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Article

Influence of “protective” symbionts throughout the different steps of an aphid–parasitoid interaction

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Abstract

Microbial associates are widespread in insects, some conferring a protection to their hosts against natural enemies like parasitoids. These protective symbionts may affect the infection success of the parasitoid by modifying behavioral defenses of their hosts, the development success of the parasitoid by conferring a resistance against it or by altering life-history traits of the emerging parasitoids. Here, we assessed the effects of different protective bacterial symbionts on the entire sequence of the host–parasitoid interaction (i.e., from parasitoid attack to offspring emergence) between the pea aphid, *Acyrtosiphon pisum*, and its main parasitoid, *Aphidius ervi* and their impacts on the life-history traits of the emerging parasitoids. To test whether symbiont-mediated phenotypes were general or specific to particular aphid–symbiont associations, we considered several aphid lineages, each harboring a different strain of either *Hamiltonella defensa* or *Regiella insecticola*, two protective symbionts commonly found in aphids. We found that symbiont species and strains had a weak effect on the ability of aphids to defend themselves against the parasitic wasps during the attack and a strong effect on aphid resistance against parasitoid development. While parasitism resistance was mainly determined by symbionts, their effects on host defensive behaviors varied largely from one aphid–symbiont association to another. Also, the symbiotic status of the aphid individuals had no impact on the attack rate of the parasitic wasps, the parasitoid emergence rate from parasitized aphids nor the life-history traits of the emerging parasitoids. Overall, no correlations between symbiont effects on the different stages of the host–parasitoid interaction was observed, suggesting no trade-offs or positive associations between symbiont-mediated phenotypes. Our study highlights the need to consider various sequences of the host–parasitoid interaction to better assess the outcomes of protective symbioses and understand the ecological and evolutionary dynamics of insect–symbiont associations.

Key words: *Acyrtosiphon pisum*, defensive behaviors, *Hamiltonella defensa*, host–parasitoid interactions, protective symbioses, *Regiella insecticola*

Associations between macro and microorganisms are widespread in animals, and recent research has highlighted the roles of these microbial partners on many aspects of their hosts' biology, ecology and evolution. In particular, there is increasing evidence that microbial symbionts contribute to the protection of their host individual against natural enemies such as pathogens, parasitoids or predators (Flórez et al. 2015). In insects, bacterial symbionts have been shown to protect their hosts in different ways, either directly by producing compounds that are toxic or repellent for the natural enemies or indirectly by enhancing the immune system of their hosts or by competing with the enemy for the same host resources (Oliver et al. 2014).

The first case of protective symbiosis in insects was found in an aphid-parasitoid interaction (Oliver et al. 2003). This seminal work has stimulated a wealth of studies devoted to better understand the influence of protective bacterial symbionts on host-parasitoid interactions, including issues related to protection mechanisms, costs and benefits of symbiotic infection, and impacts on both parasitoid ecology and trophic networks (Vorbürger and Gouskov 2011; Cayetano et al. 2015; Frago et al. 2017; Monticelli et al. 2019; Oliver and Higashi 2019; Leybourne et al. 2020). Parasitoids exploit insect hosts for resources necessary for their survival and are important biological control agents of crop pests. The parasitoid life-cycle involves successive steps including (1) the attack of the host individual, during which the parasitoid female oviposits (i.e., lays an egg with its ovipositor) on or in the encountered host (i.e., the host entry stage), (2) the development of the parasitoid immature on or in the host, which consumes the host until it dies (i.e., the development stage), and (3) the emergence of the parasitoid offspring from the host (i.e., the host exit stage) (Godfray 1994). The host is however not defenseless and can use a range of protective strategies to counter the parasitoid at the different steps of the parasitoid life-cycle. Defensive behaviors can be first used to avoid parasitoid's attack. When the parasitoid succeeds to lay an egg in the host (i.e., oviposition), the host immune system can preclude the development of the parasitoid immature. Finally, if physiological defenses are not sufficient to stop immature parasitoid development, the host can nevertheless have negative effects on parasitoid emergence from the host and/or sublethal effects on the emerging parasitoid, which would emerge with altered life history traits (Godfray 1994).

Microbial symbionts can contribute to the defense of their hosts by protecting them against parasitoids at these different stages of the parasitoid life-cycle. One of the best known cases of protective symbiosis against parasitoids concerns the pea aphid *Acyrtosiphon pisum*, which is associated with several secondary symbiotic species involved in parasitism protection (Oliver et al. 2003; Vorbürger et al. 2010; Cayetano and Vorbürger 2015; Donald et al. 2016; Leclair et al. 2016; Jamin and Vorbürger 2019). The protective bacterial symbiont *Hamiltonella defensa* is well known to provide resistance to pea aphids through the production of toxins encoded by the APSE associated phage (*Acyrtosiphon Pisum* Secondary Endosymbiont; Moran et al. 2005) that results in parasitoid immature development abortion (Degnan and Moran 2008; Oliver and Moran 2009; Weldon et al. 2013; Brandt et al. 2017). Even when parasitoid immatures overcome resistance conferred by the protective symbionts, these bacterial associates can negatively impact the life history traits of the emerging parasitoids by reducing their egg load, size and/or survival (Nyabuga et al. 2010; Schmid et al. 2012; Pons et al. 2019). Such sublethal effects on emerging parasitoids would induce variation in both their foraging strategies and host range (Monticelli et al. 2019). These microbial partners can also

modify host-parasitoid interactions by influencing the defensive behaviors of their hosts. Previous studies found that aphids harboring *H. defensa* exhibited less defensive behaviors than uninfected aphids when facing a natural enemy (Dion et al. 2011; Polin et al. 2014). Although this may sound counter-intuitive, reduction of defensive behaviors could be beneficial to the aphid by avoiding costly and redundant protection against parasitoids (Dion et al. 2011). However, the generality of this phenomenon across the different types of protective symbioses is little known (Dion et al. 2011; Ramírez-Cáceres et al. 2019).

Previous studies on the influence of protective symbionts on host-parasitoid interactions have in most cases focused on one of the different stages of the parasitoid life-cycle. As a consequence, we know little on the actual outcomes of the different defenses that protective symbionts may provide during the whole sequence of the host-parasitoid interaction, as well as correlations between symbiont effects on the different stages of the parasitoid life-cycle. Our study aimed at answering three questions: (1) Do protective symbionts influence the ability of their hosts to avoid and resist to parasitoids and impact the emerging parasitoids traits? (2) If so, are these symbiont-mediated phenotypes correlated or not? (3) How general are these symbiont-mediated phenotypes and patterns displayed during aphid-parasitoid interactions? To answer these questions, we used several lineages of the pea aphid *A. pisum* harboring different symbiont strains and species and measured their ability to defend themselves against the parasitic wasp during the attack, their level of resistance if parasitized, the parasitoid emergence rate and the life-history traits of the parasitoid emerging from these aphid lineages.

Material and Methods

Pea aphid lineages

Two bacterial symbionts infecting *A. pisum* were considered in this study: *H. defensa* and *Regiella insecticola*. *Hamiltonella defensa* is known to protect its hosts against one of its main enemies the parasitic wasp *Aphidius ervi* (Oliver et al. 2003; Ferrari et al. 2004; Oliver et al. 2005) through interaction with the APSE phage (Moran et al. 2005). However, this bacterial symbiont provides variable protection against parasitoids, ranging from no protection to a full protection against parasitism, which depends mainly on both symbiont and APSE strains (Oliver et al. 2005; Leclair et al. 2016). *Regiella insecticola* protects *A. pisum* against pathogenic fungi but with no evidence so far against parasitoids in the pea aphid (Oliver et al. 2003; Ferrari et al. 2004; Scarborough et al. 2005; Hansen et al. 2012). However, a strain of *R. insecticola* has been found to provide resistance against parasitoids in the green peach aphid, *Myzus persicae* (Vorbürger et al. 2010; Jamin and Vorbürger 2019). In addition, one study investigated the influence of *R. insecticola* on aphid defensive behaviors in the cereal aphid *Sitobion avenae* (Ramírez-Cáceres et al. 2019). Authors found no effect of the infection with *R. insecticola* on the defensive behaviors when aphids were exposed to ladybirds, although aphids infected with *R. insecticola* were more predated than uninfected aphids.

