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Development, specificity and sublethal effects of symbiont-conferred resistance to parasitoids in aphids

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Summary

1. One of the most exciting recent discoveries in the field of ecological immunology has been that insects employ the help of heritable symbionts as a defence against parasitoids and pathogens. Aphids commonly harbour the facultative bacterial endosymbiont *Hamiltonella defensa,* which is known to increase their resistance to parasitoids. It is unknown how this resistance develops during the aphids' ontogeny, following the transmission bottleneck between mother and offspring, and how specific symbiont-conferred defences are.

2. We addressed these issues in the black bean aphid, *Aphis fabae*, by exposing aphids of different age classes to the parasitoid *Lysiphlebus fabarum*. The susceptibility of aphids that were either naturally or experimentally infected with *H. defe*nsa was compared with that of uninfected aphids.

3. Susceptibility to parasitoids decreased with aphid age, but aphids harbouring *H. defensa* showed an earlier and/or steeper decline to lower levels of susceptibility than aphids without this symbiont. This is consistent with the hypothesis that during aphid development, symbiont-conferred resistance builds up with bacterial population growth, which we documented using quantitative PCR.

4. Parasitoids that successfully overcame the symbiont-conferred resistance still suffered from sub-lethal effects of *H. defensa*. They exhibited lower emergence, delayed development and reduced size compared to parasitoids developing in aphids without *H. defens*a.

5. The most striking result was a strong interaction on the rates of parasitism between aphid sublines infected with different isolates of *H. defensa* and the parasitoid lines they were exposed to, suggesting a high specificity of symbiont-conferred resistance.

6. Based on these results we conclude that when faced with hosts possessing *H. defensa*, aphid parasitoids are under selection to preferentially attack the youngest host stages and /or

to discriminate against symbiont-protected aphids. Furthermore, the specificity induced by *H. defensa* in the interaction between host and parasitoid is likely to have important consequences for coevolution. It may result in negative frequency-dependent selection and thus promote genotypic variation.

Keywords: *Aphis fabae*; coevolution; *Hamiltonella defensa*; *Lysiphlebus fabarum*; parasitoid; quantitative PCR, resistance; symbiosis

Introduction

Parasitoids of insects provide some of the best empirical examples of optimal host choice, such as choosing larger hosts to better provision their offspring (Salt 1941), or adjusting the offspring sex ratio to the size of available hosts (Charnov *et al.* 1981; King 1988). Because of its relevance for breeding biocontrol agents, host choice has repeatedly been addressed in aphid parasitoids of the subfamily Aphidiinae (Hymenoptera: Braconidae). These solitary parasitoids can attack all four nymphal instars as well as adult aphids. The plentiful resources provided by later host stages typically allow parasitoids to develop faster and to a larger body size (Sequeira & Mackauer 1992; Colinet *et al.* 2005), yet attacking larger aphids is more dangerous and time-consuming because of their more effective behavioral defenses (Chau & Mackauer 2000). Young aphids are easier to subdue (Chau & Mackauer 2001), but the smallest stages are most likely to die from the oviposition itself (Colinet *et al.* 2005), which is wasteful for the parasitoid. Given these trade-offs, it is not surprising that several studies found that aphid parasitoids preferentially attack intermediate instars of their hosts (Weisser 1994; Colinet *et al.* 2005; Tahriri *et al.* 2007), although this strategy is by no means universal (e.g. Chau & Mackauer 2001; Lin & Ives 2003).

Another important determinant of host suitability is of course its physiological resistance, i.e. the ability to prevent parasitoid development after oviposition. The limited evidence available suggests that physiological resistance of aphids increases with age (Walker & Hoy 2003; Xu *et al.* 2008). A specific feature of aphids is that variation for resistance to parasitoids occurs at two levels. First, natural populations of aphids exhibit significant genetic variation reflecting differences in innate immune defences (von Burg *et al.* 2008; Sandrock, Gouskov & Vorburger 2010). Second, aphids also differ in whether they are infected with facultative bacterial endosymbionts. One of these symbionts, *Hamiltonella defensa* (Moran *et*

al. 2005), has been shown to strongly increase resistance to hymenopteran parasitoids (Oliver *et al.* 2003; Ferrari *et al.* 2004; Oliver, Moran & Hunter 2005; Desneux *et al.* 2009; Vorburger *et al.* 2009). Mechanistically, this protection is related to the presence of toxin-encoding bacteriophages within *H. defensa*'s genome (Degnan & Moran 2008a; 2008b; Oliver *et al.* 2009). These toxins appear to kill the eggs or early larvae of the parasitoids.

