**56.0**	**1,228**	**73.1**
	Hymenoptera (other groups)	232	1.2	162	9.6
	Lepidoptera (adults)	21	0.1	18	1.1
	Lepidoptera (caterpillars)	85	0.4	65	3.9
	Neuroptera	9	0.0	9	0.5
Myriapoda	Diplopoda	25	0.1	19	1.3
	Chilopoda	74	0.4	67	4.0

RESULTS

A total of 20,284 specimens were retrieved from the traps and they were classified into 19 major groups representing 15 orders of Arachnida, Crustacea, Hexapoda and Myriapoda classes (Table 2).

Among these groups, ants were the most abundant, consisting in 56% of all of the collected specimens (10,501), and the most frequent, found in 73% of the traps, and these differences were averagely maintained through the entire sampling period (Table 2, Figs. IV, V).

Ants were represented by 22 species belonging to 16 genera and 3 subfamilies (Table 3).

The most abundant species, *F. cunicularia*, *L. niger* and *T.* cf. *immigrans* represented alone over 85% of the collected specimens, and among them *L. niger* was the most abundant during all the sampling dates (Figs VI, VII).

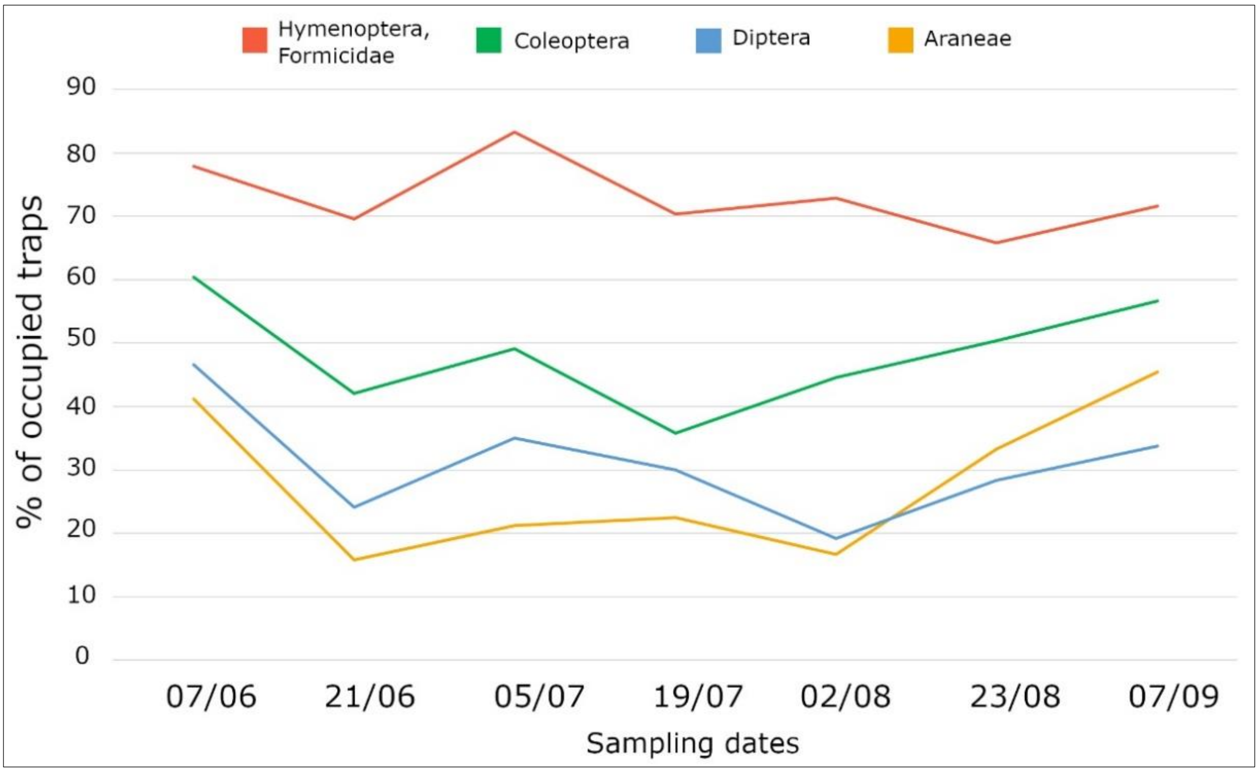

Fig. IV - Frequency of the four main arthropod groups among the traps retrieved in the seven sampling dates.

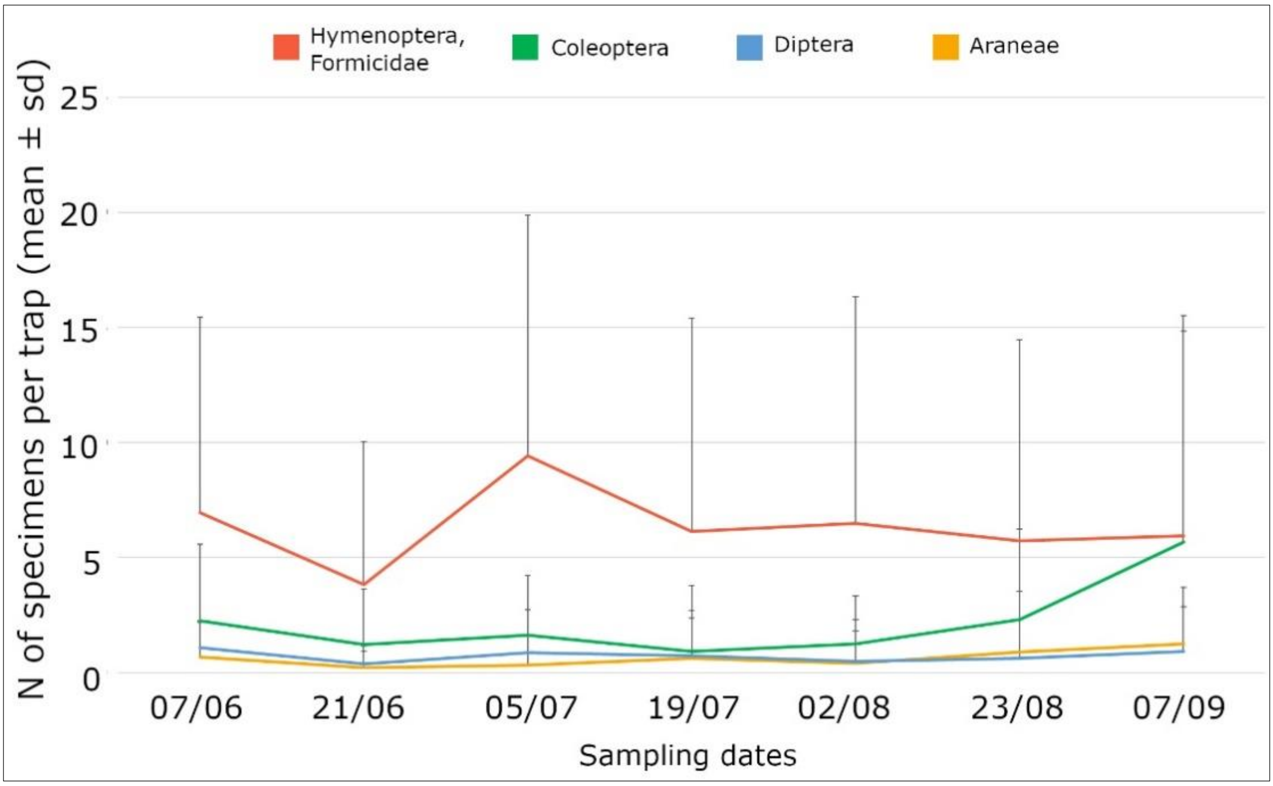

Fig. V - Arthropod specimens collected per trap during the seven sampling dates divided across the four main arthropod groups.

Table 3 - Ant species collected during the survey.

