Parasitoids as Drivers of Symbiont Diversity in an Insect Host

Authors: Nina Hafer-Hahmann¹ & Christoph Vorburger^{1,2}

1: EAWAG, Swiss Federal Institute of Aquatic Science and Technology, Überlandstrasse 133, 8600
Dübendorf, Switzerland

2: Institute of Integrative Biology, ETH Zürich, Universitätsstrasse 16, 8092 Zürich, Switzerland

E-mail: NH: nina.hafer@eawag.ch; CV: christoph.vorburger@eawag.ch

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Corresponding author: Nina Hafer-Hahmann, EAWAG, Swiss Federal Institute of Aquatic Science and
Technology & Institute of Integrative Biology, Überlandstrasse 133, 8600 Dübendorf, Switzerland; Phone:
+41 58 765 6404 ; Fax: +41 58 765 5802; E-mail: nina.hafer@eawag.ch

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Abstract

Immune systems have repeatedly diversified in response to parasite diversity. Many animals have outsourced part of their immune defence to defensive symbionts, which should be affected by similar evolutionary pressures as the host's own immune system. Protective symbionts provide efficient and specific protection and respond to changing selection pressure by parasites. Here, we use the aphid *Aphis fabae*, its protective symbiont *Hamiltonella defensa*, and its parasitoid *Lysiphlebus fabarum* to test whether parasite diversity can maintain diversity in protective symbionts. We exposed aphid populations with the same initial symbiont composition to parasitoid populations that differed in their diversity. As expected, single parasitoid genotypes mostly favoured a single symbiont that was most protective against that particular parasitoid, while multiple symbionts persisted in aphids exposed to more diverse parasitoid populations, which in turn affected aphid population density and rates of parasitism. Parasite diversity may be crucial to maintaining symbiont diversity in nature.

Introduction

Genes involved in immune defences are amongst the most diverse in any living organism. This diversity allows their owners to defend themselves against a diverse array of parasites and pathogens (hereafter parasites). In vertebrates, for example, a multitude of different major histocompatibility complex (MHC) alleles enables the specific and efficient response to parasites (Bernatchez & Landry 2003; Wegner *et al.* 2004; Sommer 2005; Milinski 2006; Piertney & Oliver 2006). Similarly, the CRISPR/Cas System (Horvath *et al.* 2010; Bhaya *et al.* 2011; Garrett *et al.* 2011; Sorek *et al.* 2013) or Ig-proteins (Watson *et al.* 2005) provide a variable component to the immune system of bacteria and insects, respectively. It seems that such diversification of the immune system is a predictable outcome of the host's evolutionary arms race with a diverse array of parasites (Litman *et al.* 2007; Messier-Solek *et al.* 2010; Ghosh *et al.* 2011). The diversity of alleles is so high that a single organism can only express a fraction of these variants. Organisms are selected to express the appropriate variants and diversity depending on the dominant parasites and their diversity (Apanius *et al.* 1997; Bernatchez & Landry 2003; Wegner *et al.* 2004; Sommer 2005; Milinski 2006; Piertney & Oliver 2006; Spurgin & Richardson 2010).

Many animals, especially arthropods, have outsourced some of their defence against parasites to protective symbionts (Brownlie & Johnson 2009; Flórez *et al.* 2015). Aphids represent the most prominent example of this. They are frequently infected with a diverse array of heritable bacterial endosymbionts that provide specific and effective protection against natural enemies such as entomopathogenic fungi and parasitoid wasps (reviewed by Oliver *et al.* 2010, 2014; Vorburger 2014; Guo *et al.* 2017; Vorburger & Perlman 2018). Different aphid species and populations vary in their