Just like the obligate endosymbiont of aphids, *Buchnera aphidicola*, facultative symbionts are maternally transmitted with high fidelity to either eggs (during the sexual generation) or live-born nymphs (during the asexual generations). Each of these transmissions imposes a bottleneck on the bacterial population (Mira & Moran 2002), which then grows back to its normal size during the aphid's development. It is thus possible that the protective effect of defensive endosymbionts such as *H. defensa* is initially weak in newborn nymphs and only builds up as the bacterial population grows inside the developing aphid. Based on this reasoning, we hypothesize that age effects on susceptibility to parasitoids differ between aphids with and without defensive endosymbionts.

We carried out two experiments to test this hypothesis in the black bean aphid, *Aphis fabae* (Scopoli) (Fig. 1), exposing different age classes of aphids with and without *H. defensa* to the parasitoid *Lysiphlebus fabarum* (Marshall). In the first experiment, we compared aphid clones that were naturally infected or uninfected with *H. defensa*. Such comparisons can provide correlative evidence for *H. defensa*'s role in the change of susceptibility with aphid age, but they do not allow a clean separation of host genetic variation and symbiont-conferred effects. Therefore, the second experiment was carried out with artificially created sublines of a single aphid clone that were either uninfected or infected with two different isolates of *H. defensa*, in which we also documented the build-up of symbiont populations in the course of aphid development using quantitative PCR. Finally, we were also able to assess the

specificity of symbiont-conferred resistance by exposing the different isolates of *H. defensa* in the same genetic background to multiple lines of the parasitoid.

Materials and Methods

INSECTS

The black bean aphid, *Aphis fabae*, is an important pest on many crops throughout the temperate regions of the northern hemisphere (Blackman & Eastop 2000). It is a host of several aphid parasitoids, the most important of which is *Lysiphlebus fabarum* (Starý 2006). Exceptionally among aphid parasitoids, *L. fabarum* reproduces by thelytokous parthenogenesis in most populations (Belshaw *et al.* 1999; Starý 1999; Sandrock & Vorburger 2011). This is very valuable for experimentation, because it allows the use of genetically homogeneous parasitoid populations founded by single parthenogenetic females (isofemale lines). Females of *L. fabarum* oviposit a single egg into aphids. The larva then hatches and develops through several instars inside the still active host, which is only killed before parasitoid pupation. Metamorphosis takes place within a cocoon spun inside the host's dried remains, forming a so-called 'mummy' from which the adult wasp emerges.

EXPERIMENT 1 - NATURALLY INFECTED APHIDS

The six clones of *A. fabae* used in this experiment represented a subset of those used in a recent study of genotypic and endosymbiont-conferred variation for susceptibility to parasitoids in this species (Vorburger *et al.* 2009). All six clones differed in their multilocus microsatellite genotypes. Four of these clones (A06-323, A06-327, A06-329 and Af6) were infected with the defensive endosymbiont *H. defensa* (designated H+) and exhibited high resistance to *L. fabarum* when tested as 2-3 days old nymphs (Vorburger *et al.* 2009). The

other two clones (A06-333 and A06-407) did not harbour *H. defensa* (designated H-) and were highly susceptible to *L. fabarum* when tested at the same age (Vorburger *et al.* 2009) One of these clones, A06-333, harboured another bacterial endosymbiont called *Regiella insecticola* (Moran *et al.* 2005). So far, there is no evidence that this bacterium affects susceptibility to parasitoids in *A. fabae* (Vorburger *et al.* 2009), which is consistent with a study on this endosymbiont in pea aphids (Oliver *et al.* 2003). But note that a defensive strain of *R. insecticola* has been discovered in a different aphids species, *Myzus persicae* (Vorburger, Gehrer & Rodriguez 2010). As parasitoids we used a single isofemale line of parthenogenetic *L. fabarum* (labelled 07-64) that had not been used in any previous experiments. It was collected in September 2007 in Wildberg near Zürich, Switzerland, from a colony of *A. fabae* on *Chenopodium album*.