The pea aphid forms a complex of plant-adapted biotypes, each specialized on one or a few legume species (Peccoud et al. 2009; Peccoud et al. 2015). Bacterial secondary symbionts are not randomly distributed among pea aphid biotypes but instead form preferential associations with some biotypes (Henry et al. 2013; Mathé-Hubert et al. 2019). In this study, we focused on three *A. pisum* biotypes, respectively specialized on *Genista tinctoria* (dyer's broom), on *Medicago sativa* (alfalfa) or on *Trifolium* (clover), hereafter

Table 1 Natural pea aphid lineages considered in the present study

Name	Sampling location	Collection date	Biotype	Secondary symbiont
Gt1	Bugey (France)	June 2014	<i>Genista tinctoria</i>	<i>Hamiltonella defensa</i>
Gt2	Bugey (France)	June 2014	<i>G. tinctoria</i>	<i>H. defensa</i>
Ms1	Noyal (France)	September 2014	<i>Medicago sativa</i>	<i>H. defensa</i>
Ms2	Bugey (France)	August 2011	<i>M. sativa</i>	<i>H. defensa</i>
T1	Ibaraki prefecture (Japan)	May 2014	<i>Trifolium sp</i>	<i>Regiella insecticola</i>
T2	York (UK)	December 2002	<i>Trifolium sp</i>	<i>R. insecticola</i>

noted “*Genista* biotype”, “*Medicago* biotype”, and “*Trifolium* biotype”. In natural populations, *H. defensa* is fixed in the *Genista* biotype, at intermediate to high frequencies in the *Medicago* biotype and rare in the *Trifolium* biotype, while *R. insecticola* is found at high prevalence in the *Trifolium* biotype, at a lower prevalence in the *Medicago* biotype and absent in the *Genista* biotype (Peccoud et al. 2015; Leclair 2016).

For each biotype, we used two genetically distinct aphid lineages singly infected with its dominant secondary symbiont: *H. defensa* for the *Medicago* and the *Genista* biotypes, and *R. insecticola* for the *Trifolium* biotype. All strains of *H. defensa* used in this study presented the APSE phage (Leclair et al. 2016). These six natural aphid lineages came from single aphid females sampled in the field on their respective host plants (details in Table 1). They were assigned to their corresponding biotype by genotyping and comparing their microsatellite profile to a reference database (Peccoud et al. 2009; Peccoud et al. 2015). To ensure that they were only infected with either *H. defensa* or *R. insecticola*, their symbiotic status was assessed with a diagnostic PCR using specific primers for symbiont amplification (Peccoud et al. 2014). To properly assess the variation of *H. defensa* effects on aphid defensive behaviors and search for a possible link between aphid defensive behaviors (i.e., avoidance of parasitism) and protection levels against parasitoid (i.e. resistance to parasitism), we considered symbiont strains with contrasted protective phenotypes. Based on our previous study, we know that the two *H. defensa* strains from the lineages of the *Genista* biotype belong to the same genetic group and provide a complete resistance to their aphid host against *Aphidius ervi* while the two strains from the lineages of the *Medicago* biotype differ genetically and provide no protection against *A. ervi* (Leclair et al. 2016). Note that *H. defensa* strains from the lineages of the *Genista* and the *Medicago* biotypes also differ genetically. Interestingly, aphids from the *Genista* biotype infected with *H. defensa* present a particular phenotype: although no parasitoid emerges from *H. defensa*-infected aphids, these cannot produce any offspring. This peculiar phenotype has been described extensively in Leclair et al. (2016). The two strains of *R. insecticola* from the lineages of the *Trifolium* biotype belong to the same genetic group (Sochard et al. 2020), but their level of protection against parasitoids was unknown prior this study.

The specificity of symbiont-mediated phenotypes was tested by introducing symbiont strains and species into new aphid genotypes. For our experiments, we considered three types of aphid lineages: the “cured lineages”, the “control lineages”, and the “new lineages”. First, we created uninfected lineages (hereafter called “cured lineages”) by removing the secondary symbiont in the six natural aphid lineages using antibiotic treatments (Sochard et al. 2020). Second, each cured aphid lineage was re-infected with its original secondary symbiont (hereafter called “control lineages”) as described in Sochard et al. (2020). Third, *H. defensa* and *R. insecticola* from the six natural lineages were introduced in the cured

lineages to create lineages with the same genotype but different symbiont strains (hereafter called “new lineages”). The symbiont strains and aphid genotypes were crossed as followed: aphids from the *Genista* and the *Trifolium* biotypes received *H. defensa* strains of the *Medicago* biotype and aphids from the *Medicago* biotype received the symbionts of both the *Genista* and the *Trifolium* biotype in exchange. Transfer of some symbionts in new aphid hosts did not work (Sochard et al. 2020) and a few lineages were lost between their creation and the experiments (Gt2-Hd^{Ms1}, Gt2-Hd^{Ms2} and Ms2-Hd^{Gt2}), so we could not test all possible combinations. Finally, by comparing the control lineages with the cured ones, we measured the effect of the secondary symbionts on the different steps of the host-parasitoid interactions (i.e. from parasitoid attack to emergence of its offspring if any) while by comparing the control lineages with the new ones, we tested whether the symbiont-mediated phenotypes are general or specific to host-symbiont association.

All these artificial aphid lineages (i.e., 6 “cured lineages” + 6 “control lineages” + 13 “new lineages”—Table 2) were kept and maintained at least 20 generations before experiments to remove the antibiotics effect and allow symbiont colonization in new aphid host individuals after transfer (Table 1). All aphid lineages were reared on broad bean, *Vicia faba*, the universal host plant of *A. pisum* biotypes, at 20°C, 70% of relative humidity and 16:8 L: D photoperiod, before and during the experiments.

Parasitoids

For our experiments, we used the solitary parasitoid wasp *Aphidius ervi* (Hymenoptera: Braconidae). The mass rearing of *A. ervi* started from at least 50 males and females produced by Koppert Biological Systems®. Parasitoids were produced on a pea aphid lineage free of any secondary symbiont and highly susceptible to *A. ervi* parasitism (Leclair et al. 2016). For the experiments, parasitoid females were standardized: 2–3 days after emergence, mated and fed (honey and water). Before each experiment, the parasitoid female was exposed to one third-instar larva of the tested aphid lineage for oviposition experience.