symbiont repertoire (reviewed by Oliver *et al.* 2014; Vorburger 2014; Flórez *et al.* 2015; McLean *et al.* 2016; Zytynska & Weisser 2016; Guo *et al.* 2017), and the symbionts usually occur at intermediate frequencies. Different symbiont species or different strains of the same symbiont species can co-occur within the same aphid species, population, or even individual (reviewed by Oliver *et al.* 2014; McLean *et al.* 2016; Zytynska & Weisser 2016; Guo *et al.* 2017), raising the question of how this diversity is maintained. Similar to other forms of immunity, protection by endosymbionts usually comes at a cost to the host. In the absence of parasites, aphids carrying protective symbionts tend to suffer from reduced competitive ability or lower reproduction and longevity (Oliver *et al.* 2008; Vorburger & Gouskov 2011; Cayetano *et al.* 2015; Heyworth & Ferrari 2015; Parker *et al.* 2017). Parallels between protective symbionts and other specific immune defences based on diverse nuclear alleles suggest that similar evolutionary forces should be at work (Hafer & Vorburger 2019). The diversity of parasites may thus drive and maintain the diversity of protective symbionts as well. Indeed, there is good evidence that selection by parasitoids increases frequencies of protective symbionts in aphid populations (Herzog *et al.* 2007; Oliver *et al.* 2008; Xie *et al.* 2015; Käch *et al.* 2018). This symbiont-conferred resistance seems to be specific. Different strains of the defensive endosymbiont *Hamiltonella defensa* (Moran *et al.* 2005) are unequally protective against different parasitoid species (Cayetano & Vorburger 2015; McLean & Godfray 2015; Leclair *et al.* 2016), or even against different genotypes of the same parasitoid species (Rouchet & Vorburger 2012; Schmid *et al.* 2012; Cayetano & Vorburger 2013, 2015; Cayetano *et al.* 2015; Vorburger & Rouchet 2016). This implies that different parasitoids may select for different symbionts in their host populations. Here we take a step further to test whether symbiont diversity is indeed maintained by parasitoid diversity, using an

experimental evolution approach. We exposed experimental aphid populations containing multiple strains of *H. defensa* to more or less diverse populations of parasitoids over multiple generations, and we followed the symbiont frequencies in the aphid populations. The prediction was that symbiont diversity should be maintained under selection by multiple parasitoid genotypes, whereas with only one parasitoid genotype, only the symbiont that provides the best protection against this particular parasitoid should persist. Our findings support these predictions, highlighting the crucial role parasites may play in maintaining the diversity of immune defences, also when provided by symbionts. In the absence of any parasitoids, symbionts decreased and could be expected to be lost eventually due to the costs they impose on their hosts.

Material & Methods

Insects

The black bean aphid, *Aphis fabae*, is an important pest of broad bean (*Vicia fabae*) and sugar beet (*Beta vulgaris*). We used four different lines of a single *A. fabae* clone which originated from a single female collected in St. Margrethen, Switzerland from *Chenopodium album* in 2006 (clone A06-405). The four lines differed only by their infections with the endosymbiotic bacterium *H. defensa*, a well-known protective symbiont that confers increased resistance against parasitoid wasps in several aphid species (Oliver *et al.* 2003; Schmid *et al.* 2012; Asplen *et al.* 2014). Three lines were created by experimentally introducing one of three genetically distinct *H. defensa* strains by microinjection (strain IDs H15, H76, and H402). The fourth line did not receive any *H.*

defensa (Cayetano *et al.* 2015). All aphid lines have been maintained clonally on broad beans at ca. 18-20°C and a 16/8 h light/dark regime prior to use in this experiment.

As parasitoids we used three asexual isofemale lines of *Lysiphlebus fabarum*, a specialised and frequent parasitoid of *A. fabae* and other species mostly of the genus *Aphis* (Tomanović *et al.* 2018). These lines originated from single females collected near Zürich, Switzerland in 2007 from *A. fabae fabae* (IL07-64), near Geneva, Switzerland in 2009 from *A. fabae cirsiacanthoides* (IL09-348), and from *A. fabae fabae* near Orbe, Switzerland in 2009 (IL09-369). For simplicity, we refer to these parasitoid wasp lines as W64, W348 and W369 hereafter. We chose lines that were known from previous work to differ in their ability to infect aphids protected by various strains of *H. defensa*. W64 is able to parasitize aphids harbouring H15 and H402 but does poorly on aphids with H76. W369 can parasitize aphids with H15 and, to a lesser extent, H76, but not aphids with H402. W348 can parasitize aphids with H402 and, less successfully, H76, but has never been tested on aphids with H15 (Cayetano *et al.* 2015; Vorburger & Rouchet 2016). Since their collection these wasps have been reared on symbiont-free *A. fabae* of a different line than the one used in this study at ca. 22°C with a 16 h photoperiod.

