Role of ant-tending in modulating the presence of symbiotic bacteria against parasitoids in aphids

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ABSTRACT

Several species of parasitoid wasps have been used to control aphids in the field in order to improve the environmental sustainability of the current agricultural practices. Behavioural and physiological defences have been reported in aphids and the role of symbiotic bacteria, such as *Hamiltonella defensa*, *Serratia symbiotica* and *Regiella insecticola*, has been molecularly dissected to understand their protection against hymenopteran parasitoids. Interestingly, the relationship between the presence of defensive symbionts and the type of aphid-ant mutualism (obligate, facultative or absent) is still understudied, whereas ants could strongly influence the ecological costs of defensive symbionts. In the present paper, we performed a screening of 16 aphid species collected in Italy and compared the presence and abundance of defensive symbionts to the degree of myrmecophily of the sampled aphid populations revealing that *Hamiltonella defensa* is the most common defensive symbiont. Our data suggest a direct effect of ant-tending on the abundance of *H. defensa* such that if aphids are maintained in insectaries in the absence of ants, aphids increase the amount of this defensive symbiont making the composition of their microbiome context-dependent.

KEYWORDS: parasitoid wasps, biological control, defensive symbiosis, aphid-ant mutualism

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INTRODUCTION

Aphids represent a serious source of damage in agriculture. Hence several strategies are currently used to control them in the field [1-3]. In order to improve the environmental sustainability of the current agricultural practices, natural enemies of aphids have been frequently used in the past few decades [4-6]. In particular, parasitoid wasps belonging to the sub-family Aphidiinae (Hymenoptera: Ichneumonoidea: Braconidae) and to the genus *Aphelinus* (Hymenoptera: Chalcidoidea: Aphelinidae) proved to be very useful since they inject eggs into the aphid bodies causing their death [4, 7-8].

Since the successful development of the parasitoid larvae is always fatal to parasitized aphids, they have evolved both behavioural (such as kicking or dropping off the plant to avoid parasitoid oviposition) [9-10] and physiological (such as preventing parasitoid development after oviposition) defences [4, 11-12].

According to literature data, behavioural resistance reduces parasitoid oviposition rate, whereas physiological resistance is fatal to the parasitoid's eggs or larvae [4]. The latter strategy is based on different mechanisms, but it can also be based on the presence of bacteria that act as defensive symbionts [4, 12-15]. Moving from the first evidence of defensive symbionts reporting that experimental infection with the two species of facultative bacterial symbionts *Hamiltonella defensa* and *Serratia symbiotica* increased the resistance of the pea aphid *Acyrthosiphon pisum* (Harris) to the parasitoid

Aphidius ervi [16], there are now several papers reporting that symbiont-conferred resistance to parasites and pathogens is an important and common phenomenon not only in aphids, but also in other insects [4].

The protective mechanism of *H. defensa* is not due to the bacteria alone, but the major role seems to be played by temperate bacteriophages, called *Acyrthosiphon pisum* secondary endosymbionts (APSEs), that encode toxins that can kill the parasitoid egg and larvae [13-14, 16-18]. In contrast to the comprehension of the defensive role of *H. defensa*, it is still unclear how the protection against parasitoids is gained through *S. symbiotica*.

Regiella insecticola is a third bacterial species which confers protection against the parasitoid *Aphidius colemani* (Viereck) and it seems to be also involved in the protection against entomopathogenic fungi [19-20]. Sequencing of *R. insecticola* genome didn't reveal the presence of APSE phages, but suggested that the resistance to parasitoid was due to five categories of pathogenicity factors [21]. Hence as a whole, it appears that different symbionts have found diverse solutions to the same evolutionary challenge.