Experimental design

The aim of the experiment was to measure the effects of single infection by either *H. defensa* or *R. insecticola* on the ability of aphids to avoid and to resist to parasitoid infection and on the life-history traits of parasitoid emerging from aphid hosts. For this purpose, fifteen third-instar larvae from a given pea aphid lineage were introduced singly into a glass Petri dish (40 × 12 mm, Steriplan®) containing a leaf disk of *V. faba*. Few hours later, these single aphid individuals were successively exposed to an *A. ervi* female. The observation began once the parasitoid was introduced into a Petri dish and ended after aphid stabbing or after 5 minutes if no attack happened. Behaviors of both aphid and parasitoid were recorded. When the parasitoid had a contact with the aphid, the event was recorded

Table 2 Artificial pea aphid lineages used in the present study

Lineage code	Aphid genotype	Type of lineage	Secondary symbiont (SS)	Symbiont strain
Gt1-Hd ⁻	Gt1	Cured	No SS	–
Gt1-Hd ^{Gt1}	Gt1	Control	<i>Hamiltonella defensa</i>	Gt1
Gt1-Hd ^{Ms1}	Gt1	New	<i>H. defensa</i>	Ms1
Gt1-Hd ^{Ms2}	Gt1	New	<i>H. defensa</i>	Ms2
Gt2-Hd ⁻	Gt2	Cured	No SS	–
Gt2-Hd ^{Gt2}	Gt2	Control	<i>H. defensa</i>	Gt2
Ms1-Hd ⁻	Ms1	Cured	No SS	–
Ms1-Hd ^{Ms1}	Ms1	Control	<i>H. defensa</i>	Ms1
Ms1-Hd ^{Gt1}	Ms1	New	<i>H. defensa</i>	Gt1
Ms1-Hd ^{Gt2}	Ms1	New	<i>H. defensa</i>	Gt2
Ms1-Ri ^{T1}	Ms1	New	<i>R. insecticola</i>	T1
Ms1-Ri ^{T2}	Ms1	New	<i>R. insecticola</i>	T2
Ms2-Hd ⁻	Ms2	Cured	No SS	–
Ms2-Hd ^{Ms2}	Ms2	Control	<i>H. defensa</i>	Ms2
Ms2-Hd ^{Gt1}	Ms2	New	<i>H. defensa</i>	Gt1
Ms2-Ri ^{T1}	Ms2	New	<i>R. insecticola</i>	T1
Ms2-Ri ^{T2}	Ms2	New	<i>R. insecticola</i>	T2
T1-Ri ⁻	T1	Cured	No SS	–
T1-Ri ^{T1}	T1	Control	<i>Regiella insecticola</i>	T1
T1-Hd ^{Ms1}	T1	New	<i>H. defensa</i>	Ms1
T1-Hd ^{Ms2}	T1	New	<i>H. defensa</i>	Ms2
T2-Ri ⁻	T2	Cured	No SS	–
T2-Ri ^{T2}	T2	Control	<i>R. insecticola</i>	T2
T2-Hd ^{Ms1}	T2	New	<i>H. defensa</i>	Ms1
T2-Hd ^{Ms2}	T2	New	<i>H. defensa</i>	Ms2

as an encounter. The outcome of an encounter was either a success (e.g., egg-laying) or a failure. A successful attack corresponded here to an encounter with an ovipositor insertion which, in *A. ervi*, leads to a single egg injection into the host (Mcbrien and Mackauer, 1990). Conversely, any encounter between an aphid and a parasitoid that did not lead to ovipositor insertion was counted as an unsuccessful attack. An encounter can occur with or without aphid defensive behaviors. Here, we considered three classes of aphid behavioral defenses: (1) aphid aggressive behaviors (i.e. quick legs or body motions), (2) escaping by walking and (3) emission of cornicular secretions (i.e. secretion containing alarm pheromone, that can glue the parasitoid in case of contact, Wu et al. 2010). After this observation, the parasitoid female was then transferred to a new Petri dish containing another single aphid from the same lineage. The experiment ended when the 15 single aphids of the same lineage were exposed to the same female parasitoid. The individual aphids which were successfully attacked by the parasitoid female (i.e., ovipositor insertion) were counted and transferred on a single *V. faba* plant and kept under laboratory conditions. From the counting, we calculated the rate of successful attack by dividing the number of aphids successfully attacked by the total number of aphids exposed to the parasitoid (i.e., 15). After 14 days, aphids successfully attacked were inspected in order to measure the mummification rate. This latter was estimated by dividing the number of aphid mummies (i.e., dead aphids containing a parasitoid immature) by the total number of aphids successfully attacked transferred onto the host plant and recovered at that time (i.e. dead or missing individuals were excluded from analyses). All aphid mummies from the same experiment were then isolated in a Petri dish and parasitoid offspring emerging from these mummies were left into the device until they died. Once the number of emerging parasitoids counted, we calculated the parasitoid emergence rate by dividing the number of

emerging parasitoids by the total number of aphid mummies isolated in the Petri dish. We then sexed parasitoid offspring and measured the size of their left-hind tibia (the tibia length is a good fitness proxy in parasitic wasp—Godfray 1994). The parasitoid females used in parasitism experiments (i.e., the mothers) were also kept for tibia measurement. The tibia length of parasitoids was measured under binocular with a graduated scale (Leica MZ16, magnification: $\times 16$). For each aphid lineage, the experiment was conducted five to seven times (i.e., 75–105 of aphid individuals exposed to *A. ervi* attack per lineage).

Statistical analyses

All statistical analyses were carried out using R version 3.6.2 (R Core Team 2019).

Univariate analyses

Because only two aphid genotypes per biotype were used, we did not test for a biotype effect on response variables in our statistical analyses. Also, as aphid genotype and aphid symbiotic status are not crossed factors, all response variables were analyzed separately for each pea aphid genotype of a given biotype.

All proportion data (i.e., the occurrence of each class of aphid defensive behaviors: aggressiveness, escaping by walking or emission of cornicular secretions; and the successful attack rate, mummification rate, emergence rate, and sex-ratio of parasitoids) were analyzed as binary response variables using Generalized Linear Mixed Models (*glmer* function of the lme4 package; Bates et al. 2015) with a binomial error distribution (logit link function). In all binomial models, the symbiotic status of aphids was fit as fixed factor whereas the parasitoid ID was fit as a random factor to include data substructure (i.e., some experimental aphids were exposed to the same parasitoid female). The tibia size of the emerging parasitoids (i.e.,

Table 3 Estimated regression coefficients (β), standard errors (SE), and P -value associated with symbiotic status tested on defensive behaviors and rate of parasitoid successful attack

Pea aphid genotype	Symbiotic status	Variables											
		Aggressive behaviors			Escape behaviors			Cornicular secretions			Successful attack rate		
		β	SE (β)	Pr(> z)	β	SE (β)	Pr(> z)	β	SE (β)	Pr(> z)	B	SE (β)	Pr(> z)
Gt1	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdGt1	−1.14	0.41	0.005**	−0.53	0.36	0.138	0.04	0.47	0.927	0.47	0.64	0.463
	Infected with HdMs1	−1.04	0.42	0.013*	−0.70	0.37	0.056	0.05	0.48	0.918	−0.23	0.57	0.686
Gt2	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdGt2	−1.03	0.50	0.037*	−0.48	0.42	0.255	0.02	0.38	0.948	1.05	0.72	0.147
	Infected with HdMs2	−0.85	0.41	0.040*	0.28	0.36	0.441	0.53	0.44	0.226	0.52	0.64	0.416
Ms1	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdMs1	−1.93	0.78	0.013*	−1.21	0.44	0.007**	−1.18	0.48	0.013*	0.91	0.58	0.058
	Infected with HdGt1	−1.79	0.78	0.022*	−0.92	0.45	0.040*	−0.66	0.42	0.119	0.38	0.52	0.242
Ms2	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdMs2	−1.81	0.78	0.020*	−1.76	0.53	<0.001***	−0.67	0.40	0.089	0.77	0.49	0.116
	Infected with HdGt1	−2.32	0.76	0.002**	−2.05	0.52	<0.001***	−0.81	0.40	0.045*	0.77	0.49	0.116
T1	Uninfected	0.00			0.00			0.00			0.00		
	Infected with RiT1	0.02	0.56	0.963	0.43	0.46	0.350	−0.44	0.37	0.235	0.56	0.78	0.470
	Infected with HdMs1	−0.78	0.46	0.091	−0.17	0.40	0.675	−0.46	0.34	0.185	−0.09	0.64	0.884
T2	Uninfected	0.00			0.00			0.00			0.00		
	Infected with RiT2	−2.08	1.08	0.054	−0.13	0.59	0.830	0.06	0.35	0.872	0.26	0.54	0.624
	Infected with HdMs1	−2.62	1.06	0.013*	−0.49	0.58	0.395	−1.06	0.39	0.007**	−0.14	0.51	0.778
	Infected with HdMs2	−2.68	1.05	0.011*	−0.46	0.58	0.425	−0.63	0.37	0.083	−0.06	0.52	0.900