Setup of the selection experiment

At the start of the experiment, we prepared 30 aphid populations with the same composition in insect rearing cages (24.5 x 24.5 x 24.5 cm; BugDorm-4F2222; MegaView Science, Taiwan). The cages contained three pots with ca. 2-weeks old broad bean plants (*Vicia faba*, 2 plants per pot), and the aphid populations comprised equal proportions of *H. defensa*-free (H-), H15, H76, and H402 infected aphids. We added 9 adult aphid females of each line to every cage (3 per pot), i.e. 36 adults in total. Aphids could move

freely between plants within their cage. After allowing the aphids to reproduce for four to six days, we applied six different treatments to the aphid populations (five replicate cages per treatment). Treatments consisted either of no wasps (NoWasp), wasps of a single asexual line (W64, W348, or W369), a simultaneous mix with equal proportions of the three wasp lines (WMixSim), or a sequential mix of the three wasp lines, i.e. wasps from a single line in each generation, but alternating lines between generations (WMixSeq; Fig. 1A). In the first generation, the wasp treatments were applied by adding 12 female wasps of the appropriate line(s) to each cage. This number was increased to 18 wasps per cage for all subsequent generations. After the addition of wasps, we left the populations undisturbed for 11-12 days (see Table S1 for a detailed overview of the timing in each generation). The wasps have a short lifespan of only two to three days (Satar *et al.* 2019), and successfully parasitized aphids are killed and become easily recognizable as mummies within 7-9 days after parasitoid oviposition. We then collected 36 healthy adult aphids from each cage to serve as parents of the next aphid generation. To ensure that they were a representative sample of the surviving aphids we took 12 adults from each pot and we took care to take them from different places on the plants to avoid taking offspring of a single mother. If we were unable to obtain 12 aphids from one pot, we collected the remaining aphids equally from the other pots, and we substituted with the oldest nymphs we could find if we were unable to recover 36 healthy adults. The collected aphids were used to inoculate plants for the subsequent generation by adding 12 aphids to each new pot in a fresh cage (Fig. 1B). Experimental evolution continued for 10 generations, although we lost one replicate evolution line of treatment WMixSeq, because the aphid population went extinct after 8 generations.

Data collection

During each transfer, we counted the number of mummies, we roughly estimated the number of aphids by counting aphids in groups of ca. 10 individuals, and we measured the total stem length of all plants in the old cages. Additionally, at the end of the 2nd, 6th, and last (10th) generation, we collected 15 unummified aphids per cage (5 aphids from each pot) to estimate aphid population composition. Aphids were stored at -20°C until further analysis.

Aphid DNA was extracted using high salt extractions following the protocol in (Sunnucks & Hales 1994), albeit adapted to a 96 deep well plate format (Gouskov *et al.* 2016). Subsequently, DNA was used for diagnostic PCRs (see Ferrari *et al.* 2012) to test for the presence of *H. defensa* and the obligate endosymbiont of aphids, *Buchnera aphidicola*, with symbiont-specific primer pairs amplifying parts of the bacterial 16S rRNA gene. Since *B. aphidicola* is present in all individuals, this served as a control for the success of the DNA extraction. Samples that were negative for *B. aphidicola* were discarded. Initially, we conducted separate PCRs for *B. aphidicola* (forward primer: 16SA1; AGAGTTTGATCMTGGCTCAG (Fukatsu & Nikoh 1998) & reverse primer: Buch_16S_Afab_R: CTTCTTCGGGTAACGTCAAGAA) and *H. defensa* (forward primer: 10F: AGTTTGATCATGGCTCAGATTG & reverse primer: T419R: AAATGGTATTCGCATTTATCG) (Ferrari *et al.* 2012), but during the course of this work switched to a different reverse primer for *B. aphidicola* (Buch_R_CV2: CCCCCACTTTRGTTTTTCAAC) which resulted in a shorter product enabling us to multiplex the PCRs. We confirmed that results were consistent between both primer pairs. For aphids positive for *H. defensa* we additionally amplified part of the *murE* gene (forward primer: murE16F: ACTAACGGGAAAACCACTAATAC & reverse primer:

