



The aphid facultative symbiont *Serratia symbiotica* influences the foraging behaviors and the life-history traits of the parasitoid *Aphidius ervi*

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With 5 figures and 1 table

Abstract: Aphids (Hemiptera: Aphididae) maintain intimate relationships with a variety of symbiotic bacteria that can be important drivers of their evolutionary ecology. In addition to the obligate endosymbiont *Buchnera aphidicola*, aphids may harbor a series of facultative symbionts that can affect their physiology, as they may be involved in heat resistance, nutrition, reproduction and defense against parasitoids. Since the presence of facultative symbionts in aphids can be disadvantageous for parasitoids, it is hypothesized that these insects have developed strategies to adjust their responses to the presence of these bacteria. In this study, experiments were conducted to determine whether the presence of the facultative symbiont *Serratia symbiotica* in the pea aphid *Acyrtosiphum pisum* affects the development and the life-history traits of the generalist parasitoid *Aphidius ervi* (Hymenoptera: Braconidae). Behavioral assays were also performed to determine whether the infection status of host aphids influences the foraging behavior of the parasitoids. It was showed that the presence of *S. symbiotica* had negative effects on the development and the life-history traits of emerging parasitoids. It was found that parasitoids preferentially orient themselves to uninfected aphid hosts, probably through chemical clues, and that they adjust their foraging behavior by investing more time in patches composed of uninfected aphid hosts. In light of these results, it is assumed that the presence of *S. symbiotica* alters host aphid quality, which in turn influences the choice of the female parasitoids for oviposition as well as the quality of the emerging parasitoids. This study highlights the ability of parasitoid wasps to modify their perception and behavior towards aphids infected with facultative symbionts and how these microorganisms interfere with host-parasite relationships.

Keywords: Aphid-parasitoid, fitness costs, foraging, resistance, *Serratia symbiotica*, symbiont

1 Introduction

Insects can maintain various relationships with endosymbiotic bacteria that can greatly affect their ecology and evolution (McFall-Ngai et al. 2013; Fisher et al. 2017). As sources of metabolic innovations, some of these symbiotic bacteria

have become essential for the host survival and reproduction (Douglas, 1998; Zientz et al. 2004; Hosokawa et al. 2010), while others are facultative because they deliver fitness benefits only in the context of specific ecological conditions (Oliver et al. 2010; Feldhaar 2011). There are a growing number of examples showing that insect-associated bacte-

ria can play a significant role in the structure and dynamic of insect-based food webs, particularly by interfering with prey-predator and host-parasite interactions (Costopoulos et al. 2014; Gerardo & Parker 2014; Leroy et al. 2011; Oliver & Martinez 2014; McLean et al. 2016; Brandt et al. 2017; Hafer-Hahmann & Vorburger 2020). However, despite evidence that facultative symbionts may interfere with these biotic interactions, it is still unclear how their presence affects the behaviour of predators or parasites toward their prey or hosts.

Aphids are remarkable models for addressing this issue, particularly in the context of host-parasite relationships, as they are parasitized by many species of parasitoid wasps and can harbour a wide range of facultative symbionts in addition to their ancient obligate nutritional partner *Buchnera aphidicola* (Douglas, 1998; Oliver et al. 2010; Russell et al. 2017). Phenotypic effects associated with facultative symbionts include resistance to heat stress (Montllor et al. 2002; Burke et al. 2010), body color modification (Tsuchida et al. 2010), host plant exploitation (Wagner et al. 2015), manipulation of host reproduction (Simon et al. 2011) and protection against parasitoid wasps and entomopathogenic fungi (Oliver et al. 2003; Łukasik et al. 2013b; Brandt et al. 2017). Since facultative symbionts can influence the expression of their host phenotype, they may play a key role in aphid evolution and diversification (Oliver et al. 2010; Feldhaar 2011). However, while facultative symbionts may be associated with a selective advantage under specific stressful conditions (e.g. thermal stress and parasitic pressure), many reported cases show that hosting symbionts often results in fitness costs, including increased development time of infected aphids, reduced longevity and fecundity (Oliver et al. 2008; Vorburger & Gouskov 2011; Polin et al. 2014; Zytynska & Weisser 2016; Skalcjac et al. 2018; Pons et al. 2019; Zytynska et al. 2021). Thus, facultative symbionts produce side effects that may affect the costs and benefits balance they confer to their host. The persistence and stability of bacterial facultative symbioses in aphid populations depends on the net outcome of the interaction between aphids and their symbionts (Leclair et al. 2016; Oliver & Higashi 2019; Zytynska et al. 2021).

Of all the symbiont-associated phenotypes described in aphids, protection against parasitoid wasps has probably been the most studied over the last 15 years. For instance, improved protection against parasitoids has been demonstrated in aphids hosting *Hamiltonella defensa* (Oliver et al. 2009, 2003), *Serratia symbiotica* and *Regiella insecticola* (Oliver et al. 2003; Vorburger et al. 2010; Pons et al. 2019). The mechanisms underlying this protection are not yet well understood and differ between symbiont species and strains (Oliver et al. 2014). In the case of *H. defensa* strains harboring APSE (*Acyrtosiphon pisum* secondary endosymbiont) bacteriophages in their genome, this protection is based on the production of phage-encoded toxins that hinder the development of parasitoid larvae (Oliver et al. 2009; Weldon et al. 2013; Brandt et al. 2017; Chevignon et al. 2018). However,

the APSE phage is not present in all aphid symbionts and it is not yet known how certain symbionts (e.g. *S. symbiotica* and *R. insecticola*) offer similar benefits. One hypothesis is that these protective effects may be due to the fitness costs associated with the presence of symbionts that make the host less suitable to a proper development of parasitoid larvae (Pons et al. 2019). These studies suggest that facultative symbionts could interfere with the larval development of associated parasitoids and that these bacteria are an important source of heritable variations under selective pressures (Oliver et al. 2008, 2012; Polin et al. 2015; Sanders et al. 2016; Hafer-Hahmann & Vorburger 2020). In addition to these developmental aspects, very little is known about the impact of facultative symbionts on parasitoid biology and foraging behavior of females.