Subfamily	Species	Occupied sites (N=10)	Tot. Ind.	% Ind.	Branches traps (B)
Formicinae	*Camponotus aethiops* (Latreille, 1798)	1	4	0.0	
	Formica cinerea Mayr, 1853	4	19	0.2	X
	***Formica cunicularia* Latreille, 1798**	**10**	**1,169**	**11.1**	**X**
	Lasius emarginatus (Olivier, 1792)	2	4	0.0	X
	Lasius fuliginosus Latreille, 1798	3	4	0.0	
	Lasius myops Forel, 1894	3	7	0.1	X
	***Lasius niger* Linnaeus, 1758**	**10**	**6,170**	**58.7**	**X**
	Plagiolepis pygmaea (Latreille, 1798)	8	62	0.6	X
	Polyergus rufescens (Latreille, 1798)	3	64	0.6	
Myrmicinae	*Aphaenogaster subterranea* (Latreille, 1798)	5	9	0.1	
	Crematogaster scutellaris (Olivier, 1792)	4	4	0.0	X
	Messor ibericus Santschi, 1931	8	727	6.9	X
	Myrmica sabuleti Meinert, 1861	3	3	0.0	
	Myrmica specioides Bondroit, 1918	3	5	0.0	X
	Myrmecina graminicola (Latreille, 1802)	7	114	1.1	
	Pheidole pallidula (Nylander, 1849)	7	334	3.2	X
	Solenopsis fugax (Latreille, 1798)	9	132	1.2	X
	Strongylognathus testaceus (Schenck, 1852)	3	3	0.0	
	Temnothorax italicus (Consani, 1952)	5	9	0.0	
	Temnothorax unifasciatus (Latreille, 1798)	4	5	0.0	X
	***Tetramorium* cf. *immigrans* Santschi, 1927**	**10**	**1,643**	**15.6**	**X**
Ponerinae	*Hypoponera eduardi* (Forel, 1894)	3	5	0.0	

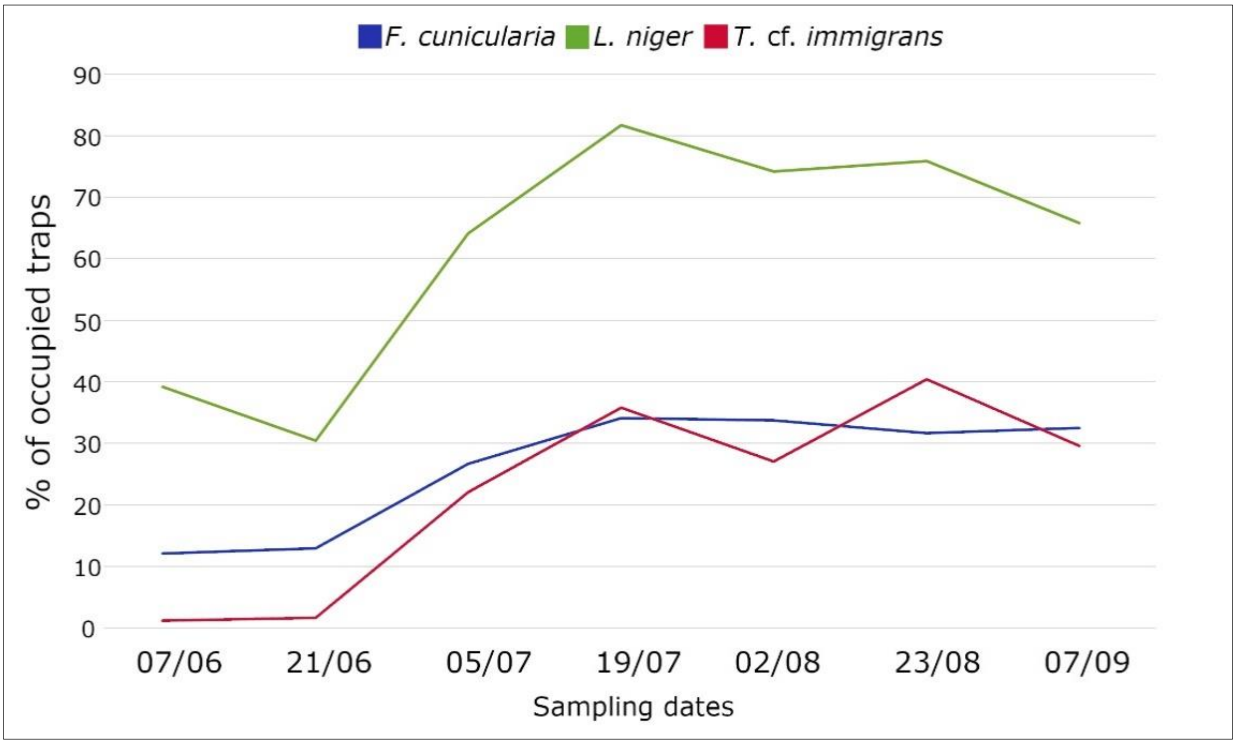

Fig. VI - Frequency on traps during the seven sampling dates of the three ant species detected in all orchards.

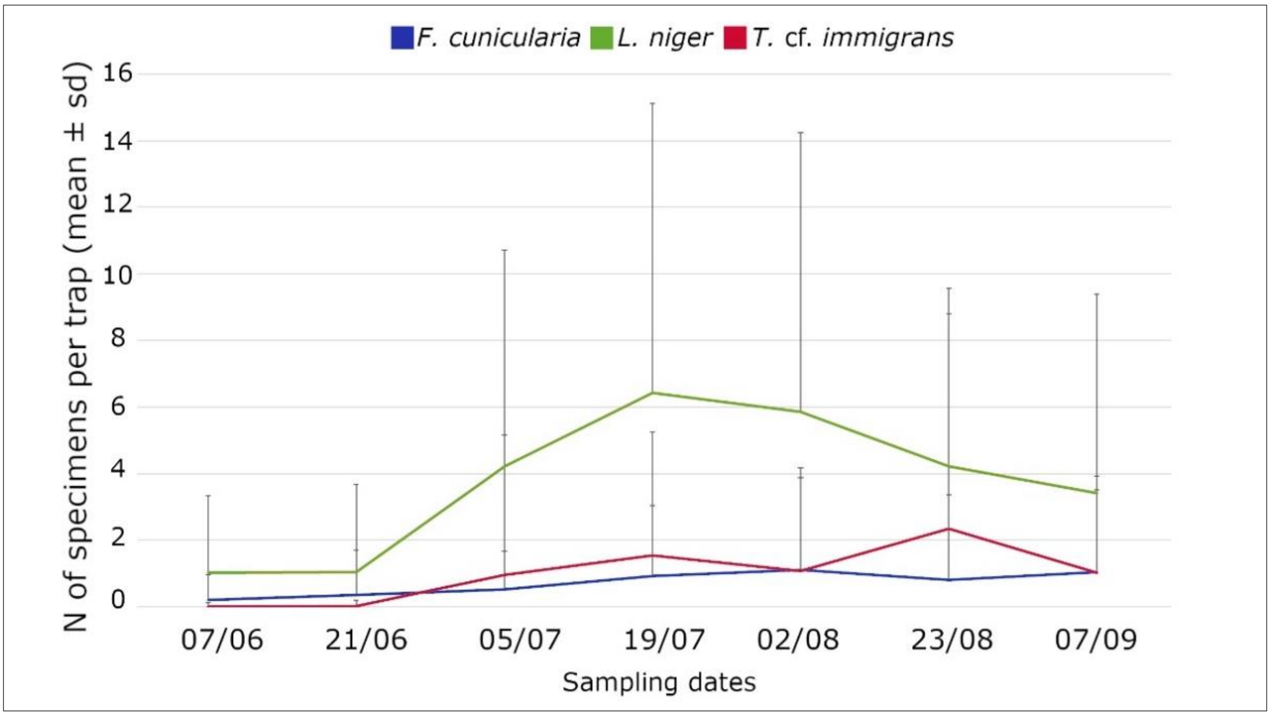

Fig. VII - Ant specimens collected per trap during the seven sampling dates for the three species detected in all vineyards.