murE936R: TTGAGAATGTCAGCGGTAATC) (Henry *et al.* 2013) and sent the amplicons to Microsynth (Balgach, Switzerland) for sequencing. The *murE* sequences differ between the three strains we used (Cayetano *et al.* 2015), enabling us to determine the strain present in each *H. defensa* positive aphid.

Statistical Analysis

All analyses and plot generation took place in R, version 3.6.0 (R Core Team 2019). To investigate treatment effects on population dynamics, we analyzed the number of aphids and the number of mummies and mummification rate as a function of the factors treatment, generation and their interaction, using linear mixed models (LMM) from the *lme4* package (Bates *et al.* 2015). Cage (replicate) was included as a random effect to account for the non-independence of successive counts from the same replicate populations. We also analyzed plant size with a LMM, as it may be affected by aphid population size. If the treatment $\times$ generation interaction was not significant ($p > 0.05$), it was removed from the models. The variance among treatments was further partitioned into the difference between cages with and without parasitoids, with three or one wasp genotype, and the variation among all parasitoid treatments, using linear orthogonal contrasts. To conform with the assumption of normality, aphid and mummy numbers and mummification rate were transformed using the *transformTukey* function from the *rcompanion* package (Mangiafico 2019) with a lambda of 0.75 for aphids, 0.375 for mummy number, and 0.325 for mummification rate.

To investigate changes of the aphid population composition in response to our treatments, we analyzed the proportions of *H. defensa*-infected aphids and of aphid lines with each *H. defensa* haplotype (H15, H76, H402) with similar LMMs. To conform to the assumption of normality proportions were transformed by using the arc-sine of its

square root except for the proportion of H15 where we used a power of 0.1 prior to calculating arc-sine. Because *H. defensa* became fixed in some treatments at the end of the experiment, we added one individual from each line to one replicate of every generation in the dataset prior to analysis to avoid problems with model fitting. If anything, this should have reduced differences and hence made comparisons more conservative. Each model was followed with a type III analysis of variance (anova) using *lmerTest* to obtain p-values for fixed effects with Satterthwaite's degrees of freedom method (Kuznetsova *et al.* 2017). Significant differences between treatments with wasps were followed up with post hoc tests using *emmeans* (Lenth 2019).

Finally, to analyze changes in the diversity of aphid lines (i.e. *H. defensa* absence or haplotype), we calculated the Shannon index for each replicate (including the additional individual in one replicate from each line, see above) using the R package *vegan* (Oksanen *et al.* 2019). The Shannon index was then used as response in a linear mixed model similar to the ones described above. We also calculated beta diversity across replicates of the same treatment with *divEst* by estimating diversity and partitioning it into alpha and beta diversity (*entropart* package; Marcon & Hérault 2015). *divEst* calculates bootstrap confidence intervals by drawing simulated communities from a multinomial distribution that follows the observed frequencies which allowed us to calculate lower and upper 95% confidence intervals.

Results

Parasitoid wasps selected strongly against *H. defensa*-free aphids, such that they virtually disappeared over time in all treatments containing wasps ($p < 0.0001$, Fig. 2A, Tables 1A, S2). In the treatment without wasps, on the other hand, the *H. defensa*-free

aphids increased over time and reached over 80% by the end of the experiment (p<0.001, Tables S3-S4). The presence of wasps diminished aphid numbers (p<0.0001, Fig. 3A, Tables 1F, S6), but this did not entail significant variation in plant size among treatments (p=0.1608, Fig. 3B, Tables 1G, S6).