Despite the strong selective advantage of defensive symbioses for aphids, it is really surprising that most of the surveys published up until now have found that defensive bacteria occur at low or intermediate frequencies and for this reason aphids possessing defensive symbionts do not go to fixation in natural populations [22-24]. As reviewed by Vorburger [4], this result could be due to the balance of selective benefits and costs conferred by the symbionts as well as to the balance between symbiont losses and gains that determine their frequency in a population. Different elements could be part of this balance as, for instance, it has been observed that symbiont-conferred resistance against parasitoids is reduced under heat stress suggesting that defensive symbionts of aphids may be suppressed or even eliminated during hot summer days [25-26]. It seems that aphids have to face a high cost to harbour defensive bacterial species that result in the shortening of their lifespan as a consequence of the metabolic demands imposed by the presence of defensive symbionts or in view of the costs of immune activation in their presence, or due to "collateral damages" to the host resulting from the symbiont's production of toxins and other pathogenic factors [4].

Interestingly, at present, very few papers have analysed the effects of predators and mutualists (such as ants) on the presence and abundance of defensive symbionts in aphids [27-28]. According to literature data, about 25% of the aphid species is involved in a facultative or obligate mutualistic relationship with ants (myrmecophily). The interaction between ants and aphids, based on the protective role of ants against natural enemies of aphids, is a well-studied example of symbiosis. In exchange for protection, they provide ants with honeydew, a sugar-rich solution that aphids sometimes enrich with amino acids in order to make it more attractive for the ants [29]. This long-lasting relationship (dating back 23-38 million years ago) affected co-evolutionary patterns of both interacting parties and had a keystone role in shaping the ecological dynamics in several natural and agro-ecosystems [30-32]. According to the different degrees of involvement in the symbiotic habit, co-evolutionary pathways led to morpho-functional and behavioural adaptation in both ants and aphids. Concerning the latter, a morphological trait considered as a clear adaptation to myrmecophily is the presence of special long hairs in the anal region suitable to hold a droplet of honeydew to facilitate the "milking" by ants. Moreover, myrmecophilous aphids present morphological traits correlated with a protected environment ensured by ant-tending (e.g. shorter cornicles, shorter legs and a longer rostrum). An alternative hypothesis is that these traits pre-existed (at least in a less specialized form) the ant-aphid relationship and acted as an exaptation favouring the establishment of mutualism [30, 33-34].

Despite the relevance of ant-aphid-parasitoid interactions, the relationship between the presence of defensive bacterial symbionts and the type of aphid-ant mutualism (obligate, facultative or absent) has been reported in very few papers [28]. The aim of the present research is therefore to perform a screening of 16 aphid species collected in Italy relating the presence and abundance of the defensive symbionts *H. defensa*, *S. symbiotica* and *R. insecticola* to the degree of myrmecophily of the sampled populations. Lastly, we compared the abundance of the defensive symbiont *H. defensa* in field-aphid populations with facultative ant mutualisms immediately after sampling and after the permanence

of the same populations in the insectary in the absence of ants in order to verify if the presence/absence of ants may modulate the abundance of some symbionts in the aphid microbiome.

MATERIALS AND METHODS

Live aphids belonging to 16 species were sampled in Modena (Italy) in May 2014 from different host plants (Table 1). Sampling was performed by beating onto a tray held underneath the branch for aphids on tree leaves or by bringing the aphids back to the laboratory alive on a piece of the host plant in case of herbaceous hosts [35]. We collected 20 individuals at each sampling site (from a single leaf when possible) for three consecutive weeks in order to have our evaluation as triplicates.

Table 1. List of the collected aphid species. Ant mutualism: untended (-), facultative tending (+) and recurrent tending (++).