continuous response variable) was analyzed using a General Linear Mixed Model (*lme* function of the nlme package; Pinheiro et al. 2019). In this model, the symbiotic status of aphids was fit as fixed factor along with the tibia size of the parasitoid mother as covariate, whereas the replicate ID was fit as random factor (i.e., some parasitoid offspring developed on the same aphid host plant and environmental conditions).

For each model, estimated regression coefficients were subsequently analyzed using z -tests to assess the difference of each lineage with the reference category of the factor (e.g., “cured lineage”) and comparisons among the different lineages were performed by changing the reference category (Zuur et al. 2009). Because all comparisons provided consistent results, only comparisons between each of the infected lineage and the uninfected lineage are reported in the manuscript (i.e., “cured lineage” used as reference category; cf. Tables 3 and 4). Data with no variability (e.g., all aphids of a given lineage resisted against parasitism) were not included in models as coefficients cannot be estimated correctly.

Multivariate analysis

To visualize the global effects of symbionts on the aphid–parasitoid interactions and possible association or trade-off between these symbiotic effects, we conducted a principal component analysis (PCA) considering all eight variables measured in our experiments: the proportion of aphids expressing aggressive behaviors (1), escape behaviors (2) and excreting cornicular secretions (3); the parasitoid successful attack rate (4); the aphid mummification rate (5); the

parasitoid emergence rate (6); the sex-ratio (7) and the tibia size (8) of the emerging parasitoids. In this PCA, we considered the mean value of the eight variables for each aphid lineages. Therefore, we excluded the aphid lineages infected with Hd^{Gt1} or Hd^{Gt2} which conferred a complete protection to their hosts. The PCA analysis was computed using the package *ade4* (Chessel et al. 2004) and plotted using the package *RVAideMemoire* (Hervé 2019) in R. The correlation matrix associated with the PCA was represented using the package *corrplot* (Wei and Simko 2017).

Results

Effects of symbionts on aphid avoidance of *A. ervi* parasitism

In both aphid genotypes from the *Genista* biotype, the symbiotic status of aphids did not influence aphid escaping behavior and emission of cornicular secretions (Figure 1, Table 3). However, aphids harboring their own strain of *H. defensa* showed on average 14% less aggressive behaviors during attack compared to uninfected ones (Figure 1). Introducing either Hd^{Ms1} or Hd^{Ms2} in Gt1 also resulted in a similar reduction of aphid aggressiveness. In both aphid genotypes from the *Trifolium* biotype, while the infection with *R. insecticola* did not influence the aphid defensive behaviors, introducing the two strains of *H. defensa* from the *Medicago* biotype induced a reduction of the aphid defensive behaviors depending on the genotype (Figure 1, Table 3). Aphids infected with Hd^{Ms2} showed reduced aggressive behaviors in T2 and reduced escaping behaviors and

Table 4 Estimated regression coefficients (β), standard errors (SE), and P -value associated with symbiotic status tested on mummification rate, parasitoid emergence, and parasitoid life-history traits

Pea aphid genotype	Symbiotic status	Variables											
		Mummification rate			Emergence rate			Sex-ratio			Tibia size		
		β	SE (β)	Pr(> z)	β	SE (β)	Pr(> z)	β	SE (β)	Pr(> z)	B	SE (β)	Pr(> z)
Gt1	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdGt1	—	—	—	—	—	—	—	—	—	—	—	—
	Infected with HdMs1	−0.04	0.42	0.912	−0.53	1.02	0.602	1.43	1.29	0.268	−4 ^e −03	0.04	0.913
Gt2	Infected with HdMs2	−0.78	0.41	0.055	−0.78	1.06	0.463	0.26	1.26	0.838	−0.07	0.04	0.108
	Uninfected	—	—	—	—	—	—	—	—	—	—	—	—
	Infected with HdGt2	—	—	—	—	—	—	—	—	—	—	—	—
Ms1	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdMs1	−0.55	0.41	0.183	0.04	0.55	0.942	0.41	0.76	0.593	0.01	0.03	0.706
	Infected with HdGt1	—	—	—	—	—	—	—	—	—	—	—	—
Ms2	Infected with HdGt2	—	—	—	—	—	—	—	—	—	—	—	—
	Infected with RiT1	−1.00	0.41	0.014*	1.18	0.74	0.111	−0.67	0.77	0.380	2 ^e −03	0.04	0.964
	Infected with RiT2	−0.06	0.44	0.892	0.04	0.55	0.949	0.51	0.76	0.499	0.02	0.03	0.623
T1	Uninfected	0.00			0.00			0.00			0.00		
	Infected with HdMs2	−0.50	0.59	0.396	−1.05	0.57	0.065	−1.95	1.27	0.125	−0.02	0.04	0.695
	Infected with HdGt1	—	—	—	—	—	—	—	—	—	—	—	—
T2	Infected with RiT1	0.26	0.61	0.665	−0.23	0.62	0.716	−0.52	1.09	0.632	9 ^e −03	0.03	0.766
	Infected with RiT2	1.19	0.71	0.095	0.41	0.76	0.588	−0.16	1.14	0.891	0.01	0.03	0.731
	Uninfected	0.00			0.00			0.00			0.00		
T2	Infected with HdMs1	−0.08	0.35	0.828	−0.52	0.56	0.351	−0.41	0.69	0.556	0.02	0.03	0.407
	Infected with HdMs2	−0.31	0.33	0.347	−0.98	0.56	0.079	−1.18	0.74	0.113	0.02	0.03	0.525
	Uninfected	0.00			0.00			0.00			0.00		
T2	Infected with RiT2	−0.34	0.37	0.358	−1.01	0.54	0.063	−0.77	1.08	0.478	0.02	0.03	0.628
	Infected with HdMs1	−0.27	0.37	0.453	0.69	0.68	0.316	−0.91	1.04	0.385	−4 ^e −03	0.03	0.912
	Infected with HdMs2	−0.19	0.37	0.597	0.24	0.65	0.713	−1.05	1.10	0.343	−9 ^e −03	0.04	0.796

cornicular secretions in T1 compared to uninfected lineages, while aphids infected with Hd^{Ms1} underwent a reduction in aggressive behaviors and cornicular secretions only in T2 (Figure 1, Table 3). In both aphid genotypes from the *Medicago* biotype, aphid symbiotic status affected differently the defensive behaviors depending on the genotype. Infection with the original strain of *H. defensa* resulted in 10% and 19% less aggressive and escaping behaviors, respectively, compared to uninfected aphids in both genotypes and in 63% less cornicular secretions in Ms1 only (Figure 1, Table 3). Introducing symbiont strains or species (i.e. Hd^{Gt1}, Hd^{Gt2}, Ri^{T1} and Ri^{T2}) into both aphid genotypes from the *Medicago* biotype also affected defensive behaviors but depending on the type of defensive behavior and the aphid genotype. In Ms1, aphids infected with strains of *H. defensa* from the *Genista* biotype showed reduced aggressiveness and evasion compared to uninfected ones, and aphids infected with Hd^{Gt2} further emitted less cornicular secretions. For *R. insecticola*, while Ri^{T2} did not affect aphid behavioral defenses, Ri^{T1} decreased both escaping behaviors and cornicular secretions. In Ms2, Hd^{Gt1} decreased the three defensive behaviors while the two strains of *R. insecticola* affected none of them (Figure 1, Table 3). Finally, whatever the aphid genotype, the rate of successful parasitoid attack was not affected by the symbiotic status of the encountered aphid: neither the infection with the original symbiont nor the infection with new symbiont strains or species influenced the number of aphids successfully attacked by *A. ervi* females (Figure 1, Table 3).

