Journal of Evolutionary Biology, 2025, **0**, 1–12
https://doi.org/10.1093/jeb/voaf083
Advance access publication 26 June 2025
Research Article

JOURNAL OF Evolutionary Biology
eseb

A test of specific adaptation to symbiont-conferred host resistance in natural populations of a parasitoid wasp

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Handling editor: Alejandro Gonzalez-Voyer, Associate editor: Carita Lindstedt-Kareksala

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Abstract

Parasitoids are important natural enemies of insects, imposing strong selection for the evolution of resistance. In aphids, the heritable endosymbiont *Hamiltonella defensa* is a key determinant of resistance, making symbiont-conferred defence a potential target for specific adaptation by parasitoids. We tested this hypothesis in the aphid parasitoid *Lysiphlebus fabarum* and four of its host species, *Aphis fabae fabae*, *A. hederae*, *A. urticata*, and *A. ruborum*. The parasitoids show host-associated genetic differentiation indicative of host specialization, and each of these aphid species harbours their own 1–3 distinct strains of *H. defensa*. We introduced eight *H. defensa* strains from all four aphid species into a common host background (a laboratory strain of symbiont-free *A. fabae fabae*) and then tested the ability of 35 field-collected *L. fabarum* lines from the same four hosts to parasitize the *H. defensa*-carrying aphids. The origin of symbionts affected parasitism success, with strains from *A. fabae fabae* and *A. hederae* conferring strong protection, and strains from *A. urticata* and *A. ruborum* providing virtually no protection. For one strain each from *A. fabae fabae* and *A. hederae*, we found a signature of specific adaptation by parasitoids, as parasitoids able to overcome their protection mostly came from the same hosts as the symbiont strains. Two other strains were so strongly protective that they permitted very little parasitism independent of where parasitoids came from. While not fully conclusive, these results are consistent with specialized parasitoids adapting to certain defensive symbionts of their host species, supporting the notion of symbiont-mediated coevolution.

Keywords: adaptation, defensive symbiosis, *Hamiltonella defensa*, aphids, parasitoids, transfection

Introduction

Hymenoptera is probably the most speciose of all animal orders (Forbes et al., 2018). Composed to a large extent of parasitoid wasp species, this group underwent extensive diversification resulting from the high host specialization required by the parasitic lifestyle (Forbes et al., 2018). This specialization is an expected outcome of antagonistic coevolution that occurs when two species engage in a tight host–parasite relationship (Engelstädter & Bonhoeffer, 2009; Kawecki, 1998). Several studies investigated reciprocal adaptations and evolutionary dynamics between one host and its parasite in various ecological contexts, based on different genetic models, such as the gene for gene model or the matching alleles model (for an overview see Agrawal & Lively, 2002). However, many insects host beneficial symbionts protecting them against natural enemies (Brownlie & Johnson, 2009; Gerardo & Parker, 2014), including parasitoids (e.g., Dedeine et al., 2001; Hunter et al., 2003; Oliver et al., 2003; and reviewed in Haine, 2007). Hence, parasitoid species must often fight joint defences from two entities at the same time—the host and the symbiont—which increases the range of targets for parasitoid counter-adaptations (Kwiatkowski et al., 2012). In such cases, the main selection target will likely depend on the strength of protection conferred by the host's own defences compared to the strength of protection conferred by the host's symbiont.

In aphids, symbiont-conferred defence can indeed be a key determinant of variation in resistance to parasitoids. *Hamiltonella defensa* is a heritable bacterial endosymbiont found in numerous aphid species (Moran et al., 2005), and aphids carrying this symbiont are often highly resistant to parasitoids (Asplen et al., 2014; Cayetano & Vorburger, 2015; Oliver et al., 2003; Vorburger et al., 2010). However, *H. defensa*-conferred defences are unequally effective against different parasitoid species. Wu et al. (2022) showed that *H. defensa* strains often—but not always—provide the strongest protection against the most common parasitoid species of their normal host species. This also applies to the black bean aphid, *Aphis fabae fabae*, in which naturally occurring strains of *H. defensa* provide protection against the dominant parasitoid *Lysiphlebus fabarum*, but not against other—less common—parasitoids exploiting black bean aphids (Gimmi & Vorburger, 2024). *Hamiltonella defensa*-conferred resistance even shows additional specificity: particular strains of *H. defensa* may protect strongly against some genotypes of *L. fabarum*, but not or only weakly against others, with these patterns varying across symbiont strains (reviewed in Vorburger, 2014). Genotype-by-genotype interactions between parasitoids and *H. defensa* can therefore explain a large proportion of the variation in parasitism success (Cayetano & Vorburger, 2013; Gimmi & Vorburger, 2021; Schmid et al., 2012). Multi-generation experiments in caged laboratory

Received November 28, 2024; revised April 30, 2025; accepted June 25, 2025

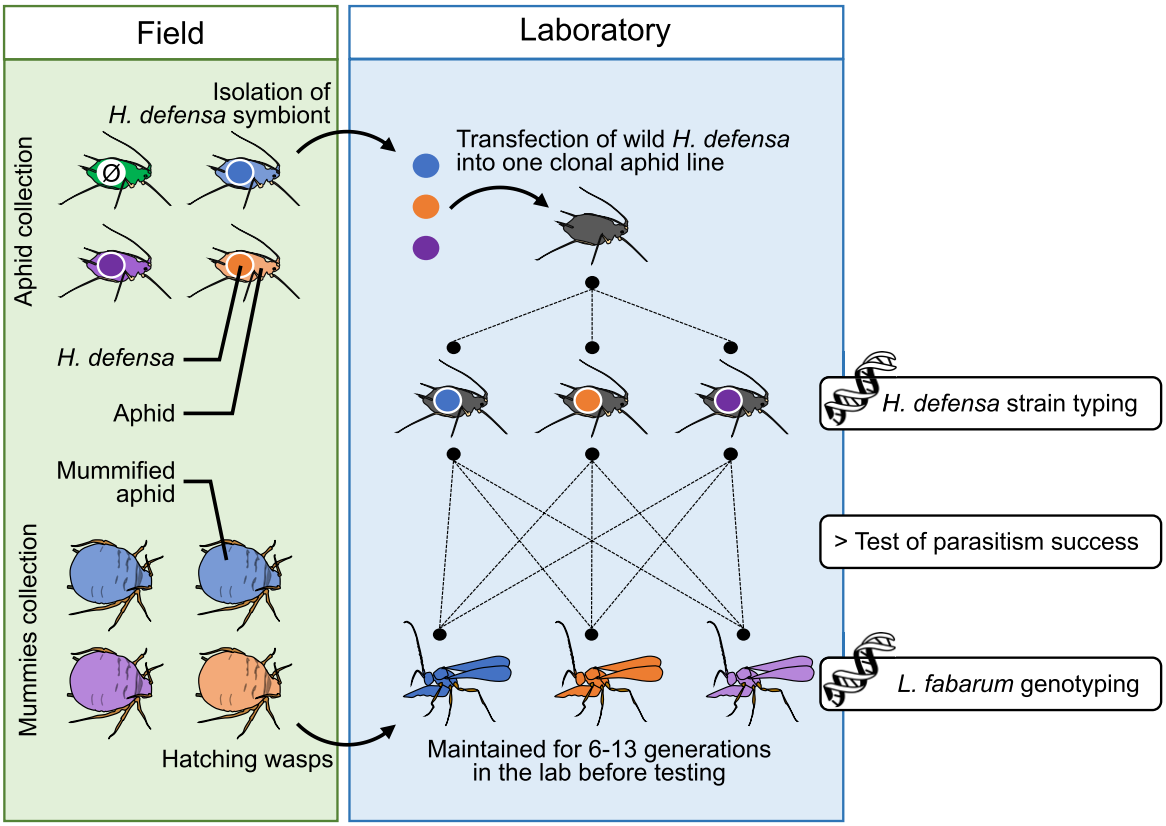

Figure 1. Overview of the experimental plan. Colours represent different species (for aphids) or different original aphid host species (for *H. defensa* and for *L. fabarum* wasps).