Aphid species	Plant host	Sampling site	Ant mutualism	Ant species	Presence of predators	Presence of mummies
Acyrthosiphon pisum	*Prunus persica*	44°38'22,90''N 10°48'40,46''E	-	-	-	+
Aphis craccivora	*Bellis perennis*	44°38'45,35''N 10°56'04,18''E	++	*Crematogaster scutellaris*	-	-
Aphis fabae	*Plantago lanceolata*	44°37'11,47''N 10°56'04,18''E	++	*Plagiolepis pygmaea*	-	-
Aphis gossypii	*Cucurbita pepo*	44°38'22.69''N 10°48'39.39''E	-	-	*Harmonia axyridis, Scymnus* sp.	+
Aphis nerii	*Hibiscus sp.*	44°38'45,35''N 10°56'04,18''E	+	*Crematogaster scutellaris*	*Harmonia axyridis, Scymnus* sp.	+
Aphis passeriniana	*Salvia sp.*	44°36'32,92''N 10°58'34,09''E	++	*Crematogaster scutellaris*	-	-
Aphis pomi	*Malus domestica*	44°38'16.91''N 10°48'41.91''E	-	-	-	-
Aulacorthum solani	*Hibiscus sp.*	44°36'32,92''N 10°58'34,09''E	-	-	*Harmonia axyridis, Scymnus* sp.	+
Chaitophorus populeti	*Populus alba*	44°37'11,47''N 10°56'04,18''E	+	*Tetramorium caepitum*	-	-
Chaitophorus salicti	*Salix fragilis*	44°37'11,47''N 10°56'04,18''E	+	*Lasius psammophilus*	-	-
Dysaphis plantaginea	*Malus domestica*	44°36'32,92''N 10°58'34,09''E	+	*Lasius* sp.	-	-
Macrosiphum euphorbiae	*Bellis perennis*	44°36'32,92''N 10°58'34,09''E	-	-	*Harmonia axyridis, Coccinella septempunctata*	-
Macrosiphum rosae	*Rosa sp.*	44°37'53,96''N 10°56'40,58''E	-	-	*Harmonia axyridis, Coccinella septempunctata*	-
Myzus cerasi	*Prunus avium*	44°38'22,90''N 10°48'40,46''E	-	-	*Coccinella septempunctata, Coccinella bipunctata*	+
Myzus persicae	*Lavandula officinalis*	44°37'11,47''N 10°56'04,18''E	-	-	-	+
Toxoptera aurantii	*Celtis australis*	44°38'45,35''N 10°56'04,18''E	-	-	*Harmonia axyridis, Adalia bipunctata*	+

For each aphid species, we recorded the presence of mummies, ants and predators weekly for 6 weeks. In particular, in order to assess the degree of association with ants, aphid colonies were monitored once a week for 1 hour; the presence of ants and their behaviour were recorded with particular attention to antennal contacts with aphids and the collection of honeydew from them. Ants associated with aphids and aphid predators (such as ladybugs) present in the aphid colonies were photographed and/or sampled for a later identification. With respect to the pattern of interactions engaged with ants [31], aphids were classified according to the following definitions: i. untended (ants were never observed collecting honeydew or having direct contacts with aphids); ii. facultatively tended (ants were only occasionally recorded in the aphid colonies but, when present, they were observed collecting honeydew and having contacts with them); iii. recurrently tended (ants were always detected patrolling aphid colonies intensively), having antennal contacts with aphids and collecting honeydew from them.

Immediately after sampling, morphological examination was carried out by macerating individual aphids in KOH (potassium hydroxide) and mounting them on microscope slides making determination possible using specific dichotomous keys [1-2]. In a few problematic cases, aphid species were distinguished by cytochrome oxidase I (COI) DNA barcoding with the standard 3% sequence divergence, which normally led to species boundaries, separating individuals collected from different host plants. For molecular determination, DNA extraction was performed immediately after sampling by whole genome DNA extraction from single aphid using the SW Genomic DNA extraction kit (Promega) according to the manufacturer's instructions. The amplification of COI sequences was performed using the primers LepF and LepR (according to the procedure reported by Footit *et al.*) [36]. Sanger sequencing was performed using the primer LepF at the BMR Genomics (Padua, Italy) and the obtained COI sequence aligned using the identification tool freely available at the Barcoding of Life Database (BOLD) (http://www.boldsystems.org/index.php/ IDS_OpenIdEngine).

The presence of *H. defensa*, *S. symbiotica* and *R. insecticola* was screened by polymerase chain reaction (PCR) using specific primers according to Erickson *et al.* [27] and thereafter visualizing PCR products in 1.8% agarose gels stained with EtBr. All samples were also screened for *Buchnera aphidicola* as a positive control, since this bacterium is commonly present as primary symbiont in aphids, according to Abbot and Withgott [37].