The choice of an aphid host by a female parasitoid is the result of a trade-off between maximizing fitness gain per egg laid and the time spent searching (Mackauer et al., 1996). Laying eggs in aphids infected by facultative symbionts can be costly for the parasitoids due to possible subsequent defective development of the offspring. The infection status of the host determined by the presence of symbiotic bacteria can therefore be an important indicator of host quality for the parasitoids. Female parasitoids thus should benefit from behavioral strategies to discriminate aphid hosts according to their infection status. Very few studies have been conducted to examine the foraging behaviors of parasitoid wasps as a function of the presence of facultative symbionts in aphid hosts. Oliver et al. (2012) showed that *Aphidius ervi* tends to superparasitize aphids infected with *H. defensa* to overcome protective effects and is able to distinguish infected from uninfected aphids based on the quantity of (E)- β -Farnesene (EBF) emitted by these insects (Oliver et al. 2012). Łukasik et al. (2013) showed that *A. ervi* and *Ephedrus plagiator* oviposited preferentially on uninfected hosts when given the choice between genetically identical individuals with or without *H. defensa* (Łukasik et al. 2013a). These studies suggest that parasitoid wasps adjust their oviposition behavior to the presence of the defensive symbiont *H. defensa* in aphid hosts. However, these studies focused on one symbiont species known to induce direct protection against parasitoids through phage toxins, but there are no data regarding the response of parasitoids to the presence of other facultative symbionts frequently associated with aphids.

In this study, we investigated the effect of *S. symbiotica*, one of the facultative symbionts most commonly associated with aphids, on the entire sequence of the aphid (here *A. pisum*)-parasitoid (here *A. ervi*) interaction (i.e., from the foraging behavior of the parasitoid to the consequences on the survival of its offspring). We hypothesized that (i) aphid clones, depending on the presence of the facultative symbiont, represent two different host resources for female parasitoids, with consequences on the development and survival of parasitoid larvae, (ii) female parasitoids perceive the infection status of the encountered hosts through chemical signa-

ture, and (iii) they adjust their foraging behavior accordingly by avoiding aphids harbouring the facultative symbiont and by leaving patches composed of such hosts more quickly. The strain of *S. symbiotica* that was used in this study has already shown an ability to confer moderate protection against parasitoids (Oliver et al. 2006, 2003). To test these hypotheses, the impact of the presence of *S. symbiotica* in *A. pisum* on the biology of the generalist parasitoid *A. ervi* was first assessed. It was then examined whether the odours emitted by the aphids are used by the parasitoid to discriminate the infection status. Finally, a series of experiments were conducted to determine how the parasitoid adjusts its foraging behavior (oviposition behaviour and patch time allocation) according to the presence of *S. symbiotica* in *A. pisum*.

2 Materials and methods

2.1 Insect rearing

All experiments were performed with pea aphid *A. pisum* clones provided by Dr. Nancy Moran (University of Texas, Austin, TX, USA). The clone Tucson was originally collected in 1999 from *Vicia faba* in Tucson (Arizona, 1999) and is naturally infected with a *S. symbiotica* strain associated with heat-stress resistance and with some protection against parasitoids, as described by (Oliver et al. 2003; Burke et al. 2010; Oliver et al. 2006). The uninfected Tucson sub-colony was established in 2005 from the Tucson pea aphid through curing of *S. symbiotica* by a heat-shock treatment (Burke et al. 2010). The infection status of both subclones was verified using diagnostic PCR (see (Renoz et al. 2015) for a full description of the method). Aphids were reared on *V. faba* seedlings at 20°C with a photoperiod of 16L: 8D to ensure parthenogenetic reproduction.

A large sample (about 300 mummies) of *A. ervi* (Hymenoptera: Braconidae) was supplied by Viridaxis S.A. (Belgium – <https://www.viridaxis.com>). *A. ervi* was reared on synchronized third-instar of a *M. persicae* clone uninfected with facultative symbionts to avoid adaptation of parasitoids to *A. pisum* prior to the experiments. The larvae were maintained on bean plants (*V. fabae*) in climate chambers at 20 ± 1°C, 60 ± 10% RH with a photoperiod 16L: 8D. *A. ervi* was reared for three generations before starting the experiments.

2.2 Does infection status influence the development and the life-history traits of emerging parasitoids?

To test the hypothesis that aphid clones that differ only in the presence of *S. symbiotica* represent different resources for the female parasitoids, the consequences of the infection status of aphids on the development and the quality of the parasitoids were determined. Prior to the experiments the 24-h-old mated females were subjected to oviposition experience by exposing them to five uninfected third-instar *Myzus persi-*

cae aphids in a Petri dish (diameter 5 cm). Any parasitoid that did not oviposit in an aphid within 5 min after introduction into the Petri dish was excluded from the experiment. Females that had an oviposition experience were then individually randomly assigned to patches composed of either infected or uninfected *A. pisum* aphids. The patches were composed of ten third-instar *A. pisum* aphids (infected with *S. symbiotica* or uninfected) placed on a bean leaf that were exposed to a female parasitoid during a 20-min session. The attack behavior of parasitoids (number of ovipositor insertions) was recorded using a video camera on a light table. The aphids were then reared on *V. faba* plants in transparent plastic cylinders (pierced with small holes to avoid condensation and sealed with tulle) under controlled conditions (20 ± 1°C, 60 ± 10% RH, under a 16L: 8D photoperiod). They were inspected daily to measure the development time of the mummies, estimated by the time between attack of aphids by a parasitoid and the formation of the mummies, as well as the parasitism rate of aphids, estimated by dividing the total number of mummified aphids (i.e., dead aphids containing a developing parasitoid) by the total number of attacked aphids (Oliver et al. 2012; Leclair et al. 2016). The mummies were then inspected daily to measure the emergence rate of parasitoids, estimated by dividing the number of parasitoids emerging from the mummies by the total number of mummies. Parasitoid emergence was monitored daily to determine the sex of emerging parasitoids and their survival rate. Once dead, the parasitoids were collected in 1.5 mL Eppendorf tubes, then frozen (−20°C) and the egg load of the females was subsequently determined (only mature lemon-shaped eggs were counted to avoid confusion with the fatty structures present in the abdomen). 30 experimental replicates both for infected and uninfected aphids (i.e., 300 aphid individuals per condition) were performed. 30 female parasitoids were tested for both infected and uninfected aphids (i.e., 300 aphid individuals per condition).