Our experiment quantified the susceptibility to this parasitoid in aphids of five different age classes from all six clones of *A. fabae*. The age classes were 0-1 days old (1st instar nymphs), 1-2 days (1st to 2nd instar), 2-3 days (2nd instar), 3-5 days (3rd instar) and 5-7 days old (4th instar). The general assay was to expose groups of aphids to wasps for a fixed period of time and measure the proportion of individuals mummified (i.e. successfully parasitized) as an estimate of susceptibility to the parasitoid (Henter & Via 1995). This measure does not distinguish between pre-ovipositional defences (e.g. avoidance behavior) and physiological resistance against the parasitoid egg or larva, but previous studies have shown that it largely reflects the latter. Clonal differences in mummification rates do not arise from differences in parasitoid oviposition (Henter & Via 1995), and parasitoids seem equally likely to oviposit in aphids with and without defensive endosymbionts (Oliver *et al.* 2003). Every combination of aphid clone and age class was replicated five times, amounting to a total of 150 aphid colonies tested.

We started the experiment by splitting each aphid clone into five sublines that were maintained on caged seedlings of broad bean (*Vicia faba*, var. 'Scirocco') grown in 0.07 l plastic pots at 20°C and a 16 h photoperiod. Sublines were reared on random positions in five different plastic trays (randomized complete blocks) for one generation prior to the actual experiment. This procedure avoids confounding differences among clones with environmental maternal effects that may be carried over from the stock culture. Susceptibility to parasitoids was assayed in the second subline generation. This generation was started by allowing adults from the first subline generation to reproduce on new seedlings for a defined period of time. We used six adults for 24 h to found the youngest three age classes (0-1, 1-2 and 2-3 days old) and three adults for 48 h to found the oldest two age classes (3-5 and 5-7 days old). Setting up these test colonies was temporally staggered such that in each block, all five age classes were available for exposure to parasitoids at the same time. For this we first counted all aphid nymphs on the plants (mean colony size: 19.5 ± 7.5 SD) and then added two female *L. fabarum* to each colony. These wasps had been reared on a highly susceptible clone of *A. fabae* not included in this experiment, and they were approx. 1 – 2 days old when used. The wasps were allowed to attack the aphids for six hours and then discarded. Nine days after exposure to parasitoids, successfully parasitized aphids were clearly recognizable as mummies and counted. Six replicates had to be excluded from the analyses because the wasps escaped from the cages during the 6-h exposure period.

We analysed the proportion of aphids mummified (i.e. successfully parasitised) using a generalised linear model with the logit link function and - to account for overdispersion - quasibinomial errors. We tested for the effects of experimental block, age class, aphid clone and the age × clone interaction. As recommended by Crawley (2005) for quasibinomial fits, *F*-tests rather than χ^2-tests to compare deviances of models with and without the effects to be tested. All analyses were carried out in R 2.9.2 (R Development Core Team 2009).

EXPERIMENT 2 - EXPERIMENTALLY INFECTED APHIDS

The second experiment followed the same basic design as experiment 1 and used the same aphid age classes. However, instead of different aphid clones, we compared three sublines of a single clone of *A. fabae* that were either uninfected or experimentally infected with one of two different isolates of *H. defensa*. For this we used clone A06-407, which was also included in experiment 1. This clone is naturally uninfected with *H. defensa* or any other known facultative endosymbiont of aphids (Vorburger *et al.* 2009). A microinjection protocol as described in Vorburger *et al.* (2010) was used to generate two *H. defensa*-infected sublines of this clone, A06-407^{H76} and A06-407^{H323}. Briefly, we injected *H. defensa*-containing hemolymph from two naturally infected donor clones, A09-76 and A06-323, into 4th instar nymphs of the recipient clone using a fine glass needle attached to a microinjection pump (FemtoJet, Eppendorf). Successful transfections lead to stable, heritable infections in the recipient subline, which we confirmed by diagnostic PCR with *H. defensa*-specific primers (McLean *et al.* 2011) for the first three generations after transfection and again before use of these sublines in the experiment.