Concurrent with the increase of *H. defensa*-protected aphids in all cages with parasitoids, the number of mummies and mummification rate began to decline after the first two to three generations of selection (p<0.001, Fig. 3C-D, Tables 1H-I, S6). However, the trajectories of these declines differed among treatments (p<0.001, Tables 1H-I, S6). In two treatments with single wasp genotypes (W64 and W369), the number of mummies declined rapidly (p<0.04) and the mummification rate remained close to zero after about four generations (Fig. 3C-D, Tables S7-S8), which is indicative of highly resistant aphid populations. Accordingly, aphids grew to larger population sizes in treatments with either of these wasp genotypes, especially W369, than in the other treatments with wasps (p<0.02, Fig. 3A, Tables S8-S9). These two wasps lines indeed selected strongly for a single (but different) *H. defensa* strain (W64 for H76, W369 for H402), which became fixed in all replicates of the respective treatment (Fig. 2B-C, S1, Tables 1B-C, S3-S5). The third treatment with a single wasp line (W348) was somewhat different in that two *H. defensa* strains (H76 & H402) persisted until the end of the experiment (Fig. 2B-C, Tables S3-S5), although this was mainly due to a single replicate that became fixed for aphids with H402, whereas the other replicates became dominated by aphids carrying H76 (Fig. 4). The two *H. defensa* strains H76 and H402 were also maintained in both treatments applying all wasp lines, either simultaneously or sequentially (Fig. 2B-C, Tables S3-S5). Again, several replicates became fixed for one or the other strain (Fig. 4), but both strains persisted in both treatments. Accordingly,

261 H76 and H402 were significantly more frequent in the presence of the wasp genotype
262 against which they conferred strong resistance (W64 and W369) than in any of the
263 other treatments ($p < 0.05$, Fig. 2B-C, S1, Tables S4-S5). Strain H15 was eliminated from
264 all treatments with parasitoids, but it remained present across generations in the
265 parasitoid-free treatment and hence showed a non-significant trend to be more frequent
266 without wasps ($p = 0.0837$, Fig. 2D, Tables 1D, S2).

267 The unequal selection exerted by the different wasp treatments was also reflected in
268 aphid population diversity. Because all populations started at maximal diversity (4 lines
269 at equal proportions), Shannon diversity declined in all treatments ($p < 0.0001$, Fig. 2E,
270 Tables 1E, S2). While diversity dropped to zero in two out of three treatments with
271 single wasp genotypes, it did not drop so low in the two mixed wasp treatments, and it
272 remained highest in the parasitoid-free treatment, where no aphid line was eliminated
273 completely (Fig. 2E, S1). However the main effect of treatment on Shannon diversity was
274 not statistically significant ($p = 0.0811$, Tables 1E, S2). Beta diversity, on the other hand,
275 increased from the initial minimum for all treatments, but it returned to a minimal level
276 in the treatments where a single wasp line consistently selected for one *H. defensa* strain
277 (W64 & W369), while it stayed relatively high for the two mixed treatments as well as
278 treatment W348 and the wasp-free treatment (Fig. 2F, S1, Tables 2, S10). In those wasp
279 treatments that maintained *H. defensa* strain diversity across generations, the number of
280 mummies and mummification rate by parasitoids remained higher than in the
281 treatments that resulted in fixation of a single *H. defensa* strain (Fig. 3C-D, Tables S3-S4).

Discussion

Parasitoid wasp diversity strongly influenced endosymbiont diversity in our laboratory host populations. In the presence of wasps, *H. defensa* increased rapidly in frequency and approached fixation within just a few generations. Two wasp genotypes selected strongly for single (but different) strains of *H. defensa*, such that a higher strain diversity of symbionts was maintained when these wasps occurred in mixtures of wasp genotypes rather than alone.

Without wasps, *H. defensa* frequencies decreased in the aphid populations, with strain H15 possibly persisting somewhat longer than the other two strains (Fig. 4). It seems likely that given more time, *H. defensa* would have died out completely. This reflects the known fitness costs *H. defensa* induces by reducing reproduction and longevity of aphid hosts (Vorburger & Gouskov 2011; Cayetano *et al.* 2015; Martinez *et al.* 2018; Sochard *et al.* 2019). Costs to the host vary among different strains of *H. defensa*, which may at least partly be related to different infection intensities (titers) achieved by different strains. Our experiment confirms that these costs are strong enough to drive *H. defensa* (nearly) to extinction when they are not offset by any benefits.