In order to evaluate the effects of ant-tending on aphids, laboratory lines were initiated for the species *Aphis fabae* (Scopoli), *Aphis craccivora* (Koch) and *Aphis passeriniana* (Del Guercio) that showed a recurrent tending in the field. In this regard, 20 adults were sampled in the field on *Plantago lanceolata*, *Bellis perennis* and *Salvia* sp. plants for *A. fabae*, *A. craccivora* and *A. passeriniana*, respectively and then maintained on the same host plants at 20 °C with 16 hours light/8 hours dark in the absence of ants in the insectary. Five lines from the laboratory populations were successively started for each aphid species and 20 individuals from each line were tested after 7 and 14 days of permanence in the insectary for evaluating the *Hamiltonella* infection rate using specific primers according to Erickson *et al.* [27].

Statistical analyses were performed using the Student's *t*-test, whereas box plot graphs were obtained using the BoxPlotR tool (freely available online at the address http://shiny.chemgrid.org/boxplotr/ [38].

RESULTS

In order to analyse the distribution of symbiont-conferred parasitoid resistance in several species of aphids present in Italy, we collected aphids belonging to 16 species from different host plants (Table 1). According to experimental plan, we sampled aphid species untended by ants and facultatively/recurrently tended. In particular, 9 species (*Acyrthosiphon pisum*, *Aphis gossypii*, *Aphis pomi*, *Aulacorthum. solani*, *Macrosiphum euphorbiae*, *Macrosiphum rosae*, *Myzus persicae*, *Myzus cerasi* and *Toxoptera aurantii*) were untended by ants, 4 species (*Aphis nerii*, *Chaitophorus populeti*, *Chaitophorus salicti* and *Dysaphis plantaginea*) were facultatively tended and 3 (*Aphis craccivora*, *Aphis fabae* and *Aphis passeriniana*) recurrently tended by ants. Ants of the species *Crematogaster scutellaris* proved to be the most commonly associated ones with aphids, having been observed tending different aphid species on different host plants (Table 1).

The search for *H. defensa*, *S. symbiotica* and *R. insecticola* showed that *Hamiltonella* was the commonest symbiont among the three considered since it was present in 9 of 16 species analyzed (Table 2), whereas *S. symbiotica* and *R. insecticola* were present in just 1 and 3 of the analysed species, respectively and showed lower infection percentages (Table 2). With the exceptions of *D. plantaginea* and *A. passeriniana*, a single symbiont species was present in all of the analysed species and in both exceptions *Hamiltonella* coexisted with *R. insecticola*.

The box plot analyses of the *Hamiltonella* presence revealed high infection frequencies in aphid species that were scarcely predated by ladybugs (Figure 1a) and in species that were scarcely parasitized and did not present aphid mummies (Figure 1c). Interestingly, box plot suggested that the prevalence of *Hamiltonella* was very variable (but generally low) in aphids untended by ants, whereas among tended aphids, the prevalence of *Hamiltonella* was lower in aphids recurrently tended (Figure 1b).

In order to verify if ant presence/absence directly influenced the infection rate of *Hamiltonella* in the aphid populations recurrently tended by ants, we sampled field populations of *A. fabae*, *A. craccivora* and *A. passeriniana* and maintained them within the insectary on the same host plants in the absence of ants. As shown by the box plot analysis, the PCR screening revealed an increase in *Hamiltonella*-infected aphids in the absence of ants such that the average percentage of infected aphids grew from 21% to 36% in *A. fabae* (Figure 2a), from 23% to 39% in *A. craccivora* (Figure 2b), and from 20% to 36% in *A. passeriniana* (Figure 2c).