Effect of symbionts on aphid resistance to *A. ervi* parasitism

Considering the two aphid genotypes originated from the *Genista* biotype, the influence of symbionts on parasitism resistance strongly depended on the symbiont strain. Once successfully attacked by an *A. ervi* parasitoid female, a high proportion of aphids of the *Genista* biotype free of *H. defensa* were mummified (70–83% for Gt1 and Gt2, respectively) while lineages infected with the original strain of *H. defensa* were totally resistant to parasitism regardless of the aphid genotype (Figure 2). Introducing either Hd^{Ms1} or Hd^{Ms2} in Gt1 provided no protection against parasitoids (Figure 2, Table 4). Considering the two aphid genotypes originated from the *Trifolium* biotype, infection with secondary symbionts did not change aphid resistance to parasitism: aphids harboring either *R. insecticola* or *H. defensa* presented similar mummification rates than uninfected ones (Figure 2, Table 4). Considering the two aphid genotypes originated from the *Medicago* biotype, we confirmed the protection levels associated with symbionts assessed previously (Leclair et al. 2016): the two strains of *H. defensa* from the *Medicago* biotype showed no protection against parasitism. Introducing *H. defensa* from the *Genista* biotype in Ms1 and Ms2 genotypes protected completely their hosts against *A. ervi* parasitism (Figure 2, Table 4). In addition, all surviving aphids of these new lineages became whitish and swollen few days after the parasitoid attack and stopped reproducing. The introduction of the two strains of *R. insecticola* into new hosts (Ms1 and Ms2) resulted in different phenotypes depending on

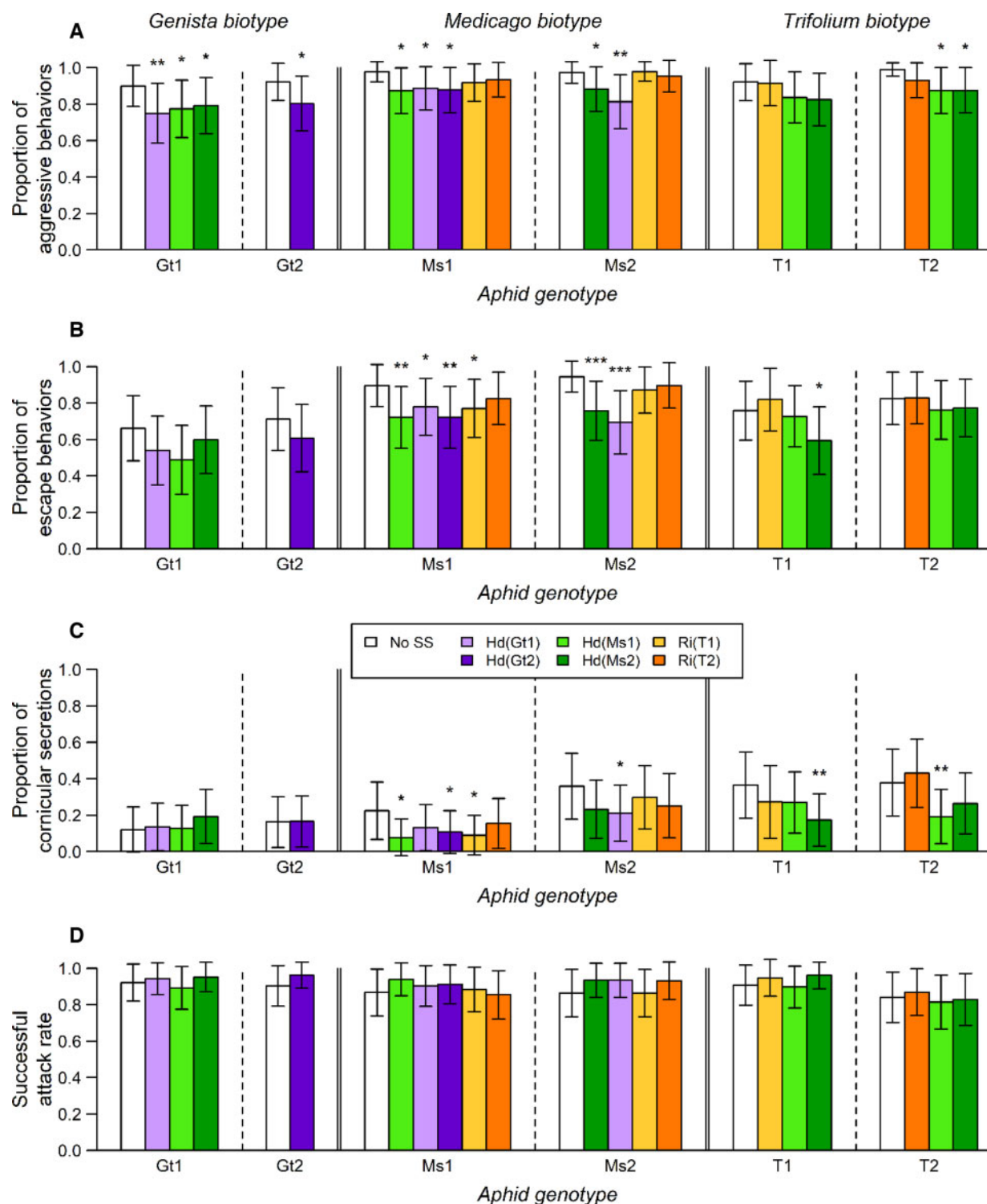


Figure 1 Effects of the symbiotic status of pea aphid individuals on three aphid defensive behaviors during the attack of an *Aphidius ervi* parasitoid female: (A) The proportion of aphids expressing legs and body movements (i.e., aphid aggressiveness); (B) the proportion of aphids walking away during or after the attack (i.e., aphid escaping); (C) the proportion of aphids emitting conical secretions, and (D) the rate of successful attack by *A. ervi* parasitoid females. Error bars correspond to standard errors. The asterisk mark shows significant differences between one symbiotic status and the cured lineage within each genotype (significance of the GLMMs parameters). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

symbiont strains and aphid genotypes. Whereas there was no difference in the mummification rate between aphids infected with Ri^{T2} and uninfected aphids, the proportion of mummified aphids was reduced in Ms1 infected with Ri^{T1} but not in Ms2 (Figure 2,

Table 4). Finally, whatever the aphid genotype, the symbiotic status of the mummified aphids did not affect the parasitoid emergence rate: neither the infection with the original symbiont nor the infection with new symbiont strains or species influenced the