The benefit offsetting the costs in our experiment was the strong resistance *H. defensa* provides against parasitoids, in agreement with previous studies on defensive symbionts in captive insect populations (Herzog *et al.* 2007; Oliver *et al.* 2008; Xie *et al.* 2015; Käch *et al.* 2018). The selection for symbiont-conferred protection led to increased population-level resistance, as shown by the corresponding decrease in mummification, albeit this decrease and hence the protective effect of *H. defensa* differed greatly between treatments (i.e. wasps). Wasp identity, composition, and diversity were crucial in deciding which *H. defensa* strains persisted. In two of the three single wasp line

treatments, namely W369 and W64, a single, but different *H. defensa* strain quickly went to fixation and any symbiont diversity was lost. The remaining strain of *H. defensa* provided effective protection as illustrated by the parallel drop in mummification and the limited impact parasitoids had on aphid numbers (Fig. 3). This confirms the previously observed specificity of *H. defensa* protection (Rouchet & Vorburger 2012; Schmid *et al.* 2012; Cayetano & Vorburger 2013, 2015; Cayetano *et al.* 2015; McLean & Godfray 2015; Leclair *et al.* 2016; Vorburger & Rouchet 2016) and shows that without any wasp diversity, *H. defensa* diversity can be lost rapidly. The remaining aphids were so well protected against the specific wasp strain that without us resupplying individuals every generation, the wasps would have been unlikely to persist (see Käch *et al.* 2018). There is good evidence that, given enough time, parasitoid populations can adapt to symbiont-conferred resistance (Dion *et al.* 2011; Dennis *et al.* 2017), but by using asexual lines and supplying parasitoids every generation anew, we precluded this possibility in the present experiment. In nature, of course, neither aphids nor parasitoids occur in closed populations. Spatial or temporal refuges can shelter *H. defensa*-free aphids from parasitism, local extinctions of parasitoids may allow their re-immigration and population growth, which in turn would favour the re-establishment of wasps. The interplay of these processes may well result in the intermediate *H. defensa* frequencies observed in nature (Russell *et al.* 2013; Smith *et al.* 2015; Vorburger & Rouchet 2016; Zytynska & Weisser 2016).

The third single wasp line treatment, W348, also selected strongly for *H. defensa*, but it did not result in the complete fixation of a single strain of *H. defensa*. This line of *L. fabarum* seems to have the ability to parasitize aphids harbouring all *H. defensa* strains included in this study (Vorburger & Rouchet 2016), although it exerted the strongest

selection for H76 (Fig. 2). Only one replicate became dominated by H402 (Fig. 4). Considering the limited number of founders and individuals transferred every generation, some degree of stochasticity in the outcome is expected when no strain is fully protective or clearly inferior in protecting hosts, such as H15, which disappeared from all cages containing wasps. Given more time, H76 may have become fixed at least in those replicates where it did not disappear initially. Nevertheless, we cannot rule out that this parasitoid's ability to overcome resistance by different symbiont strains resulted in less biased selection enabling the maintenance of symbiont diversity for a longer period, similar to a mixture of wasps (see below).

Both of the *H. defensa* strains H76 and H402 were also maintained when the parasitoids comprised a mixture of the parasitoid lines, either simultaneously or sequentially. Each strain provided effective protection against a different wasp line; their effects on host fitness were therefore more balanced in the presence of multiple lines of wasps. However, this was not evident within every cage. Many replicates became dominated by one of the strains (Fig. 4), likely reflecting the role of random drift, but across replicates two *H. defensa* strains persisted, such that beta diversity remained high in both mixed parasitoid treatments (Fig. 2E). Our findings thus show that parasitoid diversity can indeed promote symbiont diversity in host populations. In a field study manipulating plant diversity, Zytynska *et al.* (2016) observed that higher plant diversity resulted in higher symbiont diversity in aphid populations colonizing these plants. The authors speculated that this could, among other factors, be mediated by parasitoid diversity, because parasitoid diversity tends to increase with higher plant diversity (e.g. Petermann *et al.* 2010). The results of our laboratory study would indeed be consistent with this conjecture.