DISCUSSION

The presence of the three defensive symbionts *H. defensa*, *S. symbiotica* and *R. insecticola* has been evaluated in several aphid species [15, 28] suggesting that their occurrence is related to the aphid life history. However, literature data are still controversial for some species suggesting that different factors may be involved in determining their presence and abundance. For instance, data published in literature suggest that pea aphid *A. pisum* commonly hosts *Hamiltonella*

Table 2. Summary of the PCR screening looking for the presence (indicated as the proportion of infected specimens in the 20 sampled aphids) or absence (-) of *H. defensa*, *S. symbiotica* and *R. insecticola*.

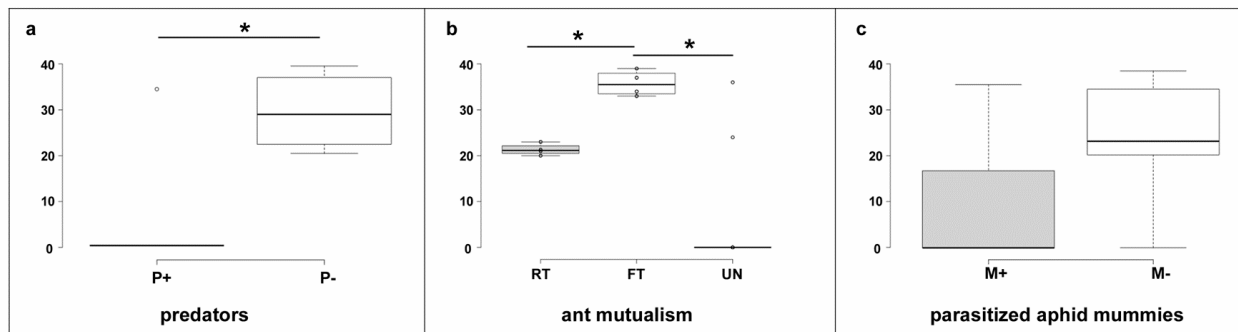| Species	H. defensa	S. symbiotica	R. insecticola	Buchnera
A. pisum	36%	-	-	+
A. craccivora	23%	-	-	+
A. fabae	21%	-	-	+
A. gossypii	-	-	-	+
A. nerii	34%	-	-	+
A. passeriniana	20%	-	11%	+
A. pomi	24%	-	-	+
A. solani	-	-	-	+
C. populeti	39%	-	-	+
C. salicti	33%	-	-	+
D. plantaginea	37%	-	12%	+
M. euphorbiae	-	-	-	+
M. rosae	-	-	-	+
M. cerasi	-	-	-	+
M. persicae	-	-	17%	+
T. aurantii	-	16%	-	+

Figure 1. Box plot analyses comparing the *Hamiltonella* infection rate in relation to predators (a) ant-aphid interaction (b) and presence of parasitized aphids (c) Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. **P+**: presence of predators. **P-**: absence of predators. **RT**: recurrently tended. **FT**: facultatively tended. **UN**: untended. **M+**: presence of parasitized aphids (mummies). **M-**: absence of mummies. Asterisk indicates that two values are significantly different at the 5% level in the Student's *t*-test.
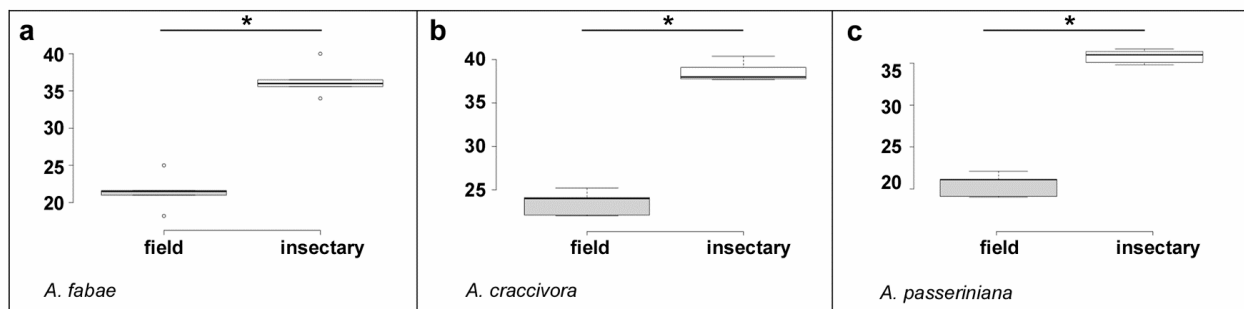