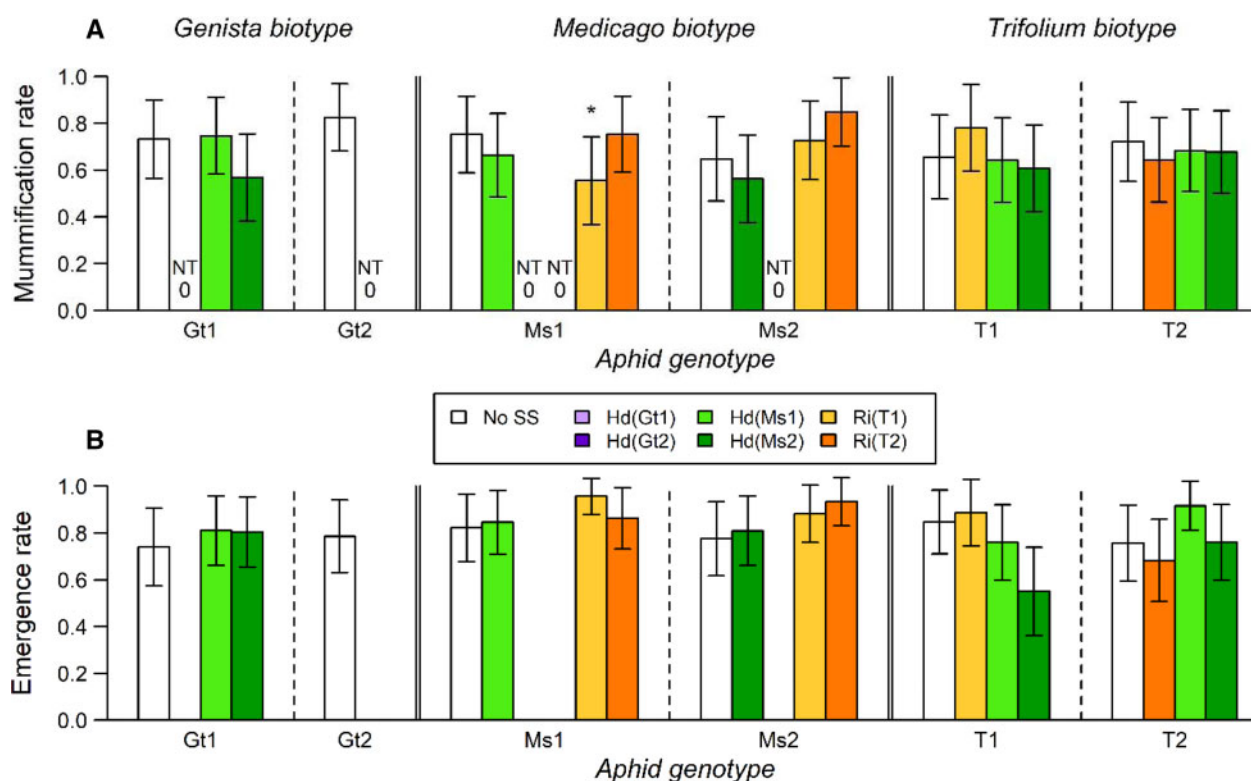


Figure 2 Effect of the symbiotic status of pea aphid individuals on both (A) the mummification rate after attack by *Aphidius ervi* parasitoid and (B) the parasitoid emergence rate. Error bars correspond to standard errors. The asterisk marks show significant differences between one symbiotic status and the cured lineage within each genotype (significance of the GLMMs parameters). NT: Not tested as no parasitism found; * $P < 0.05$.

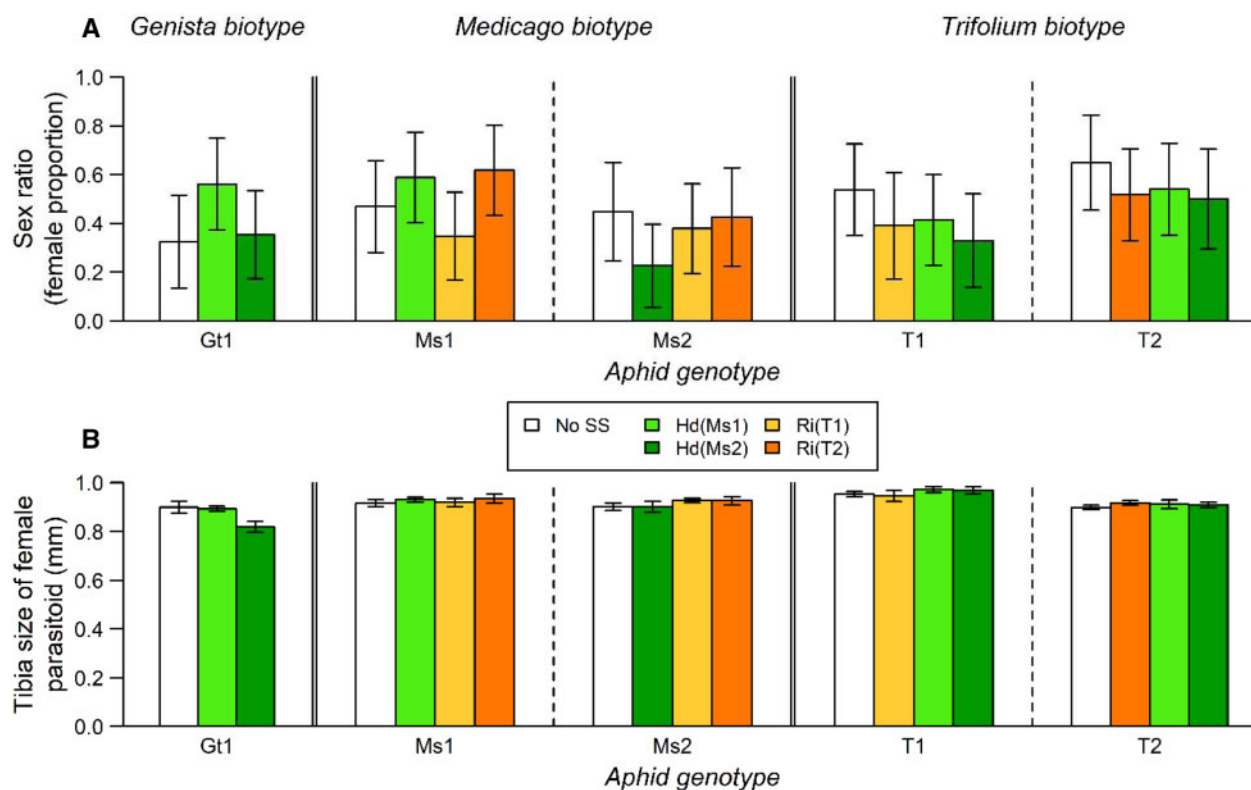


Figure 3 Effects of the symbiotic status of parasitized pea aphid individuals on life-history traits of emerging *Aphidius ervi* parasitoids: (A) sex-ratio (proportion of females) and (B) left tibia size. Error bars correspond to standard errors.