We focused exclusively on aphids infected with a single facultative endosymbiont, but in nature individual aphids can harbour multiple symbionts (McLean *et al.* 2016; Zytynska & Weisser 2016; Guo *et al.* 2017). Multiple infections are relatively rare in the black bean aphid (Vorburger & Rouchet 2016), but they can be very common in other aphid species such as the pea aphid, *Acyrtosiphon pisum*. Coinfections may alter costs and benefit in ways not necessarily predictable from single infections (McLean *et al.* 2018; Weldon *et al.* 2019), but generally the defense provided by protective symbionts is retained, albeit often at higher costs to the hosts (Doremus & Oliver 2017; Leclair *et al.* 2017; McLean *et al.* 2018).

Biologists have long used genetic interaction models to study antagonistic coevolution and the resulting maintenance of diversity. Classical examples include the gene for gene model, initially based on plant-pathogen interactions, where each resistant allele in the host has to be countered by a virulent allele in the corresponding locus of the parasite for successful infection (Flor 1956) and the matching alleles model, initially inspired by the vertebrate immune system, where the parasites require the alleles that exactly match the appropriate resistance alleles in a host (Frank 1993). Further possible models exist on a continuum between these endpoints (Agrawal & Lively 2002), and the same framework can be applied to protective symbionts (Kwiatkowski *et al.* 2012). The patterns of high specificity we observed fit well with the matching alleles model, albeit some parasitoids (e.g. W348) seem more generalised in overcoming resistance. Under a matching allele model, variation in frequencies of different parasites will result in an increase in symbionts providing resistance against the most frequent parasite type, which in turn selects for other parasites able to overcome this resistance. Cycling between different parasite genotypes (or species) and

378 different protective symbionts will ensue as long as the population size is large enough
379 or re-immigration prevents complete extinction. Such Red Queen dynamics are
380 fundamental to the co-evolution of antagonists and help maintain variation in host
381 defences, such as immune genes, as well as parasite offenses (reviewed e.g. by Ebert
382 2008; Brockhurst & Koskella 2013; Brockhurst *et al.* 2014; Strotz *et al.* 2018). Here we
383 could show that, akin to the role parasites play in shaping an animal's own immune
384 system, they can also play a crucial role in maintaining the diversity of protective
385 symbionts. An important difference remains, though. Immune genes are reshuffled and
386 inherited biparentally, such that animals have the potential to shape their offspring's
387 immune system through adaptive mate choice. By contrast, symbionts are usually
388 transmitted maternally without mixing, albeit routes of horizontal transmission for
389 heritable symbionts exist, such as being vectored by parasitoids (Gehrer & Vorburger
390 2012). Nevertheless, such events are likely to be rare and it is currently unknown
391 whether they play a significant role in this system (Hafer & Vorburger 2019).

392 Our observations have applied implications as well, because parasitoids are
393 frequently used to control pest aphids in greenhouse crops. The effectiveness of
394 parasitoid release may be strongly reduced in the presence of *H. defensa* (Käch *et al.*
395 2018), but using multiple or alternating wasp lines and resupplying them regularly may
396 maintain parasitoid diversity and hence increase the chances of successful biocontrol if
397 pests harbour multiple protective symbionts. In medicine, similar strategies are used to
398 overcome the evolution of antibiotic resistance, for example by using multiple
399 antibiotics simultaneously or sequentially (e.g. Bonhoeffer *et al.* 1997; Sun *et al.* 2010;
400 Baym *et al.* 2016).