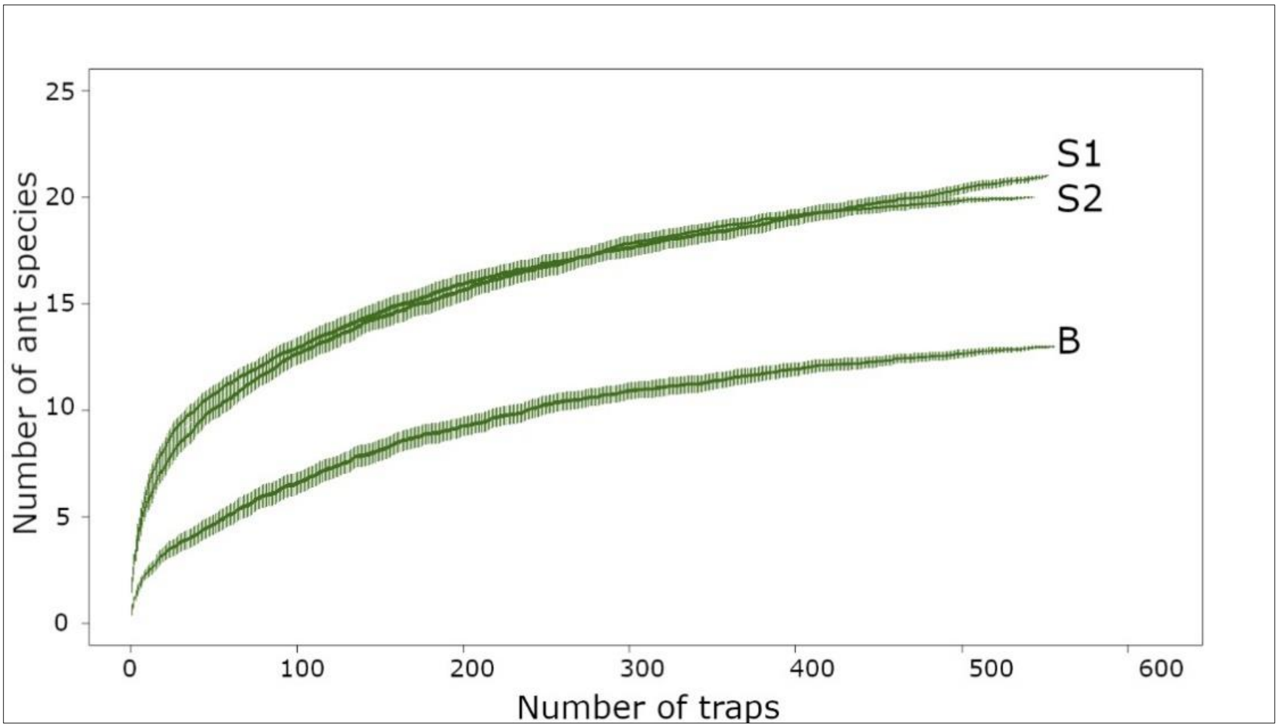

Fig. VIII - Species accumulation curves based on the number of ant species collected with traps placed in different positions. The vertical bars correspond to 30% of the standard error of the estimate.

The S1 and S2 traps yielded a comparable performance in terms of number of captured ant species (Fig. VIII), and together granted the detection of all of the species encountered during this study, while only a subset of 13 species was collected with B traps.

The number of species collected per vineyard varied from 7 to 16, while *F. cunicularia*, *L. niger* and *T.* cf. *immigrans* were found in every vineyard. Species accumulation curves showed that the sampling effort determined a clear plateau for most vineyards, with the exception of site I (Fig. IX; Table 4).

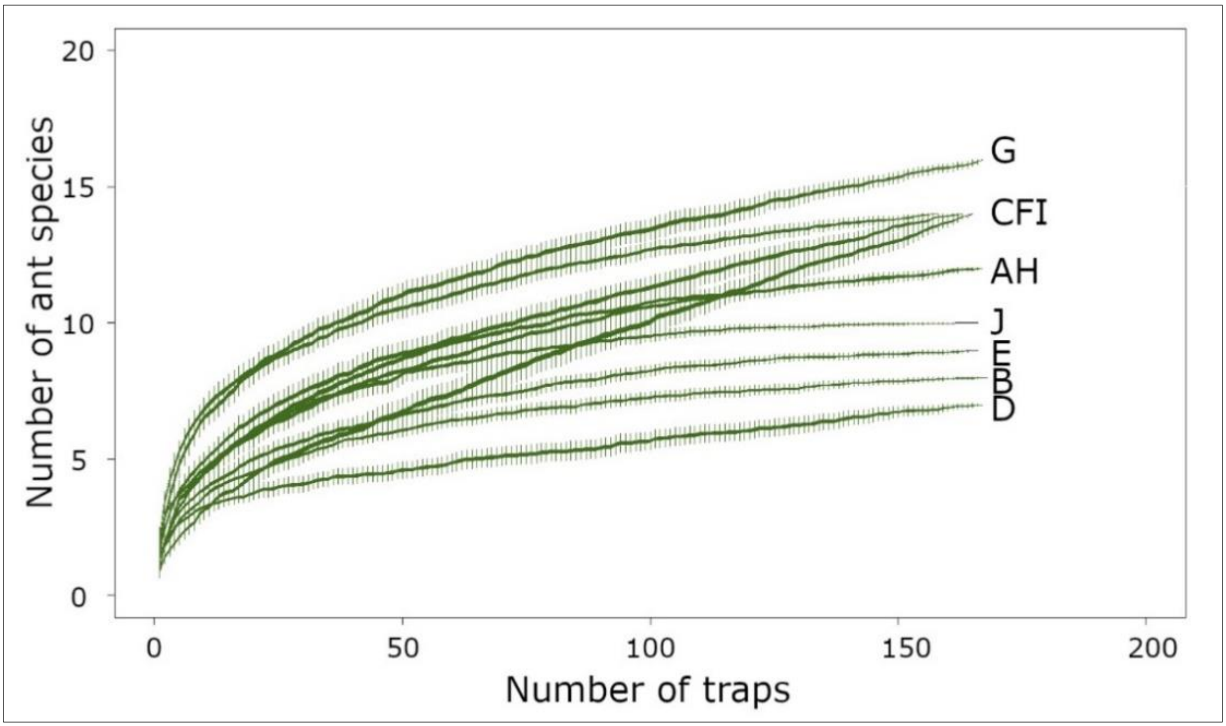

Fig. IX - Species accumulation curves based on the number of ant species collected at each site. The vertical bars correspond to 30% of the standard error of the estimate.

Table 4 - Diversity indexes of each site based on collected species and % of traps occupied by the three species present in each vineyard.

Site	**Species richness**	**Shannon index (*H*)**	**Equitability index (E_H)**	**% traps *F. cunicularia***	**% traps *L. niger***	**% traps *T.* cf. *immigrans***
A	12	1.60	.31	68	69	41
B	8	1.28	.25	3	64	26
C	14	1.87	.37	34	59	32
D	7	1.00	.19	23	77	16
E	9	1.35	.26	36	71	14
F	14	2.39	.47	23	41	33
G	16	2.46	.48	27	71	14
H	12	1.62	.32	17	55	26
I	14	1.14	.22	7	67	14
J	10	1.64	.32	28	51	11

DISCUSSION

As strongly documented from literature (e.g. AGOSTI *et al.*, 2000; LACH *et al.*, 2010), once again ants proved to be a convenient arthropod group for monitoring programs in agroecosystems, being consistently as the most abundant group in our survey. This study provides one of the few quantitative assessments conducted on the Italian ant fauna. We documented a moderately diverse fauna characterized by a high diversity of Formicinae and Myrmicinae (with a good diversity of *Lasius* genus, but also multiple species of *Formica*, *Myrmica* and *Temnothorax* genera) and by the notable absence of Dolichoderinae ants. We found an overwhelming prevalence of species characterized by very large distributions in Europe and beyond, only few Mediterranean taxa and no endemisms (JANICKI *et al.*, 2016; GUÉNARD *et al.*, 2017). This picture is not particularly different from that of recent surveys conducted on the Po Plain, but we detected no Eastern-Mediterranean species and even fewer Mediterranean or South European taxa (CASTRACANI *et al.*, 2020). Moreover, it is worth noting that three notoriously disturbance-tolerant species, *F. cunicularia*, *L. niger* and the *T.* cf. *immigrans* were the most abundant species, as it was observed elsewhere in Northern Italy (CASTRACANI *et al.*, 2020). While *T. immigrans* is probably an introduced species in Italy (CASTRACANI *et al.*, 2020), the numerical dominance of these three species likely reflects their ability to fill empty niches created by human activities (see ARNAN *et al.*, 2018; 2021). Only about half (54%) of the species we recorded was also detected on the vines themselves through the use of traps placed on their branches. Species like *C. aethiops* or *T. italicus*, which habitually visit plants (SEIFERT, 2018; GIANNETTI *et al.*, 2019), were probably only encountered on the soil just because of their low abundance. On the other hand, absence on the vines was expected for the social parasites such as the slave-maker *P. rufescens* or the inquiline *S. testaceus*, which are not active foragers. Other species such as *A. subterranea*, *H. eduardi* or *M. graminicola* were also not expected to climb into the vines because they usually forage on the soil surface or within the leaf litter (SEIFERT, 2018; GRASSO *et al.*, 2020). While *S. fugax* belongs to the same category (SEIFERT, 2018), its finding on vines was rather unexpected.