populations have indeed shown that defensive symbionts are strong mediators of the reciprocal selection between hosts and parasitoids (Hafer-Hahmann & Vorburger, 2020; Rossbacher & Vorburger, 2020). Hence, aphids are promising models to study the emergence of specific adaptations by parasitoids to frequently encountered symbionts (Vorburger, 2022).

Studies employing experimental evolution in the laboratory have shown that parasitoids can evolve specific counter-adaptations to resistance conferred by different strains of *H. defensa* (Dennis et al., 2017; Rouchet & Vorburger, 2014). What is still unclear is whether the genetic interaction between parasitoids and their hosts' defensive symbionts leads to detectable patterns of specific adaptation in natural populations of parasitoids. An earlier study on parasitoid local adaptation on a geographic scale was inconclusive; it found a nonsignificant positive correlation between the ability of *L. fabarum* to parasitize *H. defensa*-protected aphids and the mean local prevalence of *H. defensa* across several host species (Vorburger & Rouchet, 2016). In retrospect, this inconclusive result is not surprising, because the study ignored the fact that the different host species possessed different strains of *H. defensa*, which was discovered only later (Henry et al., 2022).

Here, we took this diversity of *H. defensa* strains into account, looking for potential patterns of specific adaptation by parasitoids to symbiont-conferred resistance in a system composed of four co-occurring and congeneric aphid species. These aphids are all able to carry *H. defensa*, and they are all being targeted heavily by the same parasitoid species, *L. fabarum* (Henry et al., 2022). Two characteristics of this system are especially important in the context of our study: (1) the strain diversity of *H. defensa* is strongly compartmentalized by aphid host species and limited to two or three strains in each aphid species (Henry et al., 2022) and (2) the parasitoid *L. fabarum* comprises a high diversity of asexual genotypes of which many show strong preferences for one host species only (Sandrock et al., 2011). Combined, these characteristics constitute a conducive situation for the evolution of specific adaptation to frequently encountered defensive symbionts. Within a geographically restricted area of Switzerland, we first sampled and described the strain diversity of *H. defensa* in the four aphid species as well as the genotypic diversity of the *L. fabarum* parasitoids exploiting them. We then tested for specific adaptation in parasitoids by measuring the parasitism success of multiple field-collected *L. fabarum* lines against multiple field-sampled *H. defensa* strains from different aphid host species, in a full factorial design. To estimate *H. defensa*-conferred protection unconfounded by host background, we transfected all *H. defensa* strains into the same *H. defensa*-free aphid clonal line of *A. fabae fabae*. Because each aphid species comes with its exclusive *H. defensa* strains, our working hypothesis was that parasitoid lines specialized in one aphid host would perform better against the *H. defensa* strains carried by this species than against *H. defensa* strains from other aphid species.

Materials and methods

We present a general overview of our experimental procedures in Figure 1.

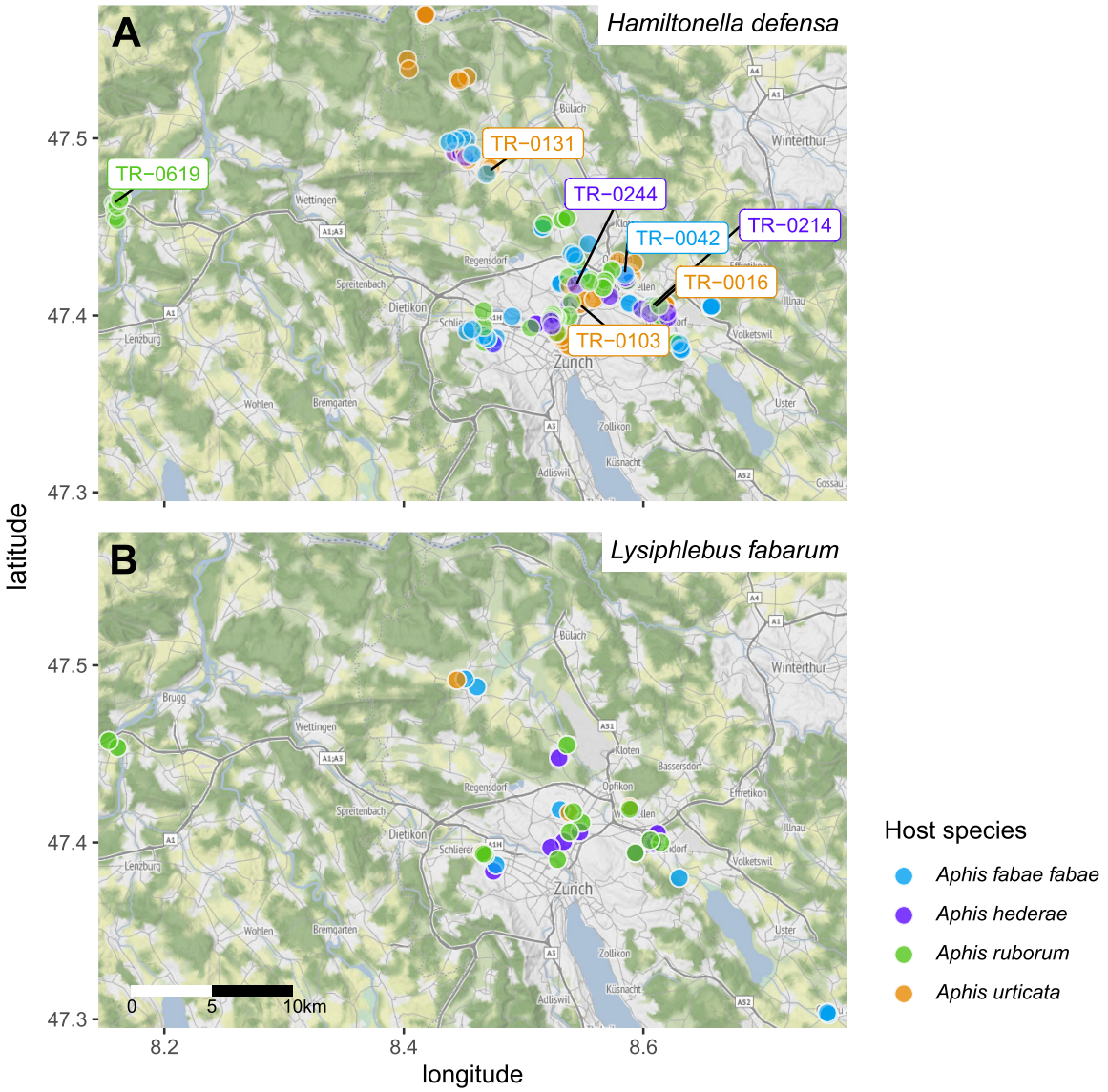

Figure 2. Field sampling maps of the *H. defensa* symbionts (A) or *L. fabarum* parasitoids (B). Each dot is a unique sample from one aphid colony. Labels of *H. defensa* samples indicate the sampling location of the successfully transfected lines. Map data are from OpenStreetMap.