2.3 Can female parasitoids discriminate infection status based on the odors emitted by the aphids?

To test the hypothesis that parasitoid females perceive aphid infection status through chemical signatures, an olfactory assay was conducted. Specifically, an all-glass Y-olfactometer (branch length: 10 cm; chamber diameter: 4 cm diameter) was used to test the orientation responses of *A. ervi* females towards the aphids as a function of their infection status. The two arms were connected to two water-containing Erlenmeyer flasks, which in turn were connected to a device ensuring a constant pressure of 2.5 bars of humid, clean air flow (133 mL/min). The olfactometer was placed on a light table to ensure uniformity of light. Prior to the experiments, the absence of positional bias for a specific branch of the olfactometer was verified by testing the preference of 30 parasitoids individually exposed to a clean air flow from both branches (without aphids). No bias was observed on

either side of the olfactometer. For the experiments, twenty third-instar aphids were put on two leaves discs (10 uninfected or 10 infected by *S. symbiotica* per leaf disc) and placed in the two peripheral chambers of the olfactometer. The aphids (infected or not) were randomly assigned to each chamber. The experiments began when a female *A. ervi* (aged between 24–48-h-old, naïve, fed and mated) was introduced into the central chamber. The experiment ended when the parasitoid entered one of the two peripheral chambers or after 20 min of observation. The test was repeated 30 times. Between each replication, the olfactometer was thoroughly cleaned with a detergent solution (RBS T105) and finally thoroughly rinsed to remove potential odor residues adsorbed on the glass and dried with a hair dryer.

To test the hypothesis that the presence of *S. symbiotica* in aphids influences the amount of EBF released, five aphids (third-instar; infected with *S. symbiotica* or uninfected) were first placed in 20 mL glass tubes and then crushed (Fischer & Lognay 2012). EBF was extracted for 2 hours with 200 μ l n-hexane containing 1.64 μ g (β)-caryophyllene (ABCR GmbH – Germany) as internal standard. The extracts were analyzed by gas chromatography with flame ionization detection at 290°C. Aliquots (1 μ l) were injected on a splitless injector maintained at 270°C (Splitless time of 1 minute). The column (Optima 5MS 30m $\times$ 0.25 mm, df = 0.25 μ m from Macherey Nagel-Düren – Germany) was programmed as follows: from 40°C (2 min) to 280°C at a constant rate of 10°C/min with a final hold of 5 min at 280°C. The quantification of EBF was performed by comparison of peak areas with that of the internal standard using a response factor of 1. Helium at a constant flow rate of 1 ml/min was used as the mobile phase. Each analysis was repeated on 8 separate batches of 5 aphids of the same age (third-instar individuals). EBF was identified by comparison of the recorded mass spectra with the Wiley and NIST data bases using a 5973 Mass detector (Agilent Technologies, Santa Clara CA, USA) coupled with an Agilent 6890 Gas chromatograph. The identification was finally corroborated by injection of pure standard EBF and comparison of retention data.

2.4 Does the aphid infection status influence the foraging behavior of *A. ervi*?

To test the hypothesis that female parasitoids adjust their behavior according to the presence of *S. symbiotica* in *A. pisum*, ten third-instar aphids (infected or uninfected) were placed on a bean leaf, and exposed to a fed and mated 24-h-old female parasitoid. Prior to the experiment, females were given oviposition experience by exposing them to five uninfected third-instar *M. persicae* in a Petri dish. Any parasitoid that did not oviposit in an aphid within 5 min of introduction in the Petri dish was discarded. During the experiment, the behavior of each tested female visiting a patch containing either 10 uninfected *A. pisum* aphids or 10 infected *A. pisum* aphids was observed. A piece of bean leaf (16 $\times$ 16 mm) was

placed in the middle of a glass Petri dish (15-cm diameter) and surrounded by a red circle (9-cm diameter), indicating the border of the patch. The behavior of each female parasitoid (deposited in the center of the patch) was recorded from the moment it entered a patch to the moment it left it, using a video camera on a light table. During each 60-min session, the following behaviors were recorded: entering the patch, leaving the patch, antennal contact (detection behavior), ovipositor contact, walking and resting. The patch residence time was defined as the cumulative time spent on the patch (containing infected or uninfected aphids) before leaving it for more than 60 seconds (Muratori et al. 2008; Wajnberg et al., 1999). At the end of each session, the tested aphids were deposited on bean plants inside transparent plastic cylinders under controlled climatic conditions (20 $\pm$ 1°C, 60 $\pm$ 10% RH, under a 16L: 8D photoperiod). Surviving aphids were dissected under a binocular microscope three days after the attack to detect the presence of parasitoid larvae and to count the number of larvae inside each aphid (used as an indicator of superparasitism). The behavior of each tested female visiting a patch containing either 10 uninfected aphids (N = 30) or 10 infected aphids (N = 24) was recorded. The behavior of 30 female parasitoids each visiting a patch containing 10 uninfected aphids and 24 parasitoids each visiting a patch containing 10 infected aphids was recorded.

3 Statistical analyses

Analyses of parasitism rate, emergence rate, as well as the proportion of emerging parasitoid females were performed by fitting generalized linear mixed models assuming a binomial error structure and a logit-link function, testing the infection status as a fixed factor. Egg load in emerging parasitoid females was analyzed by fitting a generalized linear mixed model by assuming a Poisson error structure and a log-link function, after testing for overdispersion using the DHARMA nonparametric dispersion test via standard deviation of residuals fitted versus stimulated residuals (dispersion=0.21, p=0.99) (Hartig 2019). The development time of mummies was analyzed using a generalized linear mixed model assuming a Gamma error structure and an inverse-link function. In these statistical models, the infection status was considered as a fixed factor. Moreover, as one female parasitoid attacked several aphid individuals, the parasitoid individual was considered a random factor. The sex of emerging parasitoids and its interaction with the infection status were included in the models to analyze the parasitism rate, the emergence rate, as well as the development time of mummies. The survival rate of emerging parasitoids was analyzed with a proportional hazards regression (Cox, 1972) and visualized by computing the Kaplan-Meier survival functions for the different infections and sex of emerging parasitoids (Kalbfleisch & Prentice 2002). To test whether a female par-