As parasitoids we used three different parthenogenetic lines of *L. fabarum*. In addition to line 07-64, the line already used in experiment 1, we used line 06-15, collected in May 2006 from *A. fabae* on *Vicia faba* in Sarzana, Italy, and line 09-231, collected in June 2009 from *A. urticata* on *Urtica dioica* in Sierre, Switzerland. We included three rather than a single parasitoid line in experiment 2 to better cover the variation present in parasitoid populations and to test for genetic specificity of symbiont-conferred resistance. Previous experiments suggested that the aphids' own defences against parasitoids are very general (no evidence for aphid clone x parasitoid line interactions on rates of parasitism in aphids without *H. defensa*, see Sandrock, Gouskov & Vorburger 2010), but that the presence of *H. defensa* has the potential to induce specificity in this interaction (Vorburger *et al.* 2009; Rouchet &

Vorburger, unpublished data). This could result from genetic interactions between parasitoids and the hosts' heritable endosymbionts, which would have important consequences for the coevolutionary dynamics in this system (Hamilton 1980; Carius, Little & Ebert 2001; Woolhouse *et al.* 2002).

All combinations of aphid subline, parasitoid line and aphid age class were replicated seven times in experiment 2, with replicates arranged as seven randomized complete blocks. The main response variable was again the proportion of aphids mummified, but in this experiment we also followed the fate of these mummies for blocks 4 - 7 of the experiment. We determined the rate of emergence (proportion of mummies from which parasitoids hatched), parasitoid development time (mean time from oviposition to adult emergence of all hatching mummies in a replicate) and parasitoid dry weight of three randomly selected individuals per replicate (or fewer, if less than three emerged). These measurements of parasitoid performance were taken to test if parasitoids that successfully overcome symbiont-conferred resistance and develop in aphids harbouring *H. defensa* are equally fit as parasitoids developing in aphids without this symbionts. Developing in aphids with *H. defensa* may also have sublethal effects on parasitoids (Nyabuga *et al.* 2010).

The proportion of aphids mummified and the proportion of mummies from which parasitoids emerged were again analysed using a generalised linear model with logit link and quasibinomial errors. Development time and dry weight of parasitoids were analysed with a linear model.

ESTIMATION OF *H. DEFENSA* DENSITIES IN DIFFERENT APHID LIFE STAGES

The development of *H. defensa* densities relative to aphid growth was quantified by TaqMan real-time quantitative PCR (qPCR hereafter), using an ABI 7500 Fast Real Time PCR system (Applied Biosystems). To estimate *H. defensa* densities, we quantified the copy

number of the *dnaK* gene with the following primers and probe (Chandler, Wilkinson & Douglas 2008): forward CAAGCGGATTATTAATGAACCCA, reverse TGGTGCTATTCCCTTTTCCCT, probe CGCGGCCATTGCCTACGGTTT. As an index of aphid cell number we quantified the copy number of *A. fabae*'s *EF1α* gene (Koga, Tsuchida & Fukatsu 2004), using a primer and probe set developed by Microsynth AG (Balgach, Switzerland): forward CAGCAGTTACATCAAGAAGATTGG, reverse CATGTTGTCTCCATTCCATCCAG, probe CCCAGCCGCTGTTGCTTTCGTTCC. The probes were modified with FAM as the 5'-terminal reporter dye and BHQ-1 as the 3' terminal quencher dye. We carried out the qPCR reactions in triplicate using 25 µl volumes with 5 µl of template DNA. Gene copy numbers for the *H. defensa dnaK* gene and the *A. fabae EF1α* gene were estimated in six replicate aphids from each of the five age classes for sublines A06-407^{H76} and A06-407^{H323} used in experiment 2. Aphids were frozen at -80°C until DNA extraction, which was carried out using the 'salting out' method described in Sunnucks & Hales (1996). The DNA pellet of a single aphid was resuspended in 70 µl of TE buffer and stored at -20°C before use in qPCR. Gene copy numbers were estimated from a standard curve generated with serial dilutions of a synthetic standard produced by Microsynth AG and then calculated per aphid. These values were analysed with a linear model, testing for the effects of aphid age class, *H. defensa* isolate (H76 vs. H323) and the age × isolate interaction.