Sampling

Sampling of aphids and parasitoids took place in the Swiss city of Zürich and its close surroundings from May to August 2020 (Figure 2A and B). We collected aphids (*A. urticata*, *A. ruborum*, *A. fabae fabae*, and *A. hederae*) directly from their specific host plants, stinging nettle (*Urtica dioica*), blackberry (*Rubus fruticosus*), white goosefoot (*Chenopodium album*) or common beet (*Beta vulgaris*), and common ivy (*Hedera helix*), respectively. *Aphis hederae* is very closely related to *A. fabae fabae*, whereas *A. urticata* and *A. ruborum* are more distantly related to *A. fabae fabae*/*A. hederae* and to each other (Coeur d'acier et al., 2014). One individual per aphid colony was collected. Its species, host plant, and geographic location were recorded. Each aphid sample was collected at least 5 m away from other samples to avoid collecting clones. We sampled the parasitoid wasp *L. fabarum* independently from the aphid sampling (but from the same four aphid species) by collecting visibly parasitized aphids referred to as "mummies", containing a parasitoid at the pupal stage. We attributed unique identifiers to *L. fabarum* samples (YP20-xxx) and recorded the aphid host species as well as the geographic location. Each parasitoid sample was taken at least 50 m away from other samples, also to reduce the probability of collecting offspring from the same mother. *Lysiphlebus* parasitoids are highly philopatric and tend to stay in their natal patches (Nyabuga et al., 2010). However, successful asexual lineages may still become geographically widespread (Sandrock et al., 2011), making it difficult to completely avoid sampling genetically identical individuals. To detect such repeated genotypes, we used microsatellite markers (see "Parasitoid genotyping" method). In total, we sampled 427 aphids (96, 121, 128, and 82, respectively, for *A. urticata*, *A. ruborum*, *A. fabae fabae*, and *A. hederae*) and 94 parasitized aphid colonies (15, 29, 18, and 32 by respective hosts: *A. urticata*, *A. ruborum*, *A. fabae fabae*, and *A. hederae*) (Figure 1B).

Laboratory breeding

Within 2 days after collection in the field, we transferred all aphids to individual units with their host plant. Units consisted of a pot containing soil and the appropriate host plant for each species, covered with a ventilated plastic cage. We used freshly cut, aphid-free stems of *Rubus fruticosus* for *A. ruborum* and of *Hedera helix* for *A. hederae*. *Aphis fabae fabae* and *A. urticata* samples were transferred the same way, but on lab bred *Vicia faba* (Fuego cultivar, UFA Samen, Winterthur, Switzerland) or *Urtica dioica* plants (kr13 cultivar, Sativa GmbH, Rheinau, Switzerland). We regularly transferred clonal colonies to new plants and maintained them this way for several weeks to have time to check for the presence of *H. defensa* and to genotype and identify the strains. Aphid clones without *H. defensa* or with *H. defensa* strains we already had were discarded after these checks.

From each parasitized aphid colony, a few mummies were individually stored in 2 mL Eppendorf tubes. Hatching wasps were collected, identified as *L. fabarum*, and bred as isofemale lines on a leaf disc set-up for the first generation. Only a few mummies belonged to other species than *L. fabarum*, as expected from previous samplings in the same area (Gimmi & Vorburger, 2024; Hafer-Hahmann & Vorburger, 2021). Using isofemale lines allowed us to eliminate rare wasps from arrhenotokous (sexual) lines, where unfertilized females will only produce sons. We only kept thelytokous (asexual) lines, which predominate in the field (Sandrock et al., 2011). When more than one wasp from the same parasitized colony produced female offspring, we only kept one isofemale line for further breeding. Leaf disc set-ups consisted of 5 cm petri dishes with meshed lid, containing a freshly cut disc of *V. faba* (broad bean) leaf, stuck upside down on top of a 1% agar medium. Leaf discs contained 10–20 individuals of an *H. defensa*-free *A. fabae fabae* clonal line (line A08-28 H-), which was different from the clone used later in the experiment. For each new generation of all wasp lines, we transferred around 15 individuals to a new pot containing a broad bean plant and a healthy *H. defensa*-free aphid colony. We kept lines on this set-up for 6–13 generations before testing. Many parasitoids (especially the ones originating from *A. urticata* and *A. ruborum* hosts) were not able to establish on our *A. fabae fabae* clone used for wasp rearing and were lost in the process, leading to a total of 35 well-established lines.

All animals were kept on a 16:8 hr light:dark photoperiod at 22 °C.

DNA extraction

We extracted DNA from whole aphids and parasitoids using a high salt method (Sunnucks & Hales, 1996), as described previously (Henry et al., 2022). Briefly, we crushed one individual in 8 µL of proteinase K (10 mg/mL) with a pipette tip, until obtaining a smooth homogenate. We added 300 µL of TNES buffer (50 mM Tris.HCl pH 7.5, 400 mM NaCl, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS)) to the homogenate before incubating it at 55 °C for 3 hr. Then, we added 85 µL of 5M NaCl to precipitate the proteins under vigorous manual shaking. We centrifuged the tubes for 30 min at 22,000 G to pellet the proteins and transferred the supernatant containing DNA into a clean 1.5 ml Eppendorf tube. DNA was then precipitated with ice cold 100% ethanol and centrifuged for 10 min at 22,000 G and 4 °C. We removed ethanol and replaced it with new 70% ethanol, centrifuged for 10 min at 22,000 G and 4 °C, and discarded the supernatant. Finally, we dried the pellets from the last remnants of ethanol and re-suspended the DNA in TE buffer (10 mM Tris.HCl pH 7.5, 1 mM EDTA pH 8.0).

Parasitoid genotyping

We genotyped the *L. fabarum* wild-caught lines using a multiplex protocol similar to Sandrock et al. (2007), with 10 microsatellite markers (Table S1). We determined microsatellite fragment sizes on an ABI 3130 Genetic Analyzer sequencer (Applied Biosystems, MA, USA), relative to a size standard (GeneScan-500 LIZ). We scored alleles using GeneMarker V3.0.1, based on preexisting templates of previously found alleles.