asitoid can discriminate the infection status of its aphid host, an exact binomial test was used to determine parasitoid preference for aphids according to their infection status, with the null hypothesis of no directional preference within the olfactometer. The effect of the infection status on the amount of EBF by aphids was then further tested using a general linear model, after checking the normality of the data. The number of antennal contacts, ovipositor contacts and parasitoid larvae found per aphid were analyzed by fitting linear mixed-effect models with the patch type (containing aphids infected with *S. symbiotica* or uninfected) as a fixed variable, after checking the normality of the residuals. The parasitoid individual was considered a random factor because one female parasitoid attacked several aphid individuals. Patch-leaving mechanisms of parasitoid females visiting patches containing aphids infected with *S. symbiotica* or uninfected were analyzed by a Cox proportional hazard model (Cox 1972; van Alphen et al. 2003; Lou  pre et al. 2010). This model assumes that the tendency of females to leave a given patch is the product of a basic tendency to leave it and an exponential factor (the hazard ratio), representing the effect β_i of all the covariates z_i included in the model. A hazard ratio >1 indicates that the covariate increases the tendency to leave a patch, while a hazard ratio <1 indicate that it decreases the tendency to leave the patch. The infection status of each attacked aphid was included as a fixed covariate, and antennal contact, ovipositor contact, cleaning and resting, as time-dependent covariates. All statistical analyses were performed using the software R (v3.0.1; R Development Core Team 2014) and the *lme4* package for linear mixed models, the *survival* package for survival analyses, the DHARMA package to test overdispersion, and the *Grapher* package for graphics.

4 Results

4.1 Aphid infection status affects the development of emerging parasitoids

The presence of *S. symbiotica* had a significant negative effect on the development time of mummies (GLMM, $F_{1, 246} = 28.35$, $p < 0.001$). Development time was reduced by about one day in aphids infected with *S. symbiotica* (Fig. 1A). The sex of emerging parasitoids had also a significant effect on the development time of mummies (GLMM, $F_{1, 243} = 57.79$, $p < 0.001$): females emerge later than males. Specifically, females emerging from uninfected aphids emerge later, but males emerging from aphids infected with *S. symbiotica* emerge earlier (GLMM, $F_{1, 241} = 4.82$, $p < 0.05$). The presence of the symbiont did not affect the parasitism rate (i.e., the number of attacked aphids that became mummies) of *A. pisum* aphids after the attack by *A. ervi* parasitoids (GLMM, $\chi^2 = 1.78$, $df = 1$, $p = 0.075$; Fig. 1B). Moreover, the sex of emerging parasitoids did not significantly affect the parasitism rate (GLMM, $\chi^2 = -0.93$, $df = 1$, $p = 0.35$), but males emerging from symbiotic aphids had a lower parasitism rate than other conditions (GLMM, $\chi^2 = -1.98$, $df = 1$, $p = 0.048$). The infection with *S. symbiotica* and the sex of emerging parasitoids did not significantly affect the emergence rate of parasitoids (infection status: GLMM, $\chi^2 = 0.41$, $df = 1$, $p = 0.68$; Fig. 1C; sex: GLMM, $\chi^2 = -0.23$, $df = 1$, $p = 0.82$).

4.2 Aphid infection status influences the life-history traits of emerging parasitoids

It was found that the presence of *S. symbiotica* biased the sex ratio of emerging parasitoids towards males (GLMM, $\chi^2 = 7.54$, $df = 1$, $p = 0.006$). The proportion of females was reduced by more than 10 % when the parasitoids emerged

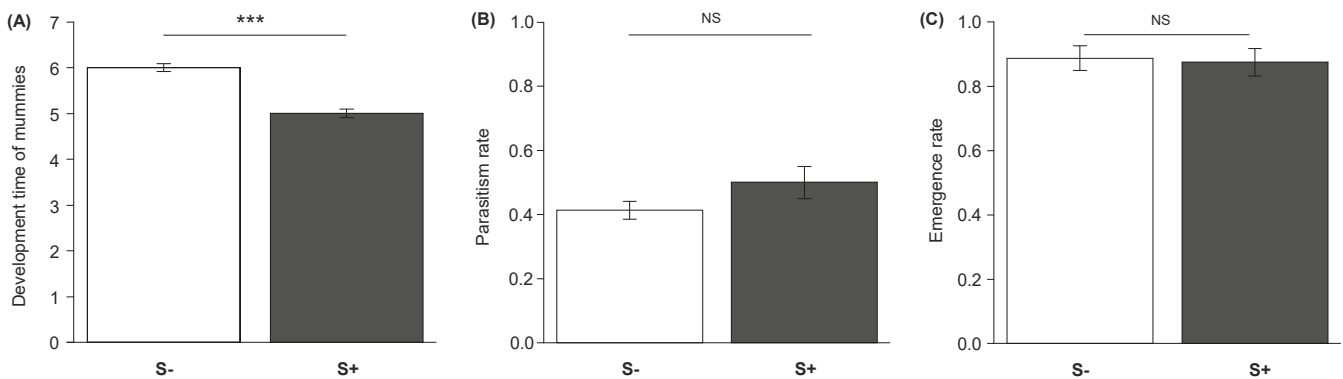


Fig. 1. Effect of *S. symbiotica* on the development time (days) of mummies (A), the parasitism rate of *A. pisum* following *A. ervi* attack (B) and the emergence rate of the parasitoids (C). The parasitism rate is the number of attacked aphids that became mummies, and the emergence rate is the number of mummies where the parasitoids have emerged. Two host conditions were used: no *S. symbiotica* infection (S-) and *S. symbiotica* infection (S+). Thirty experimental replicates were performed for each condition. Error bars depict the standard errors. Significant differences are shown (***, $P < 0.001$; N.S., no significant difference).

from aphids infected by the facultative symbiont (Fig. 2A). The presence of *S. symbiotica* had a significant negative effect on the egg load of emerging female parasitoids (GLMM, $F_{1, 136} = 533.89$, $p < 0.001$). The number of oocytes was almost halved in female parasitoids emerging from aphids infected with *S. symbiotica* (Fig. 2B). Aphid infection status also had a significant effect on the survival rate of emerging parasitoids (Cox's model, $\chi^2 = 180$, $df = 1$, $p < 0.001$; ($\beta = 2.26$, $\exp(\beta) = 9.59$, $SE(\beta) = 0.18$, $z = 12.79$)). A reduction of about 3 days was observed in the survival rate of parasitoids that emerged from infected aphids compared to those emerging from uninfected aphids (Fig. 2C). The sex of emerging parasitoids did not significantly affect the survival rate of emerging parasitoids (Cox's model, $\chi^2 = 2.16$, $df = 1$, $p = 0.14$; ($\beta = -0.23$, $\exp(\beta) = 0.8$, $SE(\beta) = 0.16$, $z = -0.53$)).