Results

EXPERIMENT 1

Host age class had a highly significant effect on the proportion of individuals that were mummified (Table 1). Older aphid nymphs were less susceptible to the parasitoid *L. fabarum* than younger nymphs (Fig. 2). There was also significant variation in susceptibility among

aphid clones (Table 1), which was not surprising given that four of them harboured a defensive symbiont. However, the protective effect of *H. defensa* became evident only in the older age classes. Up to two days of age, the proportion of aphids mummified was similar between clones with and without *H. defensa*. Two of the *H. defensa*-protected clones (A06-329 and Af6) started showing increased resistance in the 2-3 days age class, but the other two (A06-323 and A06-327) were still very susceptible at that age. Their susceptibility dropped strongly at > 3 days of age (Fig. 2a). These differences were reflected in a significant age class × clone interaction (Table 1). In the oldest two age classes, clones harbouring *H. defensa* were about three times more resistant on average than the two clones without this symbiont (Fig. 2b).

EXPERIMENT 2

Also in the second experiment, host age class had a highly significant effect on the proportion of individuals that were mummified (Table 2), again reflecting a decrease in susceptibility with age (Fig. 3a). The variation among the three sublines of *A. fabae* clone A06-407 was highly significant as well (Table 2). Experimental infection with *H. defensa* strongly increased resistance to *L. fabarum*, with one of the two isolates, H76, providing a higher level of protection (Fig. 3a). In contrast to the first experiment, the protective effect of the symbiont was already evident in aphids of the youngest age classes. Nevertheless, the development of susceptibility to parasitoids with age differed among the three sublines, resulting in a significant age class × subline interaction (Table 2). In the two sublines harbouring *H. defensa*, susceptibility dropped most strongly after two days of age, whereas in the subline without *H. defensa*, a drop in susceptibility was evident only at an age > 5 days (Fig. 3a). The three asexual lines lines of *L. fabarum* used in experiment 2 also varied significantly in the proportion of hosts they could parasitise successfully (Table 2). However,

their relative success depended strongly on the host subline (Fig. 3b), which was reflected in a highly significant subline × parasitoid interaction (Table 2). All parasitoid lines could parasitise the subline without *H. defensa*, line 06-15 being most successful on average. The subline harbouring *H. defensa* isolate H323 was more resistant on average, but this isolate provided almost no protection against one line of *L. fabarum*, 07-64. The subline harbouring *H. defensa* isolate H76 was the most resistant on average. It was almost completely resistant against parasitoid line 06-15 and very resistant against line 07-64, but line 09-231 still managed to mummify >10% of individuals harbouring this isolate of *H. defensa*. Overall, a different parasitoid line was most successful on each of the three aphid sublines.

The presence of *H. defensa* in *A. fabae* not only affected the rate of parasitism achieved by the different parasitoid genotypes, it also influenced host suitability in more subtle ways. There was a significant subline effect on all three parasitoid performance traits (Table 3). For the proportion of mummies hatching, this effect was largely due to a lower rate of emergence in the subline harbouring H76, suggesting that this isolate of *H. defensa* causes increased parasitoid mortality also at the mummy stage (Fig. 4a). There was also a significant effect of aphid age class, reflecting a slightly lower emergence from mummies when aphids were attacked at low to intermediate ages (Fig. 4a). The hosts' possession of *H. defensa* also led to a longer development time of the parasitoids, but this effect was much more pronounced for the H76 isolate (Fig. 4b), which prolonged development by approximately 1.5 days on average. The delay of parasitoid emergence caused by H76 increased with the age at which aphids were attacked (Fig. 4b), resulting in a significant age class × subline interaction (Table 3). The host subline had a significant effect also on parasitoid body size, estimated as dry weight (Table 3). Wasps developing in aphids harbouring *H. defensa* remained smaller on average than wasps developing in the *H. defensa*-free subline (Fig. 4c). The age at which

aphids were stung had a significant effect on wasp dry weight, too. Parasitoids from hosts that were attacked in the 5-7 days age class were largest on average (Table 4, Fig. 4c).