Endosymbiont identification

We used diagnostic polymerase chain reaction (PCR) to test for the presence of *H. defensa*, using specific primers (Table S 2). An additional diagnostic PCR detecting the obligate symbiont *Buchnera aphidicola*, carried by all aphids, was used as a positive control for DNA extraction. We generated amplicons using the readymade GO-taq mastermix (Promega, Madison, WI, USA), with touchdown PCR set-up as follows: 95 °C initial denaturation for 3 min, 35 cycles with 30 s of 95 °C denaturation, 30 s of decreasing annealing temperature from 65 to 55 °C on the first 10 cycles, 60 s of 72 °C elongation, and a final elongation of 6 min at 72 °C. We visualized the PCR products using capillary electrophoresis with the QIAxcel Advanced System combined with the QIAxcel ScreenGel software (Qiagen, Hilden, Germany). We removed rare samples showing no amplification of *B. aphidicola* for subsequent analysis (failed extractions). Once we knew the *H. defensa* infection status of our samples, we performed similar PCR diagnosis for five other common facultative endosymbionts (*Regiella insecticola*, *Serratia symbiotica*, *Spiroplasma sp.*, *Rickettsia sp.*, and *Arsenophonus sp.*), in all *H. defensa*-positive lines (see Table S2 for primers). For each symbiont species, we also ran positive and negative controls alongside our experimental samples. We tested for these additional symbionts because for later transfections, we only wanted to retain lines in which *H. defensa* was the sole facultative endosymbiont.

Haplotyping of *Hamiltonella defensa*

To assess the strain diversity of the *H. defensa* symbionts, we amplified and sequenced two loci (*rpoS* and *p41*) that provided sufficient resolution to discriminate local strains in a previous study (Henry et al., 2022) (Table S3). The *rpoS* gene codes for a transcription factor that is widely conserved in gamma proteobacteria, and *p41* codes for a helicase of the *A. pisum* secondary endosymbiont (APSE) bacteriophage that is present in the chromosome of *H. defensa* (Boyd et al., 2021; Degnan & Moran, 2008). We generated the amplicons with the readymade GO-taq mastermix and the same touchdown PCR settings as for the endosymbiont identification. We purified the amplicons using the Wizard SV PCR clean-up kit (Promega, Madison, WI, USA) following the manufacturer instructions. We outsourced bidirectional Sanger sequencing of the amplicons to Microsynth AG (Balgach, Switzerland). We analysed the sequenced DNA and identified the different strains of *H. defensa* using Geneious Prime version 2019.2.1 (Biomatters ltd, Auckland, New Zealand). Briefly,

all sequences from each gene were aligned using the Geneious alignment algorithm with default settings. Low-quality parts at the start and end of the sequences were trimmed, ambiguous bases were manually corrected, and both loci were concatenated. We used MrBayes version 3.2.6 to produce consensus phylogenies based on the general time reversible (GTR) substitution model, from three Markov chain Monte Carlo (MCMC) chains of 1,100,000 generations, sampled every 200 generations, and with an initial burn-in of 100,000 generations (Ronquist et al., 2012). We picked the substitution model using modeltest in PAUP* (Posada, 2003). We used the genome of one *H. defensa* strain from another aphid host, *Acyrthosiphon pisum*, as the outgroup (GenBank ref CP017613). Graphical representations of trees were made using the `phytools` package (Revell, 2021). We found eight strains across the four aphid species. The *rpoS* and *p41* sequences of these strains have been deposited in GenBank (accession numbers in Table S4). We could only use seven of them for subsequent transfections, as one strain from *A. ruborum* was unfortunately lost from the collection due to a handling error.

Symbiont transfections

To be able to compare the protection efficiency of the field-collected strains of *H. defensa*, we artificially infected a symbiont-free clone of *A. fabae fabae* (line A06-407) with each of the seven strains. We selected donors having *H. defensa* strains of interest and without co-infections by other symbionts, as we wanted to assign any differences in resistance unambiguously to *H. defensa*. We performed transfections by stabbing adult donor aphids with thin Ø 0.1 mm needles to collect *H. defensa* from their hemolymph, and immediately stabbing 3–5-day-old recipient aphids with the same needle. We allowed surviving recipient aphids to reproduce until they died and isolated their final offspring on individual leaf disc set-ups. We checked the success of each transfection by performing diagnostic *H. defensa* PCR on the second-generation offspring. We confirmed the identity of the strains and stability of the infection over ∼10 generations by performing two multi-locus sequence-typing rounds, before and after the experiments, with the same method as presented in the above section but using 10 markers instead of 2, as previously described (Henry et al., 2022) (Table S3). Four of these markers are part of the lysogenic bacteriophage APSE in *H. defensa*'s genome, which is required for the protection against parasitoids (Degnan & Moran, 2008; Oliver et al., 2009). All sequences have been deposited in GenBank (accession numbers in Table S4). We named the transfected lines TR-0016, TR-0042, TR-0103, TR-0131, TR-0214, TR-0244, and TR-0619 according to the ID of the donor line. One additional *H. defensa* strain we found in the field had already been transfected into the same recipient clone and kept in our laboratory line collection since 2009 (TR-0076). We directly used this line instead of repeating a transfection.

Parasitoid performance

We measured the reproductive performance of each parasitoid line against each *H. defensa*-infected aphid line as well as an *H. defensa*-free line of the same aphid clone, with six replicates for each ([8 transfected lines + 1 symbiont-free line] × 35 parasitoid lines × 6 replicates = 1890 measurements). All tests were performed on leaf disc set-ups. We allowed two adult aphids to reproduce on a leaf disc. After 24 hr, we removed the adults and standardized the number of nymphs to 8–12 individuals. After 24–48 hr, we introduced one 1–4-day-old parasitoid in the leaf disc set-up, and we removed it after 10 hr ± 15 min. Each wasp was only used once. We did not verify by direct observation that every aphid nymph was stabbed by the wasp, but 10 hr is more than enough time for a wasp to find and attack all aphids in such a confined space. It can therefore be assumed that most, if not all, aphids were attacked in our assay. We took note of those replicates where parasitoids had died during the tests ($N = 100$). Parasitoids were then stored at −20 °C for subsequent genotyping. After 14 days, we counted the number of mummies, and after 22 days, we counted the number of hatched parasitoid wasps. As very few parasitoids did not hatch, we only used the number of mummies in the analysis.

Statistical analysis

We analysed data in a Bayesian framework, using R (version 4.2.1; R Core Team, 2022) and the `brms` package (Bürkner, 2017) as frontends for the Stan language (Carpenter et al., 2017). Data preparation, model evaluation, and plotting relied heavily on the `tidyverse` suite of packages (Wickham et al., 2019), as well as on the `tidybayes`, `bayesplot`, and `patchwork` packages (Gabry et al., 2019; Kay, 2022; Pedersen, 2022).

We analysed parasitism success (number of mummies/number of nymphs) using a beta-binomial generalized linear mixed model (GLMM) with a logit link function. We chose a beta-binomial model because initial diagnostics we ran on the equivalent binomial GLMM revealed some evidence of overdispersion (see R scripts and datasets). Given our working hypothesis that parasitoids from a particular aphid species should be better adapted to the *H. defensa* strains occurring in that aphid species, our GLMM included the original host species of the *H. defensa* strain and the original host species of the parasitoid as fixed effects, as well as their interaction. To account for variation among *H. defensa* strains from the same host species and among wasp lines collected from the same host species, our model included random intercepts of parasitoid genotype as well as the corresponding random "slopes" of *H. defensa* strain. Finally, we included a replicate-level random effect (random intercept) to account for stochastic differences among individual assays (Harrison, 2015). Cases where the wasp died during the experiment were not excluded from the analysis, as they showed comparable parasitism rates to replicates of the same host–parasitoid combination with wasps still alive at the end of the exposure period. This is likely because the parasitoid attacks usually happen early, often in the first few minutes following the introduction of the wasp into the aphid colony.