Figure 2. Box plot analyses comparing the *Hamiltonella* infection rate in *A. fabae* (a) *A. craccivora* (b) and *A. passeriana* (c) in field-sampled populations recurrently tended by ants and the same aphid populations maintained in the insectary in the absence of ants. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. Asterisk indicates that two values are significantly different at the 5% level in the Student's *t*-test.

symbionts [39-40], whereas a survey of pea aphids in Japan reported that *A. pisum* did not host them [22]. At the same time, the survey of 75 aphid species representing 15 aphid tribes showed that the three defensive symbionts were widely distributed [39] and a similar screening (even if limited to five species) suggested that these symbionts are also present in England, but not fixed in the natural populations [41].

Recently, a large survey including more than 130 aphid species has been published [28] showing that the presence of *H. defensa*, *S. symbiotica* and *R. insecticola* is the result of a balancing selection between advantages and costs of maintaining defensive symbionts. In the present experimental

work, we screened the presence of the above-mentioned symbionts in 16 aphid species collected in Northern Italy to see if the same patterns are found in different climatic regions where diverse selection pressures may influence the establishment and maintenance of these symbioses.

According to our results, *Hamiltonella* was the commonest defensive symbiont since it was present in 9 of the 16 analyzed species. This is in agreement with previous data [28] even if we observed some differences since *Hamiltonella* is absent in the sampled Italian populations of *M. persicae, M. cerasi, A. solani, M. rosae* and *M. euphorbiae,* whereas in the UK screening these

species hosted *Hamiltonella* [28]. Interestingly, *D. plantaginea* presented *Hamiltonella* in the Italian screening, but not in the UK study [28].

S. symbiotica and *R. insecticola* were present in a few of the analysed Italian species and showed low infection percentages, contrary to a UK survey that reported the presence of these defensive symbionts also in *A. fabae*, *M. persicae*, *A. pisum*, *A. solani*, *M. euphorbiae* and *M. rosae* suggesting, as a whole, that the presence of defensive symbionts could be context-dependant. Lastly, the infection frequencies of *S. symbiotica* and *R. insecticola* were quite constant and similar to those previously reported in literature [16-28].

H. defensa has been found until now in different aphid species [16, 18, 28] and hence its common presence in the aphid species sampled in Italy is not surprising. Despite its widespread occurrence, its horizontal and vertical transmission and the strong benefits conferred, *H. defensa* does not typically reach fixation in natural populations of aphids and tend to be found at moderate frequencies [24]. The screening of more than 400 specimens from France and Switzerland revealed that *H. defensa* occurred in about the half of the analysed *A. fabae* aphids (R. Rouchet & C. Vorburger, unpublished data, cited in Lukasik *et al.* [42]. Our data reported that *Hamiltonella* was present in most of the screened species, but none of them hosted *H. defensa* in more than 40% of the collected aphids.

Also taking into account that the protection against parasitoids is crucial in providing a significant advantage for the infected clone, the absence of fixation of *Hamiltonella,* also observed in Italy, could be explained in view of the cost of hosting *H. defensa* [27, 42]. This is in agreement with the hypothesis, suggested by Henry *et al.* [28], that balancing selection plays a key role in the maintenance of protective mutualisms with symbiotic microbes.

According to our data, aphid species with low predation/parasitism rate have generally higher *Hamiltonella* infection rates confirming the defensive role of these symbionts. For instance, the absence of mummies in aphid species with high *Hamiltonella* infection rate suggests that parasitoid species are able to detect the presence of *H. defensa* in aphids and respond by avoiding to lay eggs in

Hamiltonella-infected aphids. According to literature data, parasitoid wasps *Aphidius ervi* and *Ephedrus plagiator* are able to distinguish infected from uninfected *Sitobion avenae* aphids and thus they may reduce their attacks on aphids possessing *H. defensa* [42].