In comparison to the Australian and Brazilian vineyards' ant faunas, we detected much fewer ant species. CHONG *et al.* (2011) sampled 50 vineyards from different Australian regions, achieving a vast geographic coverage, and detected 147 species, estimating each vineyard to be inhabited by 30-40 species (but recording only 5-24 species in one of the sampled regions). At the same time, ROSADO *et al.* (2012) recorded 72 species in total in Brazil, from 21 to 50 per vineyard. Our numbers are far lower and the most represented genera are different from those detected in these surveys, but this is unsurprising considering the ecological and biogeographical patterns of ant diversity worldwide: ant diversity is notoriously higher in the tropics, where genera that are dominant in the temperate ecozone have a modest presence and vice-versa (e.g. see JANICKI *et al.*, 2016; GUÉNARD *et al.*, 2017).

On the other hand, possible comparisons with vineyards of the northern temperate ecosphere, which host more similar faunas, are not particularly numerous. For example, despite several papers dealt with peculiar ant species and their role in North American vineyards (e.g. KLOTZ *et al.*, 2003; TOLLERUP *et al.*, 2004; 2007; DAANE *et al.*, 2006; 2007; NONDILLO *et al.*, 2016; TOWNSEND *et al.*, 2016; WESTERMANN et al., 2016; COOPER et al., 2019), no data on vineyard overall ant diversity are available. In some other cases, an ant species check-list is provided, but it included only species that were observed foraging on the vines (BELTRÀ *et al.*, 2017). However, some interesting comparisons may be made with data published by GONÇALVES *et al.* (2017) from Portugal and MASONI *et al.* (2017) from Central Italy. The fauna from the Portuguese sites investigated by GONÇALVES *et al.* (2017) comprises 20 species in total, slightly less than ours, but the number of species per site is averagely much higher (15-20) than in our case, and so it is the number of species (9) common to all their 6 investigated vineyards. It is also a very different fauna in both ecological and biogeographic terms, consisting prevalently of species and genera associated with xero-Mediterranean climatic conditions and with a clear Western-Mediterranean characterization (e.g. *Cataglyphis* spp., West-Mediterranean *Camponotus* species such as *C. cruentatus* (Latreille, 1802) and *C. sylvaticus* (Olivier, 1792), the Iberian subendemic *Iberoformica* genus, *Aphaenogaster iberica* Emery, 1908 from the xerothermophilous *testaceopilosa* group, *Crematogaster auberti* Emery, 1869). On the other hand, MASONI *et al.* (2017) recorded a similar number of species (19) from 10 vineyards near Florence (Tuscany, Italy), and a slightly smaller number of species per vineyard than us (5-12). The ant assemblages documented by MASONI *et al.* (2017) present some relevant similarities such as a relatively high *Myrmica* diversity and the widespread presence of *F. cunicularia* and *M. ibericus*. Concerning the latter species, MASONI *et al.* (2017) refer to *M. structor* (Latreille, 1798), but Italy most likely only hosts its cryptic sister species *M. ibericus* (STEINER *et al.*, 2018; SCHIFANI *et al.*, 2021). However, there are also relevant differences, as the reduced diversity of *Lasius*, and, at the same time, the more widespread presence of thermophilous species such as *P. pallidula* and Mediterranean *Tapinoma* species from the Dolichoderinae subfamily.

The ecological role of the overwhelming majority of ant species that inhabit vineyards across the globe, including of those we detected in our survey, is still virtually unknown. Only three ant species, the worldwide spread invasive Argentine ant *Linepithema humile* (Mayr, 1868), the South American *L. micans* (Forel, 1908) and the North American *Formica perpilosa* Wheeler, W.M., 1913 have been the subject of several studies considering them as significant pests requiring control strategies in vineyards of California and Brazil (KLOTZ *et al.*, 2003; TOLLERUP *et al.*, 2004; 2007; DAANE *et al.*, 2006; 2007; SACCHETT *et al.*, 2009; NONDILLO *et al.*, 2016; WESTERMANN *et al.*, 2016; COOPER *et al.*, 2019). On the other hand, another invasive species, the red imported fire ant *Solenopsis invicta* Buren, 1972, was deemed a positive presence due to its significant predatory action on pest species in Texas' vineyards (TOWNSEND *et al.*, 2016).

In conclusion, ant communities in agroecosystems are diverse and often species-rich, and documenting their identities is crucial to assess the possible services and disservices that different species assemblages may yield. While vineyards are worldwide spread, their ant faunas are rather different from place to place according to local climatic and biogeographic factors, so that the few available data do not show clear patterns, which could have originated from strong homogenizing ecological constraints derived from viticulture *per se*. Further investigation will be required to understand how the fauna of the vineyards from the Adige Valley compares with that of other agroecosystems or natural habitats from the same region and how different management practices may influence it. It will also be important to assess what is the role that different ant species may play in these environments to improve management practices accordingly.

AUTHORS CONTRIBUTIONS

Conceptualization: CC, DG, DAG, CI; Data collection: MD, MG, DG, FP; Specimen processing and identification: CC, MG, ES, FAS; Data curation and analysis: CC, ES; Visualization: CC, DG, ES; Writing—original draft preparation: DG, ES; Writing—review and editing: CC, MD, DAG, CI, AM, FP, FAS; Supervision: DAG, CI, AM; Funding acquisition: DAG, CI, AM. All authors have read and agreed to the published version of the manuscript.

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Section III - First data on ant diversity and distribution in Italian agroecosystems

Chapter 10

Trunk size influences species richness and functional composition of biogeographically different tree-visiting ant communities in pear orchards

Schifani, E., Giannetti, D., Castracani, C., Spotti, F.A., Mori, A., & Grasso, D.A. (2022). Trunk size influences species richness and functional composition of biogeographically different tree-visiting ant communities in pear orchards. *Redia*, 105, 163-168. http://dx.doi.org/10.19263/REDIA-105.22.20

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TRUNK SIZE INFLUENCES SPECIES RICHNESS AND FUNCTIONAL COMPOSITION OF BIOGEOGRAPHICALLY DIFFERENT TREE-VISITING ANT COMMUNITIES IN PEAR ORCHARDS

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Schifani E., Giannetti D., Castracani C., Spotti F.A., Mori A., Grasso D.A. - Trunk size influences species richness and functional composition of biogeographically different tree-visiting ant communities in pear orchards.

We investigated the diversity of ant assemblages visiting pear trees in Italy by comparing two orchards from a northern region with continental climate (Emilia-Romagna) and a southern region with Mediterranean climate (Sicily). Overall we identified 20 ant species belonging to 10 genera and 3 subfamilies. The ant communities of the two sites look significantly different in biogeographic terms, but their functional composition is similar. Moreover, we discovered that both ground and arboreal nesting species richness is positively correlated with the trunk circumference. In particular, arboreal-nesting species are limited to the deadwood richer medium and large trees. Some of the detected ant species may be useful to pest control strategies, and artificial nests may be considered to attract arboreal-nesting species in deadwood-poor trees.

Key Words: arboreal ants, myrmecology, Sicily, Emilia Romagna.