We fit our model using weakly informative priors, mostly inspired by McElreath's (2020) suggestions: Normal(0,1.5) priors for parasitoid host origin-specific intercepts; Normal(0,1) for the other fixed effects (i.e., the effects of symbionts and their interaction with parasitoid origin); Half-Normal(0,1) priors for random effect standard deviations, and a Lewandowski-Kurowicka-Joe (LKJ)(2) prior for the correlations between random effects. In addition, we used a Half-Normal(0,1) prior for the overdispersion parameter (ϕ; sensu Harrison, 2015). We ran four chains of 2000 iterations

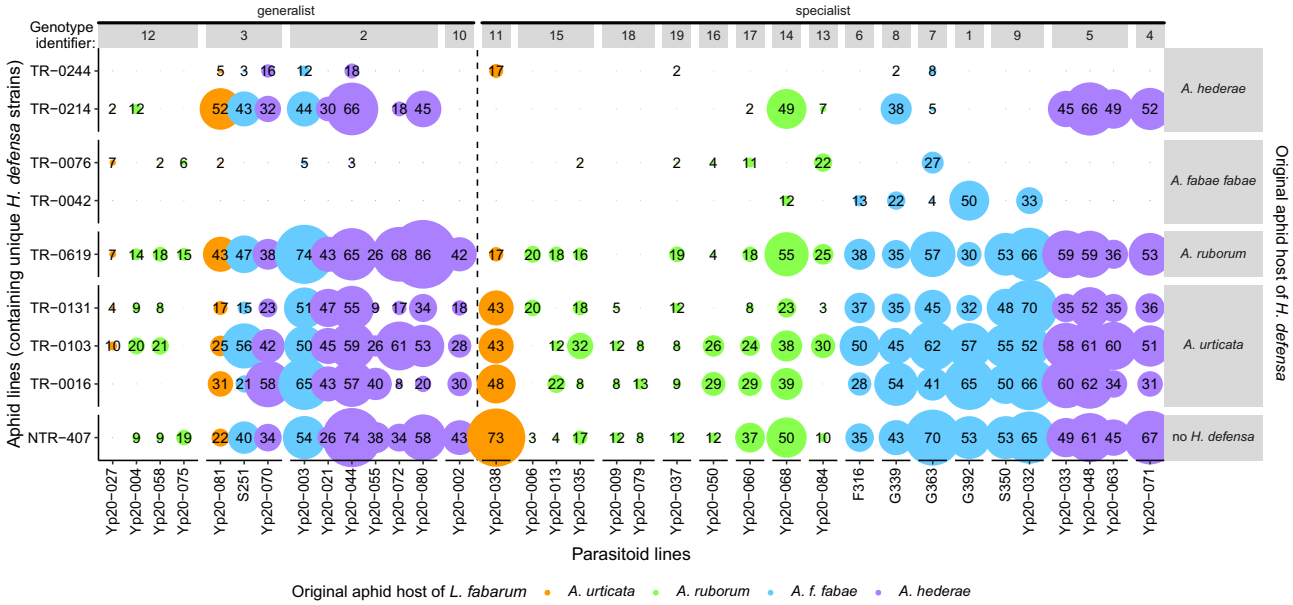

Figure 3. Parasitism success of all parasitoid lines facing all symbiont strains. Bubble size is proportional to the parasitism success, and numbers in bubbles show the average percentage of mummies ($N = 6$ replicate assays per combination). Absent bubbles indicate 0% parasitism. Parasitoid lines are ordered by host range (for simplicity, genotypes found in more than one host are called generalists, others are specialists), genotype (each identifying number stands for one distinct genotype), and by original host species of the parasitoids (bubble colours). Genotype #10 is classified as a generalist as it was sampled in two aphid species, although only one line survived and was tested in the experiment.

each, with the first half of each chain used as warm-up. We used the updated $\hat{R}$ statistic and effective sample size diagnostics developed by Vehtari et al. (2021) to evaluate our model. When posterior summaries are provided in text and figures below, they are given as means (95% Highest Posterior Density Intervals). We additionally partitioned strain-level variation (i.e., excluding clonal replicate effects and residual within-strain variation) into its aphid host components (i.e., fixed effects for both parasitoid and symbiont origins) and non-aphid host components (strain random effects), following methods defined in Johnson (2014) and Nakagawa & Schielzeth (2013). By construction, our Bayesian approach did not require any correction for multiple comparisons (Gelman & Tuerlinckx, 2000).

Results

The different *H. defensa* strains we found in the sampled aphids were mostly the same as the ones we identified with a similar sampling campaign, in the same region, the year before (Henry et al., 2022) (Figure S1). With one exception, we were able to transfer all strains into our recipient clone of *A. fabae fabae*. The new associations were stable over >10 generations. The microsatellite genotyping of the asexual *L. fabarum* detected a high genotypic diversity, with 19 unique genotypes among the 35 lines we used for the parasitism experiment. We classified the genotypes into "specialist" (15/19) and "generalist" (4/19), according to the number of host species they were found in (1 or ≥2, respectively), being aware that this crude classification does not reflect a clear ecological division, as less common genotypes collected only once would automatically fall into the "specialist" category. The *H. defensa* strains we used were a comprehensive sample of the local diversity in the field, whereas the parasitoid populations only represented a subset of the local genotypic diversity, since the sampling effort was not sufficient to reach the plateau of the rarefaction curve (Figure S2).

The general overview of the one-by-one interactions between *L. fabarum* lines and *H. defensa* strains, i.e., the percentage of successful parasitism achieved by each wasp line on aphids carrying each *H. defensa* strain (and *H. defensa*-free aphids), is provided in Figure 3. It shows a marked effect of the origin of *H. defensa* on parasitism success. Strains of *H. defensa* originating from *A. fabae fabae* (aphid lines TR-0042 and TR-0076) and *A. hederae* (TR-0214 and TR-0244) conferred resistance and reduced the average parasitism success of wasps from all origins: most $\Delta_{\text{(none—}A.\ f.\ fabae\text{)}}$ and $\Delta_{\text{(none—}A.\ hederae\text{)}}$ are negative and not overlapping 0 (see Table S5, all Δ are differences of model predictions on the logit scale), with the exception of wasps from *A. urticata* whose success was not reduced by symbionts from *A. hederae* ($\Delta_{\text{(none—}A.\ hederae\text{)}} = -0.11$ [−0.25; 0.04]). Conversely, strains from *A. ruborum* (TR-0619) and *A. urticata* (TR-0016, TR-0103, and TR-0131) provided virtually no protection against parasitism (all $\Delta_{\text{(none—}A.\ ruborum\text{)}}$ and $\Delta_{\text{(none—}A.\ urticata\text{)}}$ overlap 0, see Table S5). Besides the variation in parasitism success due to *H. defensa*, we evidenced some variation associated with the origin of parasitoid lines (Figure 3 and Figure S3). Wasp lines originating from *A. ruborum* and *A. urticata* performed worse on average than lines originating from *A. hederae* and *A. fabae fabae* (multiple $\Delta_{\text{(}A.\ ruborum\ or\ A.\ urticata\text{)—(}A.\ hederae\ or\ A.\ f.\ fabae\text{)}}$ are positive and not overlapping 0, see Table S6). Interestingly, lines with poor success were not only ineffective against symbiont-protected aphids but also against the unprotected aphids, suggesting that they were poorly adapted to the experimental clone of *A. fabae fabae* to begin with. This is consistent with the observation