4.3 Female parasitoids are able to discriminate infection status based on the odors emitted by the aphids but aphid infection status does not influence the amount of EBF released

All females expressed a choice toward one of the two branches of the olfactometer: uninfected aphids attracted a significantly higher proportion of parasitoids than aphids infected with *S. symbiotica* (Exact binomial test, $p = 0.043$, Fig. 3A). Attraction (percentage of female parasitoid) was halved for aphids infected with the facultative symbiont. However, no significant difference in the amount of EBF released by aphids was found (LM, $F_{1, 14} = 0.33$, $df = 1$, $p = 0.58$, Fig. 3B), with 199 ± 67 (SD) μg EBF/g aphid

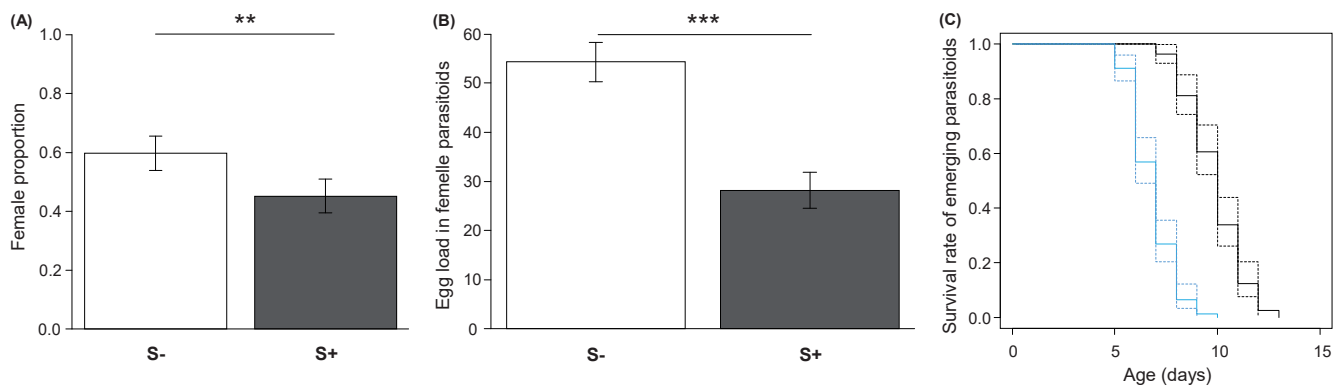


Fig. 2. Effect of *S. symbiotica* on the proportion of emerging parasitoid females (A), egg load of emerging parasitoid females (B) and survival rate of emerging parasitoids (C). Two host conditions were used: no *S. symbiotica* infection (S-) and *S. symbiotica* infection (S+). The black line corresponds to parasitoids emerging from aphids not infected with *S. symbiotica* and the blue line corresponds to parasitoids emerging from aphids infected with *S. symbiotica*. Thirty experimental replicates were performed for each condition. The upper and lower 95% confidence intervals are indicated by dotted lines. Error bars depict the standard errors. Significant differences are shown (**, $P < 0.001$; ***, $P < 0.001$).

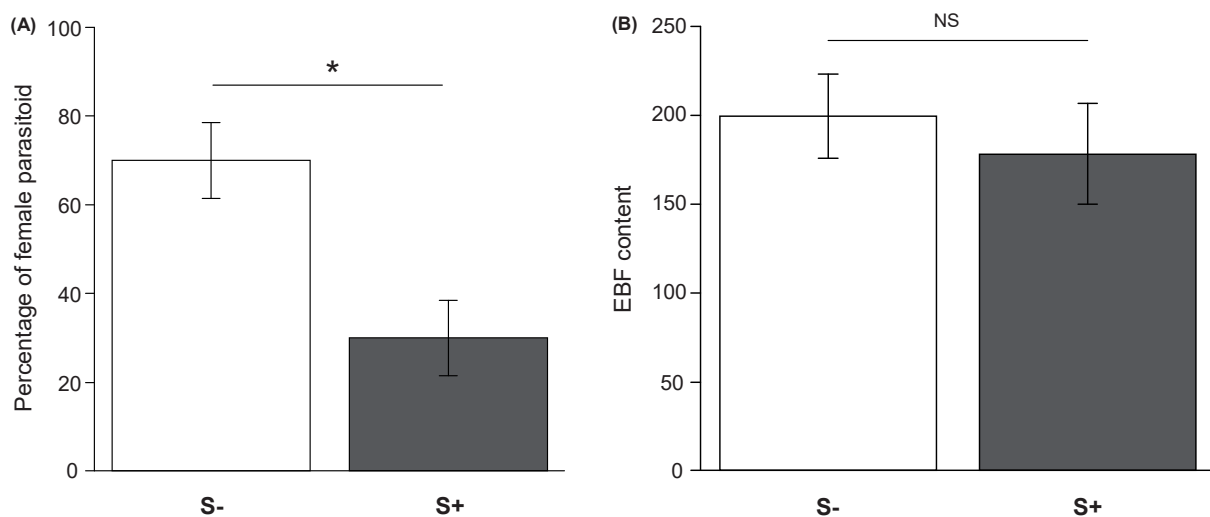


Fig. 3. Choice of female parasitoids between patches composed of uninfected (S-) ($n = 30$) and *S. symbiotica*-infected aphids (S+) ($n = 30$) (A). Amounts of EBF (μg) per gram of aphid: uninfected aphid (S-) ($n = 8$) versus infected with *S. symbiotica* (S+) ($n = 8$) (B). Error bars depict the standard errors (significant difference is shown (*, $P < 0.05$); N.S., no significant difference).

detected in uninfected aphids compared to 178 ± 80 (SD) μg EBF/g aphid in infected aphids (Fig. 3B).