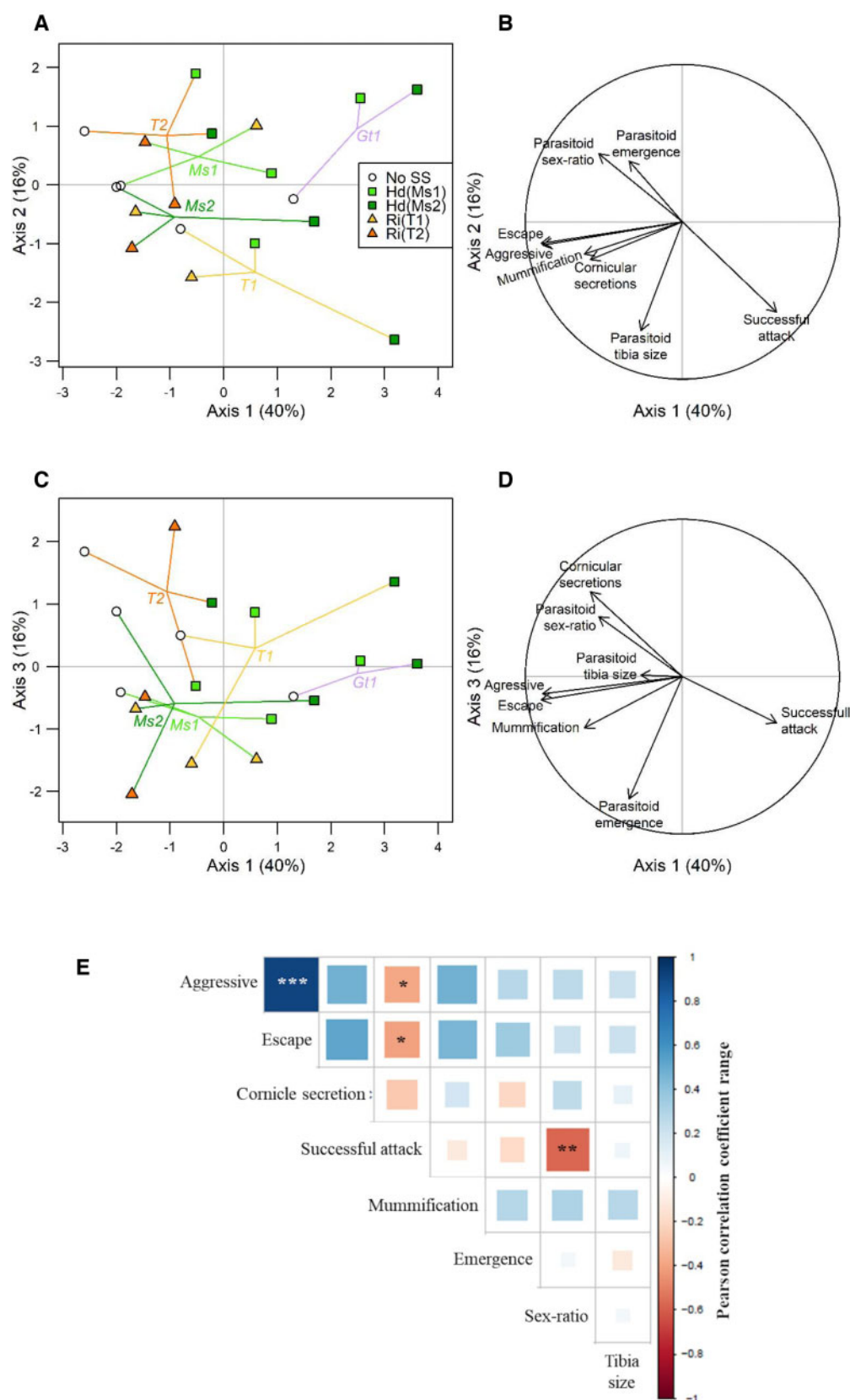


Figure 4 Principal component analysis (PCA) on the eight studied variables (see text). (A, C) On the left, score plots of the PCA showing the position of each lineage linked by their genotype for principal components 1 and 2 (A) and principal components 1 and 3 (C). (B, D) On the right, loading plots (circles of the variables) of the PCA for principal components 1 and 2 (B) and principal components 1 and 3 (D). Symbols correspond to different symbiotic status. (E) Pearson correlation coefficients matrix for the eight studied variables.

exit of the parasitoid offspring from the aphid host (Figure 2, Table 4).

Effects of symbionts on parasitoid life-history traits

As aphids infected with *H. defensa* from the *Genista* biotype were 100% resistant against *A. ervi*, all lineages carrying Hd^{Gt1} or Hd^{Gt2} were excluded from all data analyses. For the remaining lineages, the life-history traits of the emerging parasitoids did not vary according to the aphid symbiotic status, regardless of the aphid genotype. Neither the infection with the original symbiont nor the infection with new symbiont strains or species influenced the sex-ratio and tibia size of emerging parasitoids (Figure 3, Table 4).

Global analysis

The three first axes of the PCA represented about 72% of the multivariate data variation (39.5%, 16.1%, and 15.8% for principal component 1, 2, and 3, respectively). The score plots (Figure 4A and C) confirmed visually the effects of *H. defensa* on aphid defenses: aphid lineages infected with this symbiont are separated from the others along the first principal component (i.e., from the right to the left). For a given aphid genotype, the symbiotic status of aphids induced differentiation in the “phenotypic space”. For a given secondary symbiont, the dots are not grouped. So, the symbiont-mediated phenotypes depended on the host genotype and no global effects of symbiont on the aphid–parasitoid interactions was found. Furthermore, both PCA loading plots (i.e., correlation circles—Figure 4B and D) and correlation matrix associated with the PCA (Figure 4E) highlighted only a few significant correlations between variables measured. A negative correlation between the aphid behavioral defenses and the successful attack rate was found (i.e., the more the aphids defended themselves, the less the parasitic wasps attacked successfully). The sex-ratio of the emerging parasitoids was negatively correlated to the rate of successful attack (i.e., the more the wasps attacked successfully, the fewer daughters they produced—see Hardy (1992) for a review on evidence for sequence of sex allocation in parasitoids). A positive correlation between aphid aggressiveness and escaping behaviors was found. Apart from the latter, the significant correlations did not involve pair of symbiont-mediated phenotypes suggesting no association or trade-off between symbiotic effects on aphid–parasitoid interactions.

Discussion

How microbial symbionts influence animal protection against natural enemies is an important open question in ecology. Here, we tested whether protective bacterial symbionts associated with aphids influence the different steps of a host–parasitoid interaction. While we found symbiont effects on the ability of aphids to avoid and resist to parasitoid with a large variation depending on symbiont genotypes and host–symbiont associations, these symbiotic effects were not related (i.e., no trade-off between defensive phenotypes). Also, we did not detect symbiotic effects on parasitoid attack success, parasitism emergence from parasitized aphids and parasitoid offspring life-history traits.

Variation of symbiont-mediated phenotypes associated with parasitism

Consistent with previous studies, we found that aphids harboring *H. defensa* presented reduced defensive behaviors with less aggressive movements and escaping responses (Dion et al. 2011; Polin et al.

2014). However, we showed that symbiont strains varied in their effects on aphid behaviors with the two *H. defensa* strains from the *Medicago* biotype reducing all considered behavioral defenses whereas the two *H. defensa* strains from the *Genista* biotype reducing aphid aggressiveness only. Additionally, we found no effect of *R. insecticola* infection on aphid defensive behaviors, a result in line with another study on lineages of the cereal aphid *Sitobion avenae* infected or not with *R. insecticola* and exposed to a predatory ladybird (Ramírez-Cáceres et al. 2019). Considering that the magnitude of the symbiotic effect on both aphid defensive behaviors was low (i.e., reduction of 10–20% of behavioral expression) and that the impact on parasitoid attack was limited (i.e., weak correlation between aphid behavioral defenses and successful parasitoid attack rate), the relevance of such symbiotic effects in nature is questionable. The present results highlighted however that the effects of symbionts on defensive behaviors depended on symbiont strains and were not specifically associated with resistance to parasitism.