that parasitoids from *A. ruborum* and *A. urticata* were difficult to establish as isofemale lines on *A. fabae fabae* in the laboratory. To account for this base inequality among parasitoid lines, we present in Figure 4 the parasitism success estimates on *H. defensa*-protected aphids relative to the success against the unprotected aphid line. With this approach, while we confirm the same strong effects of *H. defensa* origins (Figure 4A and B and Table S7), we lose most of the previously observed effects associated with the origin of wasps. In fact, only one significant pairwise difference of parasitism rate remained between wasps from different origins when compared on aphids with symbionts from a single host species (Figure 4B and Table S8).

Complementary to these general patterns, we observed a high specificity of parasitism achieved by the different wasp lines on aphids containing the most protective symbiont strains (top four rows in Figure 3). Aphid line TR-0214, for example, carries an *H. defensa* strain from *A. hederae* providing excellent protection against most parasitoid lines, yet about a dozen wasp lines achieved rather high parasitism rates on these symbiont-protected aphids (Figure 3). Most of these wasp lines belonged to genotypes originating from *A. hederae* or from generalist genotypes found at least once on *A. hederae*. A similar pattern was observed in aphid line TR-0042, carrying an *H. defensa* strain from *A. fabae fabae*. These aphids were extremely resistant, with only six wasp lines achieving any parasitism at all, and these lines belonged almost exclusively to specialist genotypes from *A. fabae fabae* (Figure 3). While these observations suggest that specific adaptation to the *H. defensa* strains present in their main host species contributes to patterns of *L. fabarum* infectivity, the effects were relatively minor in the context of the total variation. Despite some individual parasitoid genotypes displaying clear specialization, this pattern could not be generalized to all genotypes from one origin (Figure 4C and Table S9). Overall, the symbionts' host species was a stronger predictor of parasitism rates than the wasps' host species (Figure 4 and Tables S7 and S8).

Despite its limited ecological basis, the distinction of "specialist" and "generalist" *L. fabarum* genotypes provided interesting additional insights. For example, parasitoid line Yp20-081 was collected from *A. ruborum* but did surprisingly well on the used *A. fabae fabae* clone and was even able to overcome the protection conferred by *H. defensa* strain TR-0214 originating from *A. hederae*, likely explicable by this wasp line belonging to generalist genotype 2, which was also collected from *A. fabae fabae* and *A. hederae* (Figure 3). Wasp line Yp20-003 from *A. fabae fabae* was similarly able to overcome the defences conferred by the *A. hederae*-specific *H. defensa* strain TR-0214, yet this wasp line belonged to generalist genotype 3, which was indeed collected an additional five times from *A. hederae* (Figure 3). We can also not exclude that some parasitoid genotypes collected only once and therefore classified as specialists are in fact generalists able to use other hosts from which we simply happened not to collect them. Possible examples include genotype 11 from *A. urticata* and genotype 17 from *A. ruborum*, as they were considerably more successful on the experimental clone (*A. fabae fabae*) than other wasps from the same hosts. In line with this, we observed that genotype 14 from *A. ruborum* and genotype 8 from *A. fabae fabae* surprisingly overcame the defences of *A. hederae*-derived *H. defensa* strain TR-0214 (Figure 3).

Discussion

We hypothesized that *L. fabarum* parasitoids should have higher parasitism success on hosts carrying *H. defensa* strains from the aphid species they were collected from than on hosts with strains from other aphid species. This hypothesis was based on the observations that different strains of *H. defensa* are compartmentalized within the four aphid species considered here (Henry et al., 2022), and that there is evidence for host fidelity and/or host specialization in their main parasitoid *L. fabarum* (Sandrock et al., 2011)—a situation conducive for specific adaptation to defensive symbionts by parasitoids. However, our experiment provided only partial support for this hypothesis, at best.

To our surprise, only the *H. defensa* strains from two aphid species, *A. fabae fabae* and *A. hederae*, were clearly protective against *L. fabarum*, whereas those from *A. ruborum* and *A. urticata* provided virtually no protection. Accordingly, the host from which *H. defensa* strains were obtained was an important determinant of parasitism success in our experiment. Because all *H. defensa* strains were transferred into the same clone of *A. fabae fabae*, we have to consider the possibility that we observed a lack of protection not because these strains are generally not protective, but because they were tested outside of their natural host species and less well adapted to *A. fabae fabae*. This would have been less of an issue for the *H. defensa* strains from *A. hederae*, because this species is closely related to *A. fabae fabae* (Coeur d'acier et al., 2014). We doubt that the new host species alone explains the lack of protection observed for strains derived from *A. ruborum* and *A. urticata*. There is evidence that *H. defensa* can harm parasitoid embryos even in the absence of aphid hosts (Brandt et al., 2017), and that protective strains remain effective against the same parasitoid species when they are moved between host species, even across genera (Oliver et al., 2005). However, they may not protect against the recipient species' dominant parasitoid species if that is a different one from the dominant parasitoid species of their natural host (Wu et al., 2022). Also, the *H. defensa* strains from *A. ruborum* and *A. urticata* do appear to carry the APSE phage required for protection against parasitoids (Oliver et al., 2009), since we were able to amplify and sequence parts of this phage, but this does not guarantee that their APSE is intact and functional. Hence, we cannot strictly exclude that strains from *A. ruborum* and *A. urticata* might be protective in their original host species. It would therefore be important to also test them for protection against *L. fabarum* in their natural host species, and it would further be helpful to sequence their genomes to carefully assess the state of the APSE phage in these strains.

The presumed host specialization within *L. fabarum*, so far mainly supported by genetic differentiation among wasps collected from different aphid species (Sandrock et al., 2011), was reflected in our experimental data. Parasitoid lines collected from *A. ruborum* and *A. urticata* were not only more difficult to establish on *A. fabae fabae* hosts; they also generally showed lower parasitism success in our experiments compared to lines originating from *A. fabae fabae* and *A. hederae*. This was even the case when the aphids did not carry *H. defensa*. It suggests that the parasitoids collected from *A. ruborum* and *A. urticata* were physiologically less well adapted and therefore less likely to develop in *A. fabae fabae*, because casual observations showed that the wasps did investigate and stab the aphids, as expected from lines success-