Protective symbionts are widespread throughout the animal kingdom and not only protect against parasites. Some symbionts can also protect against predators, for example (reviewed by Flórez *et al.*, 2015). Do antagonistic interactions with a variety of natural enemies also promote the diversity of symbionts in other systems? The answer to this question will depend on how targeted microbial defenses are. The specificity seen in the *H. defensa*-*L. fabarum* interaction is certainly not unique, even though its degree may be unusual. Protection by microbial symbionts frequently relies on the production of chemicals with narrow toxicity to certain predators and parasites, or with specific antifungal, antibacterial, or antiviral properties (Flórez *et al.* 2015). That the diversity of an organism's microbial protectors is shaped by the diversity of its natural enemies may therefore be a very general phenomenon.

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Tables

Table 1: Linear mixed effect models. Note that non-significant interactions have been removed from the final models and in these cases, statistics for treatment and generation are for the model without interaction. Significant p-values are highlighted in bold.

factor	df	F	p
A: Proportion of <i>H. defensa</i>			
Treatment	5,24	11.800	<0.0001
Generation	3,71	32.010	<0.0001
Treatment: Generation	15,71	8.220	<0.0001
B: Proportion of H76			
Treatment	5,23	8.040	0.0002
Generation	3,71	3.080	0.0329
Treatment: Generation	15,71	4.250	<0.0001
C: Proportion of H402			
Treatment	5,24	8.340	0.0001
Generation	3,71	0.320	0.8131
Treatment: Generation	15,71	3.780	0.0001
D: Proportion of H15			
Treatment	5,24	2.230	0.0837
Generation	3,86	33.840	<0.0001
Treatment: Generation	15,71	1.360	0.1931
E: Shannon diversity			
Treatment	5,24	2.270	0.0802
Generation	3,86	146.850	<0.0001
Treatment: Generation	15,71	0.830	0.6418
F: Aphid number			
Treatment	5,24	19.420	<0.0001
Generation	9,258	17.380	<0.0001
Treatment: Generation	45,213	1.210	0.1887
G: Plant size			
Treatment	5,24	1.750	0.1608
Generation	9,258	19.780	<0.0001
Treatment: Generation	45,214	0.610	0.9761
H: Mummy number			
Treatment	4,20	8.840	0.0003
Generation	9,177	34.510	<0.0001
Treatment: Generation	36,177	2.550	<0.0001
I: Mummification rate			
Treatment	4,19	28.950	<0.0001
Generation	9,173	18.940	<0.0001
Treatment: Generation	36,173	2.930	<0.0001

621 **Table 2: Beta diversity for each treatment in the last (10th) generation.** Estimated beta diversity and
622 Confidence intervals have been obtained through bootstrapping. For data from previous generations see
623 Table S10.

treatment	beta, measured	beta, estimated	upper CI	lower CI
NoWasp	1.54	1.54	1.23	1.76
W348	2.14	2.14	2.13	2.49
W369	1	1	1	1
W64	1	1	1	1
WmixSeq	1.60	1.62	1.49	1.83
WmixSim	1.88	1.87	1.55	2.19

Figure 1: Experimental setup. A) Aphid composition and wasp treatments. B) Timing of the experimental evolution. In each generation, uninfected aphids (white) were collected to start the next generation and analyze symbiont composition (after generation 2, 6, and 10 only), while mummified aphids (brown) were discarded. The offspring of the transferred aphids was subsequently exposed to parasitoid wasps to create selection for protective symbionts.

Figure 2: Changes in symbiont diversity and frequency of aphid lines with different *H. defensa*-haplotypes for each treatment. A: *H. defensa*-free aphids, B: *H. defensa*-haplotype H76, C: *H. defensa*-haplotype H402, D: *H. defensa*-haplotype H15, E: Shannon diversity, F: Beta diversity. Error bars indicate 95% CI. Confidence intervals for beta diversity have been obtained through bootstrapping.

Figure 3: Aphid number (A), plant size (B), mummy number (C), and mummification rate (D). Error bars indicate 95% CI.

Figure 4: Changes in frequency of aphid clones with different *H. defensa* strains (or without *H. defensa*) for each treatment (columns) and replicate (rows). Clone H- indicates *H. defensa* free-aphids, clones H15, H76 and H402 aphids with different strains of *H. defensa*.