INTRODUCTION

As a result of their evolutionary success, ants are key ecological actors in terrestrial ecosystems (Hölldobler & Wilson, 1990; Lach *et al.,* 2010; Boomsma *et al.,* 2017; Parker & Kronauer, 2021). In agroecosystems, they can be indicators of soil functioning (de Bruyn, 1999) and helpful allies for the control of pest species, including insects, fungi and weeds (Way & Khoo, 1992; Baraibar *et al.,* 2011; Offenberg 2015; Offenberg & Damgaard, 2019). However, their distribution and role in temperate agroecosystems is still insufficiently investigated. Only in recent years, ant communities of Italian agroecosystems began to be described (Castracani & Mori, 2006; Ottonetti *et al.,* 2008; La Pergola *et al.,* 2008; Campolo *et al.,* 2015; Castracani *et al.,* 2015; Masoni *et al.,* 2017; Frizzi *et al.,* 2020; Schifani *et al.,* 2020a; Giannetti *et al.,* 2021). At the same time, an increasing number of studies emphasizes the beneficial role of ants for pest control (e.g. Castracani *et al.,* 2017; Frizzi *et al.,* 2020; Schifani *et al.,* 2020a; Bulgarini *et al.,* 2021).

Ants in pear orchards have attracted significant attention due to their ability to reduce the impact of two insect and one fungal pests: the pear psyllid *Cacopsylla pyri* (L., the codling moth *Cydia pomonella* (L.), and the pear scrub *Venturia pyrina* Aderh. (Paulson & Akre, 1992; Sanchez *et al.,* 2020; Schifani *et al.,* 2020a). However, there are almost no data about ant species living in pear orchards across Europe, which species visit pear trees or which tree features make them more attractive to ants. When compared to other habitats, pear orchards may be scarce in arboreal nesting ant species (Schifani *et al.,* 2020a). Comparing ant communities visiting conspecific trees, tree size and the presence of dominant ants may be among the most significant factors shaping their composition (Tschinkel & Hess, 1999). Unlike tropical forests, temperate ones are typically inhabited by a much smaller proportion of specialized arboreal species (Tschinkel & Hess, 1999; Seifert, 2018; Klimes *et al.,* 2012; 2015). However, this proportion may be even lower in agroecosystems, where the removal of deadwood from cultivated trees can impede colonization by arboreal nesting species (Philpott & Foster, 2005). Artificial nests are sometimes used to restore the presence of tree inhabiting ants, whose predation upon phytophagous insects is sometimes highly valuable (Philpott & Foster, 2005; Philpott & Ambrecht, 2006).

In this paper we are providing a first assessment of ant diversity in Italian pear orchards by selecting two sites at different latitudes and with different climatic conditions (continental and Mediterranean). For both sites we monitored the presence of ground and arboreal nesting ant species and their relation with tree size. Not all ants climb trees, and the species foraging on the ground may still have a significant influence on cultivated plants (e.g. Grasso *et al.,* 1998; 1999; Baraibar *et al.,* 2011; Solida *et al.,* 2011; Campolo *et al.,* 2015). In the case of orchards, those species that climb cultivated trees are expected to have a more direct impact on them, but this behavioral difference is rarely recorded (Schifani *et al.,* 2020a; Giannetti *et al.,* 2021; 2022).

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MATERIALS AND METHODS

STUDY AREAS

We investigated 222 pear trees (*Pyrus communis* L.) from two organic orchards whose management was restricted to periodical lawn mowing (Fig. I). The surveys were conducted during the years 2018-2019 in the same period, to minimize potential differences between the years. Since we did not aim to evaluate the temporal variation of ant communities throughout the seasons, we chose to carry out our surveys in July which is considered a period of high ant activity for most species (SCHIFANI *et al.*, 2022a).

Site 1, Northern Italy, Emilia-Romagna, continental climate zone: Pontescodogna (Parma province) (44.7378 N, 10.1954 E, 125 m a.s.l.). This orchard includes apple, cherry, fig, peach, pear and plum trees, arranged in 15 rows and partially surrounded by a deciduous oak forest, which is part of the Regional Natural Park "Boschi di Carrega". We investigated 166 pear trees in July 2018.

Site 2, Sicily, Mediterranean climatic zone: Polizzi Generosa (Palermo province), (37.8245 N, 14.0032 E, 750 m a.s.l.). This orchard includes apple, fig, hazel, peach and pear trees, only partly arranged in rows and partially surrounded by a deciduous oak forest, which is part of the Regional Natural Park "Parco delle Madonie". We investigated 56 pear trees in July 2019.

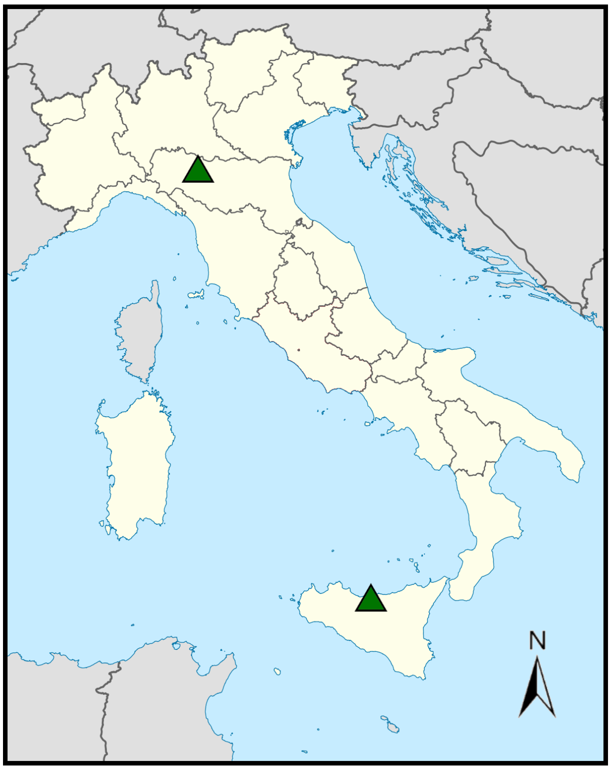

Fig. I - The two study areas investigated in Emilia-Romagna (Northern Italy) and Sicily.

DATA COLLECTED ON EACH TREE

Data sampling was organized in daily sessions from 10:00 to 12:00 am until all trees were examined. The examination of each tree was conducted through a 5 minute continuous observation period, during which all ant specimens detected on each tree were collected. They were identified by a stereomicroscope following SEIFERT (2018, 2019; 2020) and SCHIFANI *et al.* (2022). In addition, the circumference of each tree was measured 5 cm above the ground.

STATISTICAL ANALYSES

All statistical tests were performed using the software R 4.2.0 and RStudio-2022.02.2-485 (R CORE TEAM, 2022; RSTUDIO TEAM, 2022). The circumference of each tree was correlated with the number of arboreal- and ground-nesting ant species running Pearson's correlation tests.

The ant communities of the two study areas were compared using the Sørensen–Dice coefficient. To each species was assigned a chorotype following VIGNA TAGLIANTI *et al.* (1999), with the exception of the Maghrebian and SW-European distributions as suggested by PARENZAN (1994) as NAW and ESW (see also SCHIFANI *et al.,* 2021).

RESULTS

We identified 20 ant species, belonging to 10 genera of the subfamilies Formicinae (65%), Myrmicinae (30%), and Dolichoderinae (5%) (Tab. 1). Among them 5 arboreal-nesting species were found.

The similarity between ant communities identified in Emilia-Romagna (11 species) and Sicily (13 species) was 35%, according to the Sørensen–Dice coefficient, and 8 genera are shared between the two sites. In Emilia-Romagna, 73% of the identified species has a European or Eurasian distribution and 27% has a Mediterranean distribution. Conversely, in Sicily, 85% of the species identified has a Mediterranean distribution and 15% has a European or Eurasian distribution.

Pearson's correlation tests show significant positive relationships between tree circumference and the number of ant species per tree (R = 0.62, $p < 0.001$), the number of ground-nesting ants (R = 0.54, $p < 0.001$), and the number of arboreal-nesting ones (R = 0.33, $p < 0.001$) (Fig. II).