4.4 The aphid infection status influences the foraging behavior of *A. ervi*

The number of antennal contacts per aphid (LME, $F_{1, 52} = 9.37$, $p = 0.004$, Fig. 4A), the number of ovipositor contacts per aphid (LME, $F_{1, 52} = 16.23$, $p < 0.001$, Fig. 4B), and the number of parasitoid larvae per aphid (LME, $F_{1, 52} = 47.4$, $p < 0.001$, Fig. 4C) were significantly reduced when the parasitoid *A. ervi* has been confronted with aphids infected with *S. symbiotica*. The number of antennal and ovipositor contacts per aphid was reduced by about 50% when the parasitoids were confronted with aphids infected with the facultative endosymbiont. About three times more larvae were found in uninfected aphids than in infected aphids.

Cox's regression model showed that the infection status of aphids significantly influenced the patch residence time of parasitoids (Cox's model, $\chi^2 = 2.61$, $\text{df} = 1$, $p = 0.009$, Fig. 5 and Table 1). The tendency to leave the patch increased when parasitoids foraged on patches containing aphids hosting *S. symbiotica*. In addition, the frequency of antennal contacts and resting behaviours exhibited by a parasitoid were sig-

nificantly linked to the tendency to leave the patch (antennal contacts: Cox's model, $\chi^2 = 3.12$, $\text{df} = 1$, $p = 0.0018$; resting behaviours: Cox's model, $\chi^2 = 3.66$, $\text{df} = 1$, $p < 0.001$; Table 1). Each ovipositor contacts, depended on the number of ovipositor contacts previously performed on an aphid, significantly decreased the tendency of parasitoids to leave a patch (Cox's model, $\chi^2 = -2.06$, $\text{df} = 1$, $p = 0.039$; Table 1).

5 Discussion

In this study, we assessed the effects of the facultative symbiont *S. symbiotica* on the host-parasitoid interaction sequence that extends from the foraging behavior of the parasitoid to the development and life-history traits of its offspring. We showed that the presence of *S. symbiotica* in *A. pisum* affected the development and fitness of the progeny of the parasitoid *A. ervi* and influenced its foraging strategies. Parasitism of aphids infected by the symbiont resulted in reduced development time of mummies, a male-biased sex ratio and a reduced survival rate of emerging parasitoids. At the behavioral level, we found that female parasitoids spent less time in patches composed of *S. symbiotica*-infected aphids. Taken together,

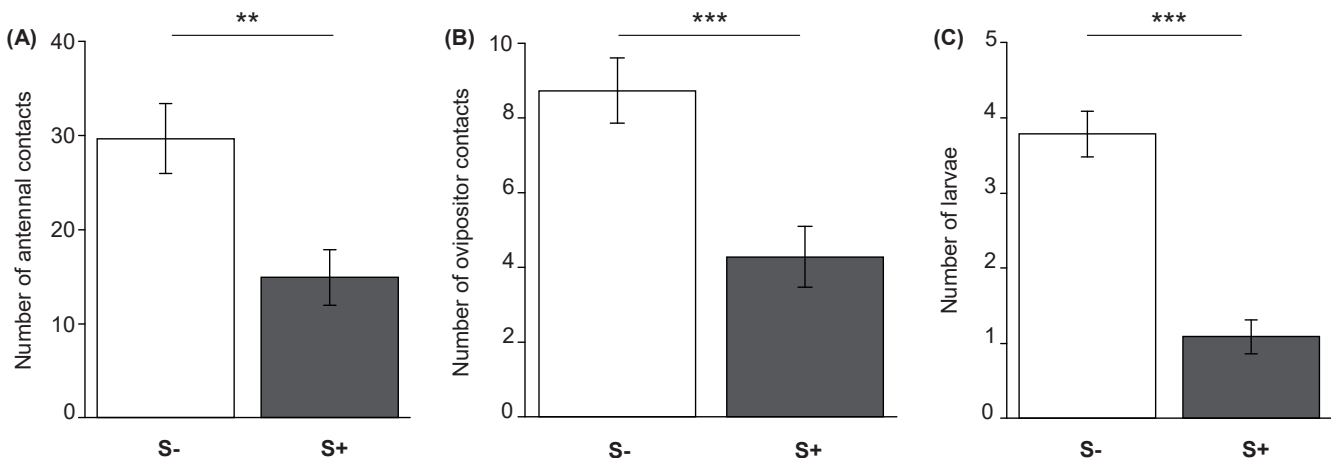


Fig. 4. Behavioral response of the female parasitoid *A. ervi* per aphid according to the aphid infection status (uninfected (S-) versus infected with *S. symbiotica* (S+)). Number of antennal contacts (A), number of ovipositor contacts (B), and number of larvae (used as an indicator of superparasitism) (C) of the parasitoid. Thirty and twenty-four experimental replicates were performed for the uninfected and infected condition, respectively. Error bars depict the standard errors. Significant differences are shown (**: $P < 0.001$; ***: $P < 0.001$).

Table 1. Different effects on the patch residence time of parasitoids. β , estimated regression coefficient of Cox's proportional hazard model; $\exp(\beta)$, hazard ratio; $\text{SE}(\beta)$, coefficient standard error; z , z-test value; P , significance of the coefficient.