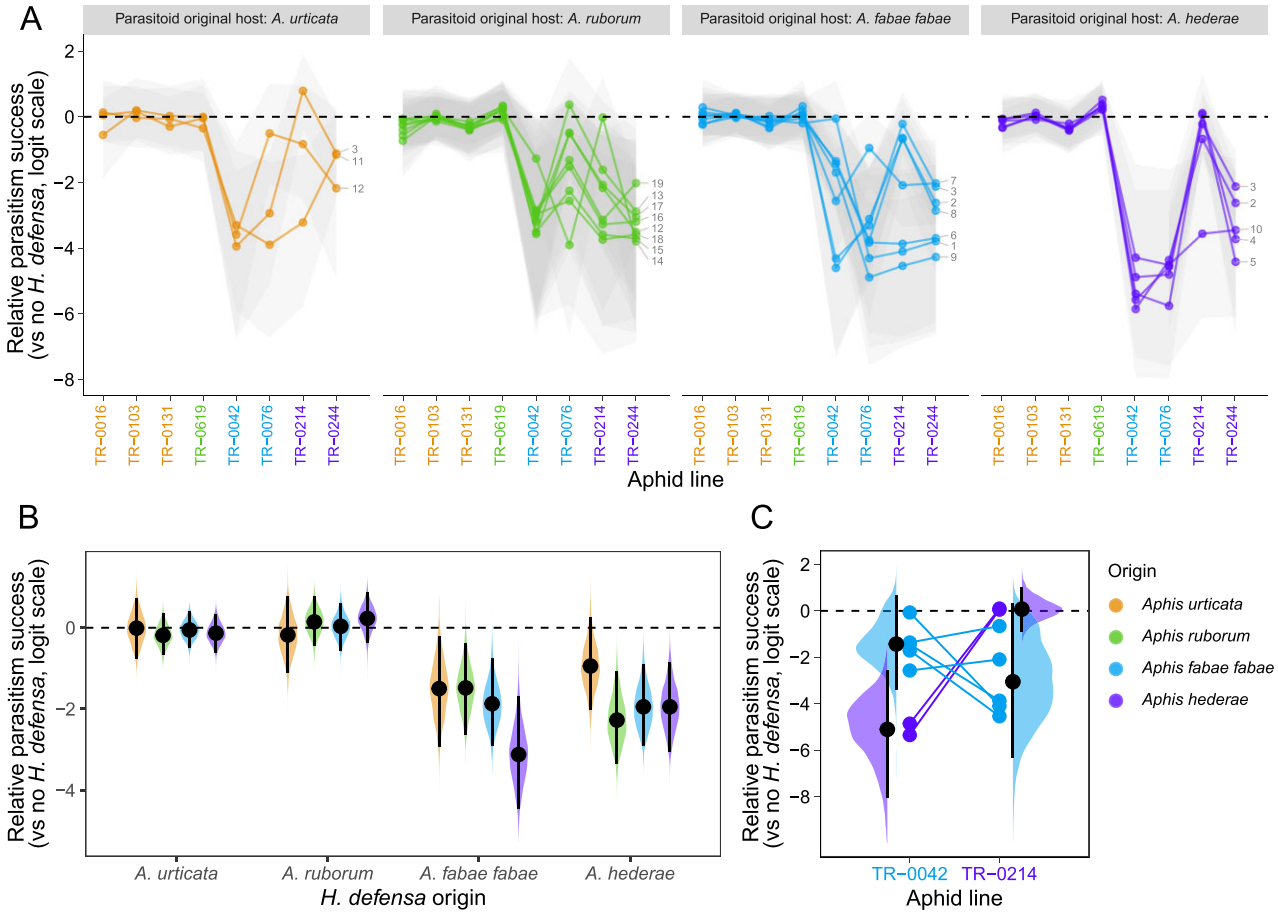

Figure 4. Predicted parasitism success of *L. fabarum* genotypes facing *H. defensa* lines relative to the success against the *H. defensa*-free aphid (represented by the dotted line on zero, log scaled). In (A), we present all *L. fabarum* genotypes × *H. defensa* lines combinations, grouped by *L. fabarum* origin, and with *L. fabarum* genotype identifiers indicated at the tip of lines. In (B), we present the posterior distribution of parasitism success, estimated by parasitoid origin × symbiont origin combinations. In (C), we present the posterior distribution of parasitism success in two selected symbiont strains showing signs of genotype-by-genotype interactions with the parasitoids, depending on their origin. We excluded from this representation all parasitoids from *A. urticata* and *A. ruborum*, as well as all the generalist genotypes. Unique genotypes are represented with coloured dots linked with lines. In (A), dots indicate the predicted mean, and grey ribbons show the 95% credible intervals. In (B) and (C), coloured shaded areas show the posterior distribution, black dots show the predicted mean, and error bars show the 95% credible intervals. In all plots, colours correspond to the origin of *L. fabarum* (inside the plot) or the origin of *H. defensa* (aphid line names on the *x* axis).

fully maintained in the lab for several generations on *A. fabae fabae* host. However, we did not quantify wasp behaviour, nor did we check oviposition success. Our experimental design can therefore not distinguish between aphids that survived because they were not attacked and aphids in which the parasitoid failed to develop. Hence, we cannot exclude that the globally lower parasitism success by *L. fabarum* collected from *A. ruborum* and *A. urticata* may in part be due to reduced interest in the unfamiliar aphids we exposed them to. What we can exclude, based on previous studies with *L. fabarum* on *A. fabae fabae*, is that in cases where we did observe protection by *H. defensa*, the reduced parasitism success was due to wasps avoiding oviposition in *H. defensa*-infected aphids. Similar numbers of eggs were observed in *H. defensa*-positive and *H. defensa*-negative aphids after the same type of assay (Dennis et al., 2017), and in another experiment where each aphid was stabbed exactly once, the protection provided by *H. defensa* was just as clear as in experiments with uncontrolled oviposition (Vorburger et al., 2013). In our analysis, we accounted for all baseline differences in parasitism success across parasitoid genotypes by quantifying *H. defensa*-induced protection relative to the success in unprotected aphids. This approach allowed us to isolate the protective effect of *H. defensa* from inherent variation among the wasp lines.

As protection against *L. fabarum* was restricted to *H. defensa* strains from *A. fabae fabae* and *A. hederae*, we have to look there for signatures of specific adaptation to symbiont-conferred resistance. While the general pattern was not overwhelmingly clear, there was at least some supportive evidence. Aphid line TR-0042, carrying the locally most prevalent strain of *H. defensa* from *A. fabae fabae* (Gimmi et al., 2023), was highly resistant to *L. fabarum*, but five of the six lines having any success at all on these aphids came indeed from *A. fabae fabae*. Similarly, aphid line TR-0214 carried a protective *H. defensa* strain from *A. hederae*, and the majority of wasp lines successfully parasitizing these aphids came from *A. hederae* or belonged to generalist genotypes also collected from *A. hederae* (Figures 3 and 4). Knowing that parasitism success of *L. fabarum* on *H. defensa*-protected aphids is largely determined by genotype-by-genotype interactions between parasitoids and the hosts' symbiont (Cayetano

& Vorburger, 2013; Schmid et al., 2012), these patterns are consistent with the hypothesis that for a given host species, selection will favour those parasitoid genotypes able to overcome this particular host's defensive symbionts. In natural communities, however, these interactions do not play out on a one-by-one basis. A parasitoid may exploit multiple host species whose relative abundance can vary in space and time, host species themselves differ in how commonly they carry *H. defensa* (Henry et al., 2022; Vorburger & Rouchet, 2016), and—as seen here—*H. defensa* harboured by different species may be unequally protective. Coevolutionary interactions are embedded in local communities that show spatial and temporal variation, forming mosaics of varying selection strength (Thompson, 2005). Expecting a perfect correspondence of traits and counter-traits between interacting species may therefore be asking too much from coevolution, as stated eloquently by Thompson (1999). This might help to explain why for the other two protective strains harboured by lines TR-0244 (from *A. hederae*) and TR-0076 (from *A. fabae fabae*), no pattern of specific adaptation by parasitoids was evident. They were so strongly protective that they permitted very little parasitism no matter where the parasitoids came from. An alternative explanation for this observation could be that the parasite is not always ahead in its coevolutionary arms race with the host. Especially in the present system, where parasitoids have smaller population sizes, slightly longer generation times, and more limited dispersal than their hosts, the tide may also turn (Gandon & Michalakis, 2002; Greischar & Koskella, 2007).