DISCUSSION

We are providing a first account of ant communities visiting pear trees in Italy. Our assessment highlighted significant diversity of species between sites at different latitudes, reflecting Italy's remarkable biogeographical complexity. The ant fauna of Northern Italy was overwhelmingly characterized by European or Eurasian taxa, while these were a small minority in Sicily where Mediterranean species were very numerous (also see CASTRACANI *et al.,* 2020; WANG *et al.,* 2022). At the same time, genera and species-groups are similar between the two investigated regions, suggesting an overall remarkable functional affinity: in both Emilia-Romagna and

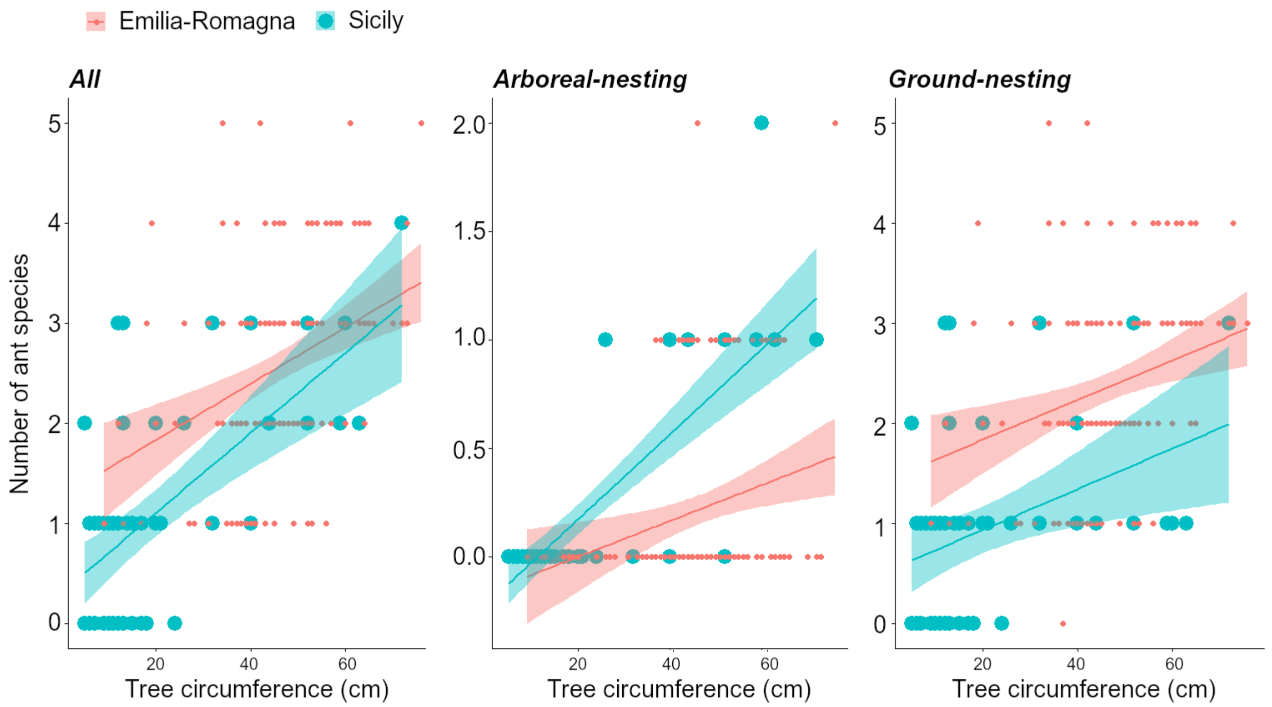

Fig. II - Results of the Pearson's correlation tests between tree circumference and the number of ants found visiting the same tree on pear trees from Northern Italy and Sicily.

Tab. 1 - Ant species visiting trees in the two study areas. Arboreal-nesting species are in bold.

Subfamily	Species	Chorotype	Visited trees	
			Emilia Romagna	Sicily
Dolichoderinae	***Dolichoderus quadripunctatus* (Linnaeus, 1771)**	TUE	8%	-
Formicinae	*Camponotus aethiops* (Latreille, 1798)	SEU	-	27%
	Camponotus gestroi Emery, 1878	MED	-	9%
	Camponotus lateralis (Olivier, 1792)	MED	-	3%
	Camponotus nylanderi Emery, 1921	ITAL	-	18%
	Camponotus piceus (Leach, 1825)	ESW	58%	30%
	***Camponotus vagus* (Scopoli, 1763)**	CEM	2%	-
	***Colobopsis truncata* (Spinola, 1808)**	TEM	3%	-
	***Colobopsis imitans* Schifani et al., 2021**	NAW	-	3%
	Formica cunicularia Latreille, 1798	ASE	38%	6%
	Lasius casevitzi Seifert & Galkowski, 2016	THYRR	-	12%
	Lasius niger (Linnaeus, 1758)	ASE	34%	-
	Lasius paralienus Seifert, 1992	EUR	51%	-
	Plagiolepis pygmaea (Latreille, 1798)	ASE	49%	18%
Myrmicinae	*Crematogaster laestrygon* Emery, 1869	NAW	-	15%
	***Crematogaster scutellaris* (Olivier, 1792)**	WME	8%	18%
	Myrmica sabuleti Meinert, 1861	TUE	1%	-
	Pheidole pallidula (Nylander, 1849)	ESW	-	3%
	***Temnothorax italicus* (Consani, 1952)**	ITAL	2%	-
	***Temnothorax mediterraneus* Ward et al., 2014**	WME	-	4%

Sicily most species belong to the subfamily Formicinae. Although counting only about half of the Italian Myrmicinae species (Schifani, 2022), this group plays a particularly important role in ant-plant interactions in temperate areas (Wilson & Hölldobler, 2009). Out of 10 occurring genera, only *Dolichoderus*, *Pheidole*, and *Myrmica* are not shared between the two investigated areas. Sicily has a larger amount of *Camponotus* and *Crematogaster* species, while the more cold tolerant genera *Lasius* and *Formica* play a more important role in Emilia-Romagna. Besides that, the two communities often host either of two closely related, often vicariant species, such as *Colobopsis truncata* and *Co. imitans*, *Lasius paralienus* and *L. casevitzi*, or at least species of the same genus with similar ecology, such as *Temnothorax italicus* and *T. mediterraneus* (Schär *et al.,* 2020; Schifani, *et al.,* 2022). In addition, *Camponotus piceus* was the sole common ant in both sites. The number of species we found in each community reflects that of comparable studies in other temperate regions (Tschinkel & Hess, 1999), and it is obviously much lower than in tropical forests (e.g. Klimes *et al.,* 2015). We found few more species in Sicily, but on average each tree hosted less ants, maybe because July is a less favorable month for ant activity under the hot and arid Mediterranean climate (Retana & Cerdá, 2000; Schifani *et al.,* 2020b).

Our results show a significant increase of both arboreal and ground nesting species when larger trees are present. This contrasts with the outcome of other studies, where the occurrence of dominant tree inhabiting *Crematogaster* spp. on larger trees reduces ant species richness (Tschinkel & Hess, 1999). The dominant arboreal species *Cr. scutellaris* seemingly does not affect the presence of other ants on the trees we investigated. Also, it is worth mentioning that at least two species (*Camponotus lateralis* and *Co. imitans*) can take advantage of the presence of *Cr. scutellaris* (Schifani *et al.,* 2022). Arboreal nesting species represent a small portion of the observed ant diversity and are almost exclusively limited to medium and large trees. The lack of sufficient deadwood for nesting in younger trees may be the main factor affecting their presence (Tschinkel & Hess, 1999; Schlaghamerský & Omelková, 2007), although the relationships between ants and deadwood availability is still little explored if compared to the studies of other insects (e.g. Campanaro *et al.,* 2011; Thomaes *et al.,* 2018). Artificial nests may be adopted to overcome this issue whenever the presence of tree inhabiting ants, such as *Cr. scutellaris*, which may play a role in pest control (e.g. Castracani *et al.,* 2017; Giannetti *et al.,* 2019), is needed (Philpott & Foster, 2005).

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Section III - First data on ant diversity and distribution in Italian agroecosystems

Chapter 11

Developing the DNA barcoding library of European ants: the species of the Italian agroecosystems

Developing the DNA barcoding library of European ants: the species of the Italian agroecosystems

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Introduction

DNA barcoding is a widespread specimen identification method that is based on sequencing a short DNA section of a specimen and comparing it to a library of sequences of already identified specimens. The ability of DNA barcoding to successfully provide species-level identification can greatly vary across taxonomic groups, since different species may sometimes share the same haplotype (barcode sharing) because of different possible mechanisms (e.g., introgression, incomplete lineage sorting). However, DNA barcoding remains a cost-effective tool across many different taxonomic groups and can also be used for the simultaneous identification of multiple taxa from the same sample (metabarcoding).