	β	$\exp(\beta)$	$\text{SE}(\beta)$	z	P
Infection status	0.98	2.66	0.37	2.61	0.009
Antennal contacts	0.0063	1.01	0.002	3.12	0.0018
Resting behaviours	0.19	1.21	0.053	3.66	<0.001
Ovipositor contacts	-0.036	0.96	0.017	-2.064	0.039

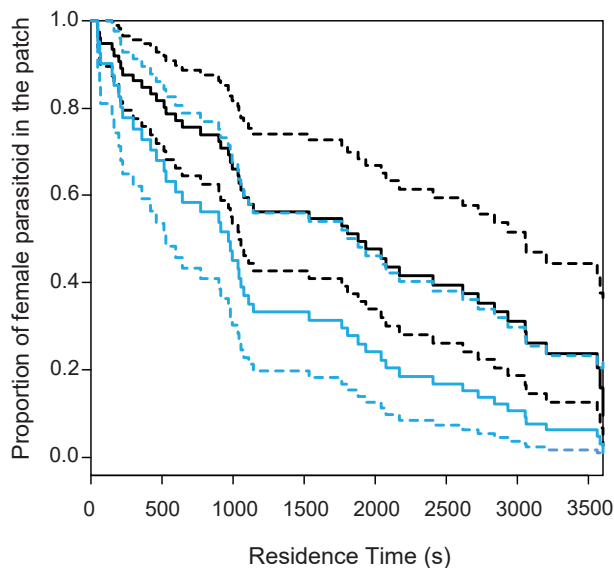


Fig. 5. Proportion of female parasitoids remaining inside the patches of aphids (uninfected versus infected with *S. symbiotica*) according to time. The black lines correspond to the parasitoids placed with aphids not infected with *S. symbiotica* and the blue lines correspond to the parasitoids placed with aphids infected with *S. symbiotica*. The upper and lower 95% confidence interval are indicated by dotted lines.

these results suggest that aphids infected with the facultative symbiont are less profitable for the parasitoids than those that are not infected. In the wild, aphids may be dispersed in the habitat or aggregated in patches and the behavioral sequence (search, location, evaluation, attack) leading to host parasitization depends on many factors, the first being the host quality (Hubbard & Cook, 1978). The value of a host can be assessed through factors such as the species, age, color and the parasitic status (Godfray & Godfray, 1994; Outreman et al. 2001; Boivin et al. 2004; Libbrecht et al. 2007; Henry et al. 2008, 2009; Oliver et al. 2012; Bilodeau et al. 2013; Cochard et al. 2019). It is now clearly established that host quality can also be influenced by associated facultative symbionts, as they can impact the physiology of infected insects (Monticelli et al. 2019; Oliver et al. 2010; Zytynska et al. 2021). Using the *A. pisum*-*S. symbiotica*-*A. ervi* system, our study confirm that bacterial endosymbionts can exert a profound influence on the foraging strategies of parasitoids within patches, with consequences on host exploitation and parasitoid life-history traits.

Our first objective was to determine whether the presence of *S. symbiotica* influences the development and the fitness of the emerging parasitoids by evaluating a series of indicators as proxies (including survival rate of emerging parasitoids, egg load and sex-ratio). In our study, we found that the infection status of aphids did not influence the rate of parasitism, although the strain of *S. symbiotica* we used has been reported to be associated with moderate protection against parasitoids (Oliver et al. 2006; 2003). These results could,

however, be a consequence of the experimental design used: the parasitism rate is low under both conditions because parasitoids may not have laid eggs in all aphids. As in previous studies (Łukasik et al. 2013a; Luo et al. 2017; Sochard et al. 2020), our results showed that infection status did not influence the emergence rate of parasitoids. However, they indicate that the presence of *S. symbiotica* in aphids alters the examined life-history traits of emerging parasitoids including the development time of the mummies, sex ratio, egg load and survival rate. We have thus shown that harboring *S. symbiotica* influences the physiological suitability of host aphids for parasitoids development and thus the quality of emerging adults. It has been shown that the sublethal effects induced by some symbiotic bacteria on developing parasitoids are due to toxins produced by APSE phages residing in the bacterial genome, which affect the fitness traits of parasitoids when the toxins do not kill them (Weldon et al. 2013; Oliver et al. 2014; Brandt et al. 2017; Monticelli et al. 2019). This phage-based protective phenotype has essentially been reported in the context of the association between aphids and the facultative symbiont *H. defensa* (Oliver et al. 2003, 2005; Oliver & Higashi 2019). Since toxin-producing APSE phages are absent from the genome of *S. symbiotica* strain Tucson (Renoz et al. in press), it is likely that these physiological effects are the result of the fitness costs associated with the presence of *S. symbiotica* that make the host less suitable for proper development of parasitoid larvae. Indeed, while facultative symbionts are often described as mutualists capable of providing benefits to their insect host depending on the ecological context (Oliver et al. 2010), there is increasing evidence that most of these bacterial partners are also associated with fitness costs for their host (Oliver et al. 2006; Vorburger & Gouskov 2011; Polin et al. 2014; Gerardo & Hurst 2017; McLean et al. 2018; Leybourne et al. 2020; Zytynska et al. 2021). The fitness costs associated with *S. symbiotica* strain Tucson have not been determined here, or in other studies. However, several studies have reported significant negative effects of other *S. symbiotica* strains, including increased insect susceptibility to insecticides (Skaljac et al. 2018) and reduced fecundity and survival rate of the infected individuals (Pons et al. 2019; Elston et al. 2020; Perreau et al. 2020). Fitness costs associated with facultative symbionts generally result in a reduction in host lifespan and reproductive rate, as well as a decrease in weight and size (Vorburger & Gouskov 2011; Pons et al. 2019).

Most studies that have investigated how facultative symbionts interfere with host-parasitoid interactions have focused on the rate of parasitism rather than the quality of emerging parasitoids. The few studies that have examined this have reported a reduced emergence rate, extended development time, and reduced parasitoid size and weight (Nyabuga et al. 2010; Schmid et al. 2012). Regarding the impact of aphid-associated symbionts on parasitoid development time, our results showed that the mummy stage duration is shortened in aphids infected with *S. symbiotica*. These results can be

explained by the quality of the hosts correlated to their size. Indeed, aphids infected with facultative symbionts are generally smaller than uninfected clones (Oliver et al. 2006; Pons et al. 2019; Skaljic et al. 2018), and since the small hosts are consumed more rapidly by parasitoid larvae, the development time may thus be shortened (Harvey 2005). It has long been known that host quality can influence sex allocation in many species of parasitoids (Charnov, 1982; Hardy, 1994). Where host quality (often measured by size) differentially affects the fitness of progeny, selection will favor mothers that manipulate sex ratio in response to current distributions of host quality (Charnov, 1982; Ode & Heinz 2002). In addition, we found that the development of parasitoids in aphids infected with facultative symbionts resulted in male-biased offspring, as assumed by Monticelli et al. 2019 (Monticelli et al. 2019). Two hypotheses can explain these results. First, as the sex-ratio was measured after adult emergence, the presence of the symbiont in the aphids may induce lower survival of female parasitoid during development because they need more reserve than males and aphids infected by the symbiont represent low-quality hosts. Second, if female parasitoids could perceive infected aphids as low-quality hosts, they may also choose to oviposit fewer female eggs in these aphids to avoid a loss of a part of their progeny. These hypotheses remain to be investigated. Interestingly, our results diverge from previous observations that showed that the sex ratio of parasitoids is not influenced by the presence of facultative symbionts (Nyabuga et al. 2010; Sochard et al. 2020). However, symbiont strains or even species and aphid strains were different. Finally, the reduced egg load in female parasitoids developing in *S. symbiotica*-infected aphids and the shorter survival rate of the emerging parasitoids we observed support the hypothesis that aphids hosting the symbiont represent hosts of lower quality and therefore less profitable hosts for the parasitoids than to those that are not infected.