Taken together, these results indicate that even when parasitoids are able to overcome the symbiont-conferred resistance of their host, they suffer from negative effects of developing in an aphid harbouring *H. defensa*.

SYMBIONT DENSITIES

Aphid age class obviously had a highly significant effect on gene copy numbers of the *A. fabae EF1α* gene ($F_{4, 49} = 35.39$, $P < 0.001$) as well as the *H. defensa dnaK* gene ($F_{4, 48} = 53.40$, $P < 0.001$). Copy numbers for either gene did not differ between the two sublines harbouring different isolates of *H. defensa* (*EF1α*: $F_{1, 49} = 1.04$, $P = 0.312$; *dnaK*: $F_{1, 48} = 1.95$, $P = 0.169$), nor were there significant age × subline interactions (*EF1α*: $F_{4, 49} = 0.23$, $P = 0.918$; *dnaK*: $F_{4, 48} = 0.31$, $P = 0.868$). Copy numbers of both genes increased exponentially with aphid age. However, the increase of *dnaK* (*H. defensa*) was steeper up to an aphid age of 2-3 days and shallower thereafter, as revealed by the log-scale plot (Fig. 5), such that the ratio of *dnaK* to *EF1α* copies was highest at the intermediate age classes (Fig. 5). Copy numbers of the *H. defensa dnaK* gene were very high and exceeded those of *A. fabae*'s *EF1α* gene between two- and more than eightfold, depending on aphid age.

Discussion

We showed that the susceptibility of *A. fabae* to its parasitoid *L. fabarum* decreases with age, and that the temporal trajectory of this decrease differs between aphids with and without the defensive endosymbiont *H. defensa*. The proportion of aphids mummified by parasitoids declined earlier and/or more steeply in aphids harbouring this defensive symbiont. We regard

this as evidence that the protective effect of *H. defensa* builds up during aphid development and is not yet fully developed at birth, presumably due to a transmission bottleneck between mother and daughter (Mira & Moran 2002). The evidence from the first experiment is correlational, because we worked with naturally infected or uninfected clones and could not separate the symbiont's effect from genetic variation among clones. This was possible in the second experiment using experimentally infected lines with the same genetic background, and they provided generally consistent results. In these lines we also documented with quantitative PCR how the symbiont population builds up rapidly during the aphid's development. Interestingly, the *H. defensa* cell number increased faster than the host cell number up to an aphid age of about three days, but more slowly thereafter, such that the ratio of symbiont to aphid gene copy numbers peaked around mid-development. However, the strength of symbiont-conferred defences was not determined by this ratio, because resistance was highest in the latest nymphal instars. The rapid increase of aphid gene copy numbers in the second half of development may be related to offspring growth. Aphid nymphs already contain the developing embryos of the next generation, which are ready to be born soon after adult ecdysis (telescoping of generations).

A marked difference between the two experiments was that in the first experiment, symbiont-conferred resistance was only evident in the intermediate and older age classes, while the experimentally infected lines benefitted from some protection against parasitoids already at the youngest age. Although the experimental protocols were very similar, we cannot exclude that this difference may be due to some environmental variation between experiments. An alternative explanation is that the protective effect depends not only on the specific isolate of *H. defensa* and the genotype of the attacking parasitoid, as clearly evident from experiment 2, but also on the host's genetic background. Possibly, the expression of symbiont-conferred resistance is particularly strong in the clone we used for the second

experiment. As yet there is no evidence for such interactions on resistance to parasitoids (Oliver, Moran & Hunter 2005), but they have been documented for other traits affected by endosymbionts (McLean *et al.* 2011).