The 2020 collection in and around Zurich recovered most of the same *H. defensa* strains in the four aphid species that had already been discovered during a larger sampling campaign in the previous year (Henry et al., 2022, see Figure S1). We can thus be confident that we had a comprehensive representation of the local *H. defensa* strain diversity in our experiment, with the exception of one abundant strain of *A. ruborum* that was lacking, unfortunately. It was remarkable that all introductions of new *H. defensa* strains into the *A. fabae fabae* clone used in the experiment led to stable, heritable infections without any obvious harmful effects on the host. Unstable or highly virulent new infections were reported in some other transfection studies (e.g., Łukasik et al., 2015; Wu et al., 2022), but these included transfers between distantly related species from different genera, whereas we only moved strains among congeneric species. The natural diversity of *L. fabarum* genotypes was less comprehensively represented in our experiment than that of *H. defensa* strains, as shown by the rarefaction curves (Figure S2). Nevertheless, they exhibited ample variation in their ability to overcome *H. defensa*-conferred resistance. It may seem surprising to find so much genotypic diversity (19 distinct genotypes among 35 lines) in a predominantly asexual species. This has also been reported in a much larger survey using the same set of markers more than a decade ago (180 genotypes among 706 samples: Sandrock et al., 2011). The high diversity of thelytokous (asexual) genotypes can be explained by the fact that thelytoky has a simple genetic basis in *L. fabarum* (Sandrock & Vorburger, 2011), such that a process termed contagious parthenogenesis (Engelstädter et al., 2011; Simon et al., 2003) can repeatedly give rise to new asexual lines from coexisting sexual populations, which are rare in central Europe but more common in southern Europe (C. Vorburger, personal observation, 2023). As an aside, three of the *L. fabarum* genotypes used in the present study were already identified among the most abundant asexual genotypes in the study by Sandrock et al. (2011), using the same microsatellite markers (data not shown). Some asexual lines can thus persist for extended periods of time in natural populations. Notably, two of those three shared lines were classified as generalist (found in mummies from different aphid species) by Sandrock et al. (2011), but were only sampled once and thus classified as specialists in our collection, highlighting the limits of this classification in case of non-exhaustive sampling.

Coming back to the observation that *H. defensa* strains from two aphid species were not protective—assuming that the lack of protection was not an artefact from moving these strains into a new host species—an obvious question is what maintains this symbiont at moderate (*A. urticata*: approx. 25%) or even high (*A. ruborum*: approx. 90%) prevalence in these aphid populations. This relatively high prevalence is even more surprising considering that hosting *H. defensa* may come at a lifespan cost (Vorburger & Gouskov, 2011). A possible explanation could be that *H. defensa* provides benefits other than resistance to parasitoids. Once a bacterial endosymbiont has evolved vertical transmission, its fitness becomes tightly linked to the host's survival and reproduction. This may favour the evolution of traits promoting host survival (Ewald, 1987; Herre et al., 1999), which include, but are not restricted to, protection against natural enemies (Jones et al., 2011; Lively et al., 2005). Other benefits provided by facultative heritable endosymbionts in aphids include increased tolerance to thermal stress (e.g., Montllor et al., 2002; Russell & Moran, 2006) or the ability to exploit specific host plants (e.g., Tsuchida et al., 2004; Wagner et al., 2015). The same symbiont may also provide multiple benefits to its aphid host (Heyworth & Ferrari, 2016). Hence, we speculate that *H. defensa* may provide additional, yet undiscovered ecological benefits to *A. urticata* and *A. ruborum*. This hypothesis is supported by increasing evidence challenging the view of parasitoids being the main drivers of balancing selection acting on protective secondary endosymbiont frequencies. For instance, Smith et al. (2021) demonstrated that parasitoids were only responsible for 10% of *H. defensa* frequency changes in natural populations of pea aphids, while temperature appeared to be the best predictor. A similar observation was made by Gimmi et al. (2023) for the black bean aphid, *A. fabae fabae*. This suggests that there are unidentified thermally sensitive costs and benefits associated with hosting *H. defensa*.

To conclude, our experiment showed that the natural host species of *H. defensa* as well as the natural host species of the wasps had a strong effect on *L. fabarum* parasitism success. The latter was largely a consequence of doing all tests in *A. fabae fabae*, to which many *L. fabarum* genotypes originally associated with other hosts were less well adapted—a reflection of the general pattern that host specialization prevails in insect parasitoids (Godfray, 1994; Smith et al., 2006, 2008). Heritable endosymbionts are an important part of the defence arsenal in several insects, including aphids (Oliver & Perlman, 2020; Oliver et al., 2014). Symbiont-conferred defences may therefore be targets of parasitoid counteradaptation (Dennis et al., 2017; Dion et al., 2011; Rouchet & Vorburger, 2014), in addition to the host-encoded behavioural, structural, and physiological defences, which are undoubtedly important as well. If different host species are associated with different defensive symbionts, as is the case in our study system, host

specialization may also entail adaptation to the host's defensive symbionts. Our test of this hypothesis was not fully conclusive, but it provided evidence of such adaptation against two protective strains of *H. defensa*, lending support to the proposition that defensive symbionts can be important mediators of host–parasite coevolution (King & Bonsall, 2017; Vorburger & Perlman, 2018).

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

Datasets of the parasitoid sampling, aphid sampling, and parasitism success experiment, as well as the R scripts used for their analysis, are all available on Zenodo (DOI: 10.5281/zenodo.8389105).

Author contributions

Youn Henry (Conceptualization [equal], Formal analysis [equal], Investigation [equal], Writing - original draft [lead], Writing - review & editing [equal]), Maxime Dahirel (Formal analysis [equal], Writing - review & editing [supporting]), Jesper Wallisch (Investigation [equal]), Sandro Ginesi (Investigation [equal]), and Christoph Vorburger (Conceptualization [equal], Funding acquisition [lead], Investigation [supporting], Writing - original draft [supporting], Writing - review & editing [equal]).

Funding

This project was funded by the Swiss National Science Foundation (grant number 31003A_181969 to C.V.).

Acknowledgments

We are grateful to Elena Gimmi for kindly sharing with us some of the parasitoid lines used in this study. We also thank Paula Rodriguez for her wise advice and help with insect collection.

Conflicts of interest

Authors declare no conflict of interest.

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Received November 28, 2024; revised April 30, 2025; accepted June 25, 2025