When aphids were attacked by *A. ervi*, the different strains of *H. defensa* provided various levels of protection against the parasitism whereas *R. insecticola* did not protect aphids, except in one case (Ri^{T1} introduced in Ms1). The strains of *H. defensa* from the *Genista* biotype precluded the development of the parasitoid while the *H. defensa* strains from the *Medicago* biotype, despite harboring the APSE phage (Leclair et al. 2016), provided no protection against *A. ervi* parasitism. The reasons for this protection variation have been already proposed in previous papers and involve aphid, parasitoid, symbiont and phage genotypes, and their interactions (Oliver et al. 2005; Oliver et al. 2009; Leclair et al. 2016; Oliver and Higashi 2019). Concerning *R. insecticola*, our results confirm previous studies on the pea aphid showing little or no influence of this symbiont on parasitism resistance (Oliver et al. 2003; Hansen et al. 2012). The only exception was when the *R. insecticola* strain from T1 was introduced in Ms1. This reduction in parasitism rate could be an indirect effect of the symbiont cost in this specific aphid lineage. In another study, *A. fabae* infected with *Serratia symbiotica* showed a reduced parasitism rate (Pons et al. 2019), which may result from size reduction of infected aphids and parasitoid preference for larger hosts. In addition, since *R. insecticola* can confer protection against parasitoids in other aphid species such as *Myzus persicae* (Vorbürger et al. 2010), we cannot exclude that some *R. insecticola* strains originating from other *A. pisum* hosts can protect pea aphids against *A. ervi* or other parasitoid species (Vorbürger et al. 2010; Asplen et al. 2014; McLean and Godfray 2015).

Like previous studies (e.g., Łukasik et al. 2013, 2015; Luo et al. 2017), our results showed that the symbiotic status of the aphids did not influence the emergence rate of parasitoids. However, bacterial symbionts could impose sublethal effects on parasitoids emerging from infected aphid hosts, such as a reduction of parasitoid body mass (Nyabuga et al. 2010; Schmid et al. 2012; Luo et al. 2017; Pons et al. 2019), a lower emergence rate (Schmid et al. 2012; Pons et al. 2019) or a delayed development (Schmid et al. 2012). Here, we found no effect of symbiont infection on the measured traits on emerging parasitoids. It has been hypothesized that sublethal effects induced by *H. defensa* are due to the toxins produced by the APSE phage of the bacteria (Oliver et al. 2009), which would affect parasitoid fitness traits when the toxin does not kill it (Monticelli et al. 2019). As these toxins differ from one APSE variant to another, we can hypothesize that in our study, the APSE phage of the *H. defensa* from the *Medicago* biotype does not produce the toxins that are effective or sufficiently harmful to *A. ervi*. Concerning the possible influence of aphid symbionts on parasitoid sex-ratio, Monticelli et al.

(2019) hypothesized that parasitoids could discriminate infected aphids as low quality hosts and choose to oviposit more male eggs, what would result in male-biased offspring. However, our results along with those of Nyabuga et al. (2010) on a wide range of pea aphid–symbiont associations did not show sex ratio bias associated with protective and non-protective symbionts.

Do symbionts induce defensive syndromes in their host?

Our results highlighted the influence of symbionts on the ability of aphids to avoid and resist to parasitoid infection and revealed specific patterns in symbiont-mediated phenotypes. The two strains of *R. insecticola* had a very limited influence on the aphid–parasitoid interaction. The two *H. defensa* strains from the *Genista* biotype decreased slightly aphid defensive behaviors, by affecting aggressiveness only, but protected completely their host against parasitoids. Finally, the two *H. defensa* strains from the *Medicago* biotype had a stronger impact on defensive behaviors but did not protect their host nor influence parasitoid traits. Considering aphids infected with *H. defensa*, both the univariate and multivariate analyses showed no association between symbiont-mediated phenotypes as the reduction of aphid defensive behaviors was not related to the level of resistance conferred by the secondary symbionts. However, in our study we could compare only a few associations and additional lineages should be examined to have a broader insight into correlations between symbiont effects at the different steps of aphid–parasitoid interactions. In Dion et al. (2011), we first reported that bacterial symbionts could reduce the defensive behaviors of their hosts and hypothesized that this reduction could be either an adaptive response to the parasitism resistance conferred by the symbiont or a cost imposed by the symbiosis. By showing an absence of link between symbiont effects on defensive behaviors and parasitism rate, our study suggests that the reduction of aphid behaviors is a by-product of the infection. Furthermore, if adaptive, the reduction of defensive behaviors should be counter-selected as it would leave aphids defenseless against other natural enemies like predators (Polin et al. 2014) or other parasitoid genotypes or species against which the symbiont is not effective (Vorburger et al. 2010; Asplen et al. 2014; McLean and Godfray 2015). Field studies are needed to test whether the reduction of defensive behaviors due to *H. defensa* infection has any consequences on aphid individuals (i.e. exposure to other natural enemies).

When are “protective” symbionts protective?

Considering our results and other studies on symbiont-mediated protection against parasitoids, we would like to discuss here what is required to qualify a symbiont as protective. The reduction of defensive behaviors caused by the infection with several symbiont species turns out more harmful than beneficial to the host, because they are more vulnerable to predators or parasitoids against which they are not protected (Dion et al. 2011; Polin et al. 2014; this study). There is also no evidence so far of any symbiont able to enhance the defensive behaviors of its host. Concerning the resistance of host against the parasitism, studies show that many symbiont species are able to protect their host against different parasitoid species (Oliver et al. 2003; Vorburger et al. 2010; Cayetano and Vorburger 2015; Donald et al. 2016; Leclair et al. 2016; Jamin and Vorburger 2019), but for the same symbiont species, the level of protection may differ greatly from one strain to another (Oliver et al. 2005; Leclair et al. 2016) and depends on the parasitoid species or genotype (Schmid

et al. 2012; Cayetano and Vorburger 2015). We also know that environmental parameters can also influence this protection, such as temperature (Heyworth and Ferrari 2016; Doremus et al. 2018) or the plant used by the host (Sochard et al. 2019). Symbionts may also protect aphids indirectly by reducing plant volatiles that are attractive for parasitoids (Frago et al. 2017). Also, symbiont-mediated protection generally induces fitness costs to their hosts, whether constitutive or induced (Vorburger et al. 2013; Leclair et al. 2016; Sochard et al. 2019), which can go to reproduction arrest in the case of the *Genista* biotype infected with *H. defensa*. Certain symbionts have also been found to affect parasitoid life-history traits once emerging from infected hosts (Nyabuga et al. 2010; Schmid et al. 2012; Pons et al. 2019). Although these sublethal effects of symbionts do not provide a direct benefit to the host as it dies at the end, they would have a negative impact on parasitoids at both individual and population scales (Monticelli et al. 2019). This large variation in symbiont-mediated types and levels of protection leads to a better definition of what a protective symbiont is. From the host individual scale, a protective symbiont would be a microbial partner that limits the development of the enemy without reducing its host's fitness drastically. Considering this definition, protective symbioses would be not so frequent and would be not always displayed by a symbiont species as a whole.

In conclusion, we showed that symbionts may influence different steps of the host–parasitoid interactions, highlighting the need to consider the whole sequence of the process to better assess the outcomes of protective symbiosis and understand the dynamics of host–symbionts in natural populations. These different effects, alone or in combination, as well as their specificity in terms of host–symbiont associations, may be added to the factors explaining why protective symbionts are only found in intermediate frequencies in host populations. Finally, our study underlines the need for a better definition of the notion of protective symbioses.

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Author contributions

All authors conceived and designed the experiments. C.S. and L.B. conducted the experiments. C.S. and Y. O. analyzed the data. Y. O. and J.-C.S. contributed to the supervision of this study. All authors contributed critically to the drafts and gave final approval for publication.

Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

The data underlying this article are available in Zenodo at <https://zenodo.org/record/4028402>.

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