The results of both experiments suggest that parasitoids could improve their success on symbiont-protected hosts by adaptive host choice. The probability of successful parasitoid development was higher in younger hosts for all aphid clones and sublines tested, but selection on parasitoids to attack young nymphs would certainly be stronger when hosts harbour *H. defensa*. It would thus be interesting to test if parasitoid populations living on hosts with a high prevalence of *H. defensa* have evolved a different host stage preference compared to parasitoids on hosts without defensive symbionts.

SUBLETHAL EFFECTS OF *H. DEFENSA* ON PARASITOIDS

In agreement with the correlative study by Nyabuga *et al.* (2010), we found that the presence of defensive symbionts in their hosts may also have sublethal effects on aphid parasitoids. Wasps that managed to develop successfully in aphids protected by *H. defensa* showed reduced emergence, prolonged development time and smaller size. Interestingly, these effects were stronger in the aphid subline harbouring isolate H76, which also provided higher resistance overall. It is possible that parasitoid mortality (i.e. aphid resistance) and the sublethal effects on surviving parasitoids have the same mechanistic basis, namely the exposure to phage-encoded toxins produced by *H. defensa* (Oliver *et al.* 2009). Thus, from a female parasitoid's perspective, the disadvantage of attacking symbiont-protected aphids is twofold. Its offspring are less likely to develop at all and if they do survive, they may suffer from reduced fitness. This should result in selection on female parasitoids to recognise and avoid hosts that harbour defensive symbionts, but as yet we are unaware of any evidence that *L. fabarum* or any other aphid parasitoid exhibits such discrimination.

It is not possible to determine whether the observed negative effect of *H. defensa* on the dry weight of emerging wasps represents an indirect effect mediated by aphid body size or a direct response to the presence of the symbiont. We did not quantify aphid body size in the present experiment, and the evidence from previous experiments is ambiguous. Comparisons of naturally infected and uninfected clones of *A. fabae* suggested a positive rather than a negative effect of *H. defensa* on aphid size (Vorburger *et al.* 2009; Castañeda, Sandrock & Vorburger 2010), but a recent study of experimentally infected lines revealed a slight negative effect (Vorburger & Gouskov 2011). Thus, we cannot exclude that wasps developing in symbiont-protected hosts are smaller because of a negative effect of *H. defensa* on aphid size.

SPECIFICITY OF SYMBIONT-CONFERRED RESISTANCE

Probably the most striking result of this study was the strong host subline × parasitoid line interaction observed in experiment 2. The level of protection provided by the two isolates of *H. defensa* depended to a large extent on the genotype of the attacking parasitoid. This stands in stark contrast to an experiment using numerous clones of *A. fabae* without *H. defensa*, which revealed ample genetic variation for resistance but no evidence for host line × parasitoid line interactions (Sandrock, Gouskov & Vorburger 2010). Because all aphids used in experiment 2 were genetically identical, it is clearly the endosymbiont that is responsible for the specificity of the interaction observed here. Facultative endosymbionts such as *H. defensa* are faithfully transmitted from mother to offspring and thus represent part of the heritable (clonal) variation available to selection by parasitoids. As a result of their genetic interaction with parasitoids, they may transform a host-parasitoid system in which resistance and infectivity behave like running speed in a predator-prey relation (Sasaki & Godfray 1999) to a system that is characterised by strong genetic specificity as observed, for example,

in *Daphnia*-pathogen interactions (Carius, Little & Ebert 2001; Luijckx *et al.* 2011). Such genotype × genotype interactions lead to negative frequency-dependent selection between hosts and parasites and thereby promote genotypic variation (Woolhouse *et al.* 2002). That endosymbionts not only provide their hosts with protection against parasitoids but also alter the reciprocal selection between hosts and parasitoids by inducing genetic specificity is remarkable. The evolutionary consequences of this effect deserve further attention.