Henry *et al.* [28] reported that *H. defensa* and *R. insecticola* were more likely to occur in aphid species that are not tended by ants suggesting that the association with ants or possessing protective symbionts may be alternative ways used by aphids to get protection from parasitoids and fungal pathogens. The basic element of the paradigm of mutualism between aphids and ants (as well as other honeydew secreting insects) is that aphids are effectively protected by ants against potential sources of danger (such as predators, parasitoids and pathogens) in exchange of food [29-30, 43-44].

This suggests, therefore, that ant-tended aphids may limit some costs related to defence since hosting *H. defensa* and *R. insecticola* is known to entail costs in some aphid species [14, 42, 45]. As a consequence, the occurrence of a symbiont in a particular aphid species may depend on the balance between the costs of infection and the benefits they provide. By reducing, but not necessarily eliminating, natural enemy pressure, ant-tending may tip this balance against symbiont presence, although other external factors may also be involved [28]. This hypothesis could also explain apparently controversial data reported in literature; for instance Erickson *et al.* [27] reported that the ant presence did not reduce *H. defensa* frequency in the aphid *Obtusicauda frigidae*. However, in this case ant-tended aphids were devoid of parasitoids even if they lacked *Hamiltonella* and hence it was not clear if this symbiont really played any role in the protection of the aphids against parasitism. However, *O. frigidae* is not involved in obligate mutualism with ants but is facultatively attended, and hence the different nature of the mutualism could also be relevant.

Diverging from previous works [27-28], we tried to go into more details on the role of ants in regulating the presence/absence of *Hamiltonella* and we distinguished three different ant-aphid interaction types (untended/facultatively tended/ recurrently tended) in place of two (tented/not-tended). According to our results, untended aphids

may greatly vary in their amount of *Hamiltonella*, but on an average they have extremely low infection rate suggesting that either the other protector species or different strategies are present to face parasitism and predation. Interestingly, we observed that *Hamiltonella* infection rates clearly differ between facultatively tended and recurrently tended aphid species suggesting that the prevalence of *Hamitonella* is lower in the aphid population recurrently tended by ants. These results suggest that the effective defence against parasitoid wasps arises from a balance between the frequency of the interactions with ants and the amount of *Hamiltonella*. If aphids are recurrently tended, the costs of the mutualism with ants could be therefore cheaper than those related to *H. defensa*-carrying. On the contrary, if the presence of ants is not stable or frequent, aphids have to increase the presence of defensive symbionts suggesting that the associations with ants and protective symbiosis are actually not effectively alternative, contrary to what was previously suggested in literature [28].

CONCLUSION

In order to better understand the effect of ant interactions on the *Hamiltonella* infection rate, we isolated field populations of *A. fabae*, *A. craccivora* and *A. passeriniana* and maintained them in the absence of ants. The analysis of the infection rates clearly evidenced an increase in the *Hamiltonella* presence in the three species suggesting that ant-aphid interaction may modulate the presence of defensive symbionts. Even if a physiological mechanism triggered by aphids cannot be *a priori* excluded, our hypothesis is that ants can discriminate infected/uninfected aphids, favouring aphids not infected by *H. defensa* since they have longer lifespan and better reproductive rate that could increase the availability of honeydew for ants. As reported in literature, ants eliminate aphids containing developing wasps [45-46] and considering that parasitoid wasps can discriminate *Hamiltonella*-hosting aphids due to their production of a larger amount of the aphid alarm pheromone (trans-β-farnesene) than uninfected aphids [16], it is also possible that ants may use trans-β-farnesene as a cue to distinguish *Hamiltonella*-infected aphids.

A better understanding of the role played by ants in the establishment and maintenance of the aphid microbiome is very important both from an eco-ethological and an applicative point of view. Indeed, the effective control of aphid population by natural enemies could be strongly affected by ants not only through the defence against predators, but also by reducing the constitutive costs that aphids have to pay for their defence against parasitoid wasps.

CONFLICT OF INTEREST STATEMENT

No conflicts of interest.

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