To detect and exploit patches, female parasitoids can use chemical clues, including alarm pheromones released by aphids (Vandermoten et al. 2012). Our olfactometer bioassay suggests that *A. ervi* females are more attracted to aphids that do not harbor *S. symbiotica*. We therefore conducted an experiment to establish whether the EBF signature is involved in this choice behavior. Indeed, EBF is an attractant for a wide range of aphid natural enemies, including parasitoid wasps (Battaglia et al. 2013; Hatano et al. 2008; Vandermoten et al. 2012). Oliver et al. (2012) have already reported that aphids infected with *H. defensa* released lower amounts of EBF than uninfected aphids (Oliver et al. 2012) and suggested that the amount of EBF released by aphids could be a cue used by female parasitoids to select the most profitable hosts (i.e., not infected with the defensive symbiont *H. defensa*). In our study, we did not detect a significant difference in the amount of EBF released by aphids as a function of their infection status. Since female parasitoids tend to preferentially orient to uninfected aphids rather than

S. symbiotica-infected aphids, it is likely that female parasitoids use other chemical cues to differentiate aphids based on their infection status. Cuticular compounds could be used over short distances (Muratori et al. 2006; Hatano et al. 2008). While EBF has been described as the main alarm pheromone in several aphid species (including *A. pisum*) allowing parasitoids to detect hosts over longer distances, other volatile substances whose biological activity has not yet been characterized could play an attractive role similar to EBF (Francis et al. 2005; Vandermoten et al. 2012).

Our results suggest that *A. pisum* infected with *S. symbiotica* are less profitable hosts for *A. ervi* females compared to uninfected ones. We have therefore tested whether the infection status of *A. pisum* influenced the behavioral sequence leading to the parasitization of aphids by female parasitoids. Indeed, our results show that *A. ervi* parasitoids adjust their oviposition behavior and patch-time allocation according to the presence of *S. symbiotica* in the aphids. This results in a sharp decrease of the patch time allocation when *S. symbiotica* is housed in aphids, but also in a reduction of the attack behavior. These findings strengthen the hypothesis that the presence of facultative symbionts is a factor that can significantly determine the value of the host for parasitoids and that the parasitoid adjusts its foraging behavior accordingly. The literature reports several cases highlighting how facultative symbionts can interfere with parasitoid decisions in host-parasitoid interactions. A notable example is the association between the pea aphid *A. pisum* and the facultative symbiont *Rickettsiella viridis* in which the symbiont changes the aphid's body color from red to green, making infected aphids more attractive to parasitoid wasps since they tend to oviposit into green morphs (Losey et al., 1997; Libbrecht et al. 2007; Tsuchida et al. 2010). Protective phenotypes against parasitoid wasps that are associated with the symbiont *H. defensa* is another striking example of how facultative symbionts can affect host-parasitoid interactions (Oliver et al. 2003, 2005, 2014; Oliver & Higashi 2019). The few studies that have examined the impact of host endosymbionts on the foraging behavior of parasitoids have focused on the interactions involving this symbiont species (Monticelli et al. 2019). It has been shown that *A. ervi* parasitoids avoid *Sitobion avenae* clones infected with *H. defensa*, preferentially ovipositing in uninfected clones (Łukasik et al. 2013a). It has also been shown that *A. ervi* tends to superparasitize *H. defensa*-infected *A. pisum* aphids to overcome symbiont-mediated defense and increase the chances of successful parasitism (Oliver et al. 2012). The results presented here show that *A. ervi* oviposits preferentially in uninfected clones. However, in contrast to Oliver et al. (2012) observations, our results show that *A. ervi* tend to superparasitize uninfected clones rather than clones infected with *S. symbiotica*. Similarly, Nyabuga et al. (2010) also did not observe a tendency to superparasitism when one or more facultative symbionts are present in *A. pisum*: they even generally observed the opposite in the case of the different symbiotic combina-

tions they tested. These contrasting results may be due to the model systems used in the different studies (i.e., aphid strains, parasitoid wasps and symbionts), as well as the different experimental approaches, and in particular the duration of host exposure to parasitoid and host density. Indeed, Oliver et al. (2012) assessed the level of superparasitism by counting parasitoid eggs six hours after oviposition while we counted the parasitoid larvae three days after oviposition and Nyabuga et al. (2001) did so five days after oviposition (Nyabuga et al. 2010; Oliver et al. 2012).

In conclusion, we showed that *S. symbiotica*, one of the most frequent facultative symbiont species in aphids, influences the different steps of the host-parasitoid interactions, from foraging behavior to parasitoid development and life-history traits. The *A. pisum*-*A. ervi* system has already been used in previous studies to decipher the mechanisms underlying defensive symbiosis. However, the impact of host symbionts should depend on a combination of factors, including the genotype of each biological model (including the strain of the bacterial symbiont) and environmental conditions. There is currently very little data on the role of insect symbionts on food web structure and community dynamics. In particular, this study suggests that aphid-associated symbionts may have cascading effects at the population and community levels, notably by influencing host exploitation and parasitoid fitness. Further studies are needed to clarify the impact of host-symbiont interactions on the selection pressures on parasitoids, as well as on the demography of these insects. This is an important issue because parasitoids are increasingly used in biological control programs, whose effectiveness could be influenced by hidden players in the interactions between insects and their natural enemies.

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