The most basic limitation to the use of DNA barcoding and metabarcoding is related to the availability of a reliable reference library. Sequences in the libraries must be correctly linked to species-level identifications, as many organisms as possible must sequenced, and for each taxon, as many representative of different populations should be included in order to cover spatial variation and assess intraspecific variation. The construction of a DNA barcoding library for European ants is a key part of the *Genetic Map of European ants* project (AntGem - https://www.mattiamenchetti.com/antgem).

To obtain a library containing at least about 80% of the over six hundred European ant species, the project produced thousands of new barcode sequences and re-analyzed those that were already available.

Here, we present an overview of the data collected for the 34 ant species that were encountered and studied through the chapters of this thesis for their presence and role in the Italian agroecosystems.

Materials and methods

DNA was extracted from one or a few legs of each ant specimen. DNA-barcoding (mitochondrial gene cytochrome c oxidase I, COI, 658 bp) data was generated at two institutes: the Centre for Biodiversity Genomics, University of Guelph, Canada, using the primers LepF1 and LepR1 (deWaard et al. 2008); the Butterfly Diversity and Evolution Lab (BDEL), following the protocol by Schär et al. (2020) and using the primers LCO1490/HC02198 (Folmer et al. 1994). In the latter case, PCR products were visualized by gel electrophoresis and sent to Macrogen Europe for Sanger sequencing. Raw sequences were edited and aligned in Geneious Prime 2020.2.4 (Kearse et al. 2012). Chromatograms and sequences have been inspected for the presence of, respectively, double peaks and stop codons.

Results

A total of 836 new sequences were produced for the 34 ant species listed in the previous chapters, which summed with the available ones that were analyzed are 1,192 sequences (from 4 to 80 per species – see Table 1). For each species, we assembled a haplotype network and a Principal Coordinate Analysis (PCoA) to illustrate intraspecific diversity patterns (Fig. 1). Furthermore, to illustrate phylogeographic patterns, we built a geographic map of each species using the colors derived from the PCoA to plot the spatial distribution of the haplotypes found across different populations (Fig. 2).

Discussion

Our results suggest that DNA barcoding may be used effectively to identify species belonging to cryptic species complexes, such as *Tapinoma subboreale* or *Tetramorium immigrans* whose morphological identification can be very demanding (requiring the use of male genitalia in the first case, and of male genitalia or several time-consuming morphometric measurements in the second – see Seifert 2012; Wagner et al. 2017). At the same time, about one-fourth of the ant species examined here showed barcode sharing and were therefore not unequivocally distinguishable by DNA barcoding only. This group included species of presumably recent divergence for which incomplete lineage sorting may be the underlying mechanism (e.g., *Colobopsis* spp., see Schifani et al. 2022) or species with complex introgression history (e.g., *Myrmica sabuleti*, see Blatrix et al. 2020). The DNA fragment used for barcoding in ants also proved to be a valid tool to explore biogeographic patterns in many species, as illustrated in the example of *Crematogaster scutellaris*. DNA barcoding appears as a promising tool to facilitate ant identification, but more caution is needed in comparison to other groups of organisms in which barcode sharing is rarer.

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Table 1. DNA barcode data collected for the 34 ant species mentioned in the previous chapters of this thesis.

Species	New barcode sequences	Total number of barcode sequences	Barcode sharing with other European ants	Max intraspecific p-dist	Median intraspecific p-dist
Camponotus aethiops	55	67		0.096	0.021
Camponotus vagus	46	50		0.070	0.005
Camponotus piceus	41	48		0.065	0.009
Camponotus lateralis	46	50		0.026	0.011
Camponotus nylanderi	11	15		0.017	0.003
Camponotus gestroi	8	8		0.115	0.107
Colobopsis truncata	38	64	Yes	0.023	0.004
Colobopsis imitans	4	14	Yes	0.025	0.002
Crematogaster laestrygon	6	6		0.055	0.051
Crematogaster scutellaris	56	61		0.032	0.021
Crematogaster sordidula	36	39		0.104	0.021
Dolichoderus quadripunctatus	30	45		0.045	0.003
Formica cinerea	16	21	Yes	0.040	0.008
Formica cunicularia	46	52	Yes	0.070	0.035
Lasius casevitzi	11	21		0.009	0.002
Lasius emarginatus	43	54	Yes	0.029	0.005
Lasius myops	29	35		0.046	0.006
Lasius niger	39	62		0.008	0.000
Lasius paralienus	14	20		0.018	0.012
Messor ibericus	23	51		0.003	0.000
Myrmica sabuleti	20	27	Yes	0.035	0.006
Myrmica specioides	15	15		0.025	0.017
Nylanderia jaegerskioeldi	11	12		0.003	0.000
Pheidole pallidula	47	52		0.135	0.103
Plagiolepis pygmaea	47	66		0.110	0.003
Solenopsis fugax	36	38		0.063	0.046
Tapinoma magnum	24	80	Yes	0.046	0.014
Tapinoma subboreale	16	18		0.023	0.014
Temnothorax affinis	15	21		0.039	0.020
Temnothorax italicus	4	4	Yes	0.006	0.003
Temnothorax mediterraneus	5	11		0.034	0.029
Temnothorax unifasciatus	11	18	Yes	0.121	0.086
Tetramorium immigrans	13	69		0.024	0.006
Tetramorium semilaeve	32	53		0.037	0.018

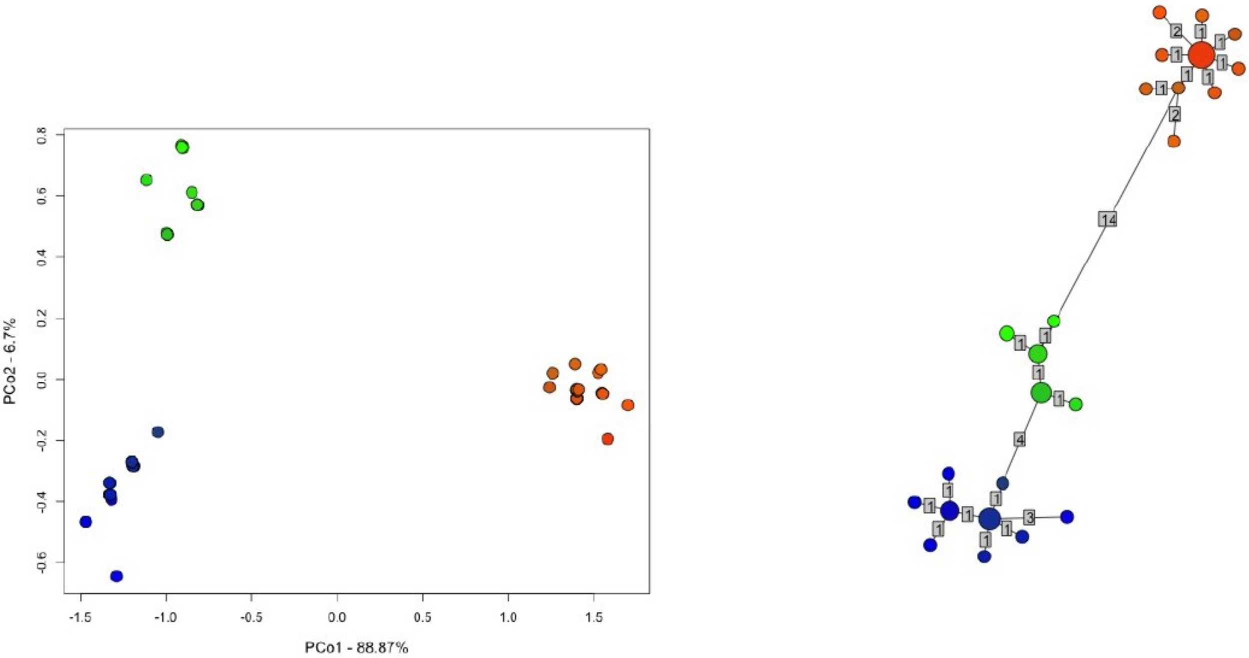

Figure 1. PCoA based on pairwise p-distances between *Crematogaster scutellaris* sequences (left). Colours match a bidimensional color space. Haplotype network of *Crematogaster scutellaris* (right).

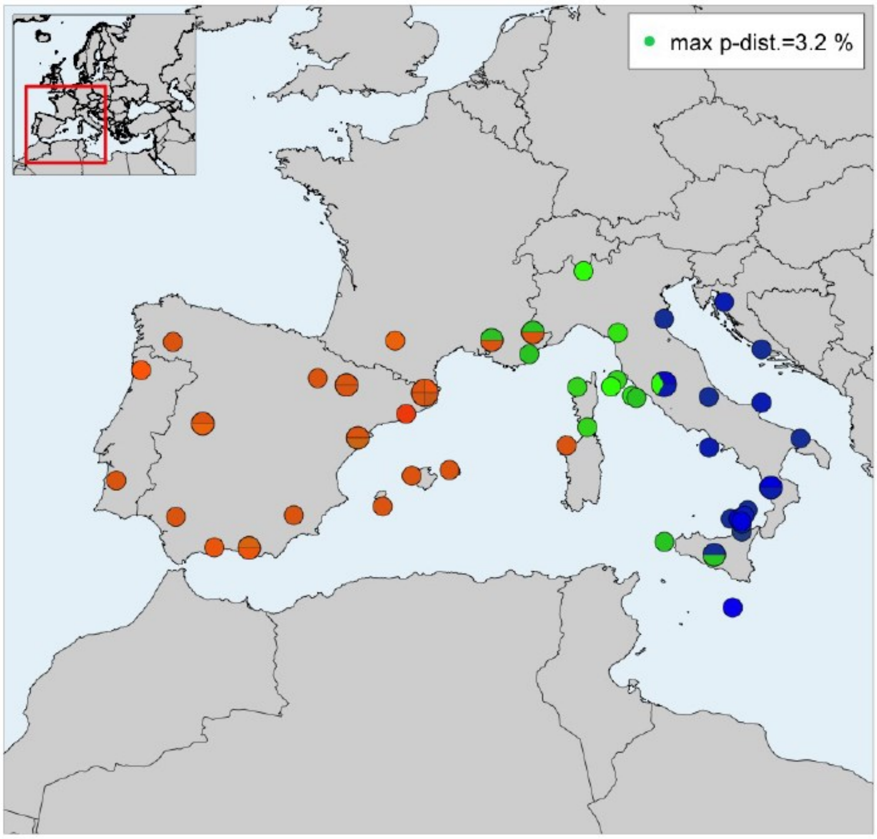

Figure 2. Genetic map of Crematogaster scutellaris (Olivier, 1792). Nearby localities of sequenced specimens are merged in pies (left). Colors match the bidimensional color space of the PCoA projection (Fig. 1 left) of p-dist between sequences (dots).

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5. DISCUSSION

The Mediterranean hosts a rich diversity of agricultural systems and ecological conditions paired with a rich diversity of arthropods. The interactions that form multitrophic networks in agricultural settings are complex and intricate, and an increasing ecological understanding of the dynamics they create is key to moving towards more sustainable agricultural practices (Wezel et al. 2009). As biological control is poised to be one of the main tools for sustainable agricultural management (Bale et al. 2008), it is important to disentangle key interactions at different trophic levels, and ants are naturally an important component of agroecosystems in the region.

Our studies contributed to deepening the knowledge of interactions between ants and key arthropod pests, describing relationships that are important to study not only for the sake of applied entomology but also from an evolutionary perspective. Throughout our investigation of interactions between ants, insect pests, and their natural enemies, we combined laboratory and field experiments that allowed us to focus more on the behavioral or the ecological aspects of these interactions respectively.

On one hand, we demonstrated how Mediterranean ants can have a significant protective role against phytophagous insect pests of agricultural concern or even fungi (Chapters 1-5), comparable to what is observed in other, better-investigated regions (Offenberg 2015; Anjos et al. 2022). The results of the field experiment described in Chapter 1 showed a very substantial reduction of fruit damage caused by the codling moth (amounting to about 3% of the fruits versus 11-28% in the other treatments) and of the leaf damage caused by the pear scab (significantly fewer black spots or extended necrotic areas on the leaves). In laboratory experiments (Chapter 2), ants – by other studies known to prey upon adult larvae or pupae – eliminated 43% of newly hatched larvae during 10-min trials, leaving eggs intact but suggesting a possible effect on the oviposition behavior of adult moth that is yet to be explored. Under laboratory conditions, very common Mediterranean ant species were able to significantly decrease the reproductive success of *Xylosandrus compactus* ambrosia beetles at different stages (Chapter 3), and some could directly enter the beetles' nests, preying upon all their brood – which is particularly interesting considering the lack of information regarding native predators of this pest in Europe. Encouraging results were also found when newly hatched green stink bugs were exposed to Mediterranean ants in laboratory experiments, increasing their average mortality from zero up to 17% (Chapter 4).

Field assessments of interactions between ants and *Xylosandrus* ambrosia beetles, or ants and stink bugs (Chapters 3-5) will be crucial to quantify the outcomes of these interactions in agricultural settings and their possible contribution to biological control. More in general, field experiments aimed at understanding ant contribution to ecological dynamics in the highly diverse agricultural settings of the Mediterranean region are still scarce. In the specific context of Chapter 1, significant effects of ants against the codling moth would only emerge when ant activity and abundance were actively manipulated by providing extra food sources, and similar manipulative tests should be conducted in other contexts to assess the cost-efficiency of these strategies.

Furthermore, we are excited to be planning additional investigations to better understand the role of indirect interactions mediated by semiochemicals, whose role was underscored in the interactions with ambrosia beetle females, deterred from laying their eggs by the chemical traces of passing ants hours before (Chapter 3).

On the other hand, in multiple cases, we measured how the validity of general paradigms, such as the generalist predatory attitude of ants towards other insects or their protective role towards their mutualist honeydew-producing partners, are subject to great variation depending on the specific identity of the species involved. The frequent concern that ants may disrupt the activity of parasitoids and predators used for biological control needs to be assessed case by case. We could not predict that ants would show no aggression against the scelionid parasitoids that attack stink bug eggs (Chapters 4-5), nor that ants would not reduce the abundance of aphid predators and parasitoids in the fava bean plantation where we worked (Chapter 6). Instead, the behavioral differences of different ant species constitute a meaningful variable that should be taken into consideration in management practices (Chapters 7-8), while exceptions to general paradigms pose interesting evolutionary questions, for instance, regarding the gradient of mutualisms developed in ant-aphid interactions. Developing effective and sustainable management practices for ant-hemipteran mutualisms will inevitably benefit from increasingly more accurate knowledge of the behavioral and ecological dynamics shaping these complex networks.

Further increasing the efforts to explore ant role in Mediterranean agroecosystems will undoubtedly benefit from advancing our understanding of the characteristics of the ant species and communities that inhabit them, with integrative approaches offering the most promising perspectives to unravel complex diversity patterns (Chapters 9-11). This can also lead to learning how different management approaches can influence these ant communities, thus influencing the different services and disservices they may provide, as already observed in other regions (Perfecto & Vandermeer 1996). It also contributes to evaluating the effects of management practices on biodiversity in agricultural systems, which is crucial to insect conservation across Europe (Wagner et al. 2021).

The study of ants' role in Mediterranean agroecosystems has just begun during the last few decades, and the diversity of these environments will make their exploration a long but fascinating journey. Many directions still must be investigated, and while complexity may, unfortunately, delay the discovery of practical answers to some agronomical problems, it will certainly reward those who seek with several cues on wider ecological and evolutionary aspects on the evolutionary history of ants and the arthropod communities they are part of.

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Index of other articles produced during the years 2020-2023

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1. Akbar, S.A., Bharti, H., Schifani, E., & Wachkoo, A.A. (2023). Taxonomic study of the ant genus *Vollenhovia* (Hymenoptera, Formicidae) in India, with two new species from the Western Ghats biodiversity hotspot. *European Journal of Taxonomy,* 908(1), 77-107. https://doi.org/10.5852/ejt.2023.908.2339
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