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517 **Figure captions**
518
519 **Fig. 1.** A winged adult and several nymphs of the black bean aphid, *Aphis fabae*, under attack
520 by two individuals of the aphid parasitoid *Lysiphlebus fabarum*. Photograph by Christoph
521 Vorburger.
522
523 **Fig. 2.** Susceptibility of five age classes of the black bean aphid, *Aphis fabae*, to the
524 parasitoid *Lysiphlebus fabarum*. (a) Means plotted separately for all six clones used in
525 experiment 1, and (b) averaged across clones that do (H+) or do not (H-) harbour the
526 defensive endosymbiont *Hamiltonella defensa*.
527
528 **Fig. 3.** Susceptibility to the parasitoid *Lysiphlebus fabarum* of one uninfected (H-) and two
529 experimentally infected sublines of *Aphis fabae* clone A06-407, harbouring two different
530 isolates of the defensive endosymbiont *Hamiltonella defensa* (H76 and H323). (a) Means
531 plotted separately for the five age classes and overall, averaged across parasitoid lines. (b)
532 Means plotted separately for each parasitoid line used in experiment 2, averaged across age
533 classes, to illustrate the strong host subline × parasitoid line interaction.
534
535 **Fig. 4.** Effects of host infection with *Hamiltonella defensa* on (a) parasitoid emergence (mean
536 proportions of mummies hatching), (b) mean parasitoid development time (time from
537 oviposition to emergence), and (c) mean parasitoid size (dry weight).
538
539 **Fig. 5.** Development with age of aphid cell numbers and endosymbiont populations,
540 quantified as copy numbers of the *Aphis fabae EF1α* gene and the *Hamiltonella defensa*
541 *dnaK* gene, respectively, using qPCR.

Table 1. Analysis of deviance table for the proportion of aphids mummified in experiment 1. The generalised linear model was a quasi-likelihood fit with logit link and binomial errors, using a dispersion parameter of 3.34 (see Material and methods).

Effect	df	Deviance	*F*	*P*
Block	4	13.24	0.974	0.425
Age class	4	262.24	19.286	<0.001
Clone	5	54.44	3.201	0.010
Age × clone	20	131.48	1.934	0.016
Residual	110	431.18		

Table 2. Analysis of deviance table for the proportion of aphids mummified in experiment 2. The generalised linear model was a quasi-likelihood fit with logit link and binomial errors, using a dispersion parameter of 3.37.

Effect	df	Deviance	*F*	*P*
Block	6	78.16	3.86	0.001
Age class	4	108.53	8.04	<0.001
Subline	2	386.68	57.33	<0.001
Parasitoid	2	35.83	5.31	0.005
Age class × subline	8	74.15	2.75	0.006
Age class × parasitoid	8	44.81	1.66	0.108
Subline × parasitoid	4	223.67	16.58	<0.001
Age class × subline × parasitoid	16	73.23	1.36	0.163
Residual	263	966.82		

Table 3. Analysis of deviance (proportion of wasps emerging) and analysis of variance results (development time and wasp dry weight) for three parasitoid performance traits. The generalised linear model for the proportions of mummies from which wasps emerged was a quasi-likelihood fit with logit link and binomial errors, using a dispersion parameter of 1.479.

	Proportion of wasps emerging				Development time				Wasp dry weight			
Effect	df	Deviance	*F*	*P*	df	MS	*F*	*P*	df	MS × 10^4	*F*	*P*
Block	3	2.65	0.60	0.619	3	0.20	0.96	0.417	3	3.58	5.33	0.002
Age class	1	9.07	6.13	0.015	1	0.09	0.44	0.510	1	5.14	7.64	0.007
Subline	2	12.29	4.15	0.019	2	13.88	67.80	<0.001	2	3.51	5.21	0.008
Parasitoid	2	4.69	1.58	0.211	2	0.10	0.50	0.609	2	1.98	2.95	0.059
Age class × subline	2	1.59	0.54	0.585	2	1.73	8.45	<0.001	2	0.55	0.82	0.446
Age class × parasitoid	2	4.78	1.62	0.205	2	0.30	1.45	0.242	2	0.01	0.02	0.986
Subline × parasitoid	3	4.50	1.02	0.390	3	0.16	0.77	0.517	3	0.39	0.59	0.626
Age class × subline × parasitoid	3	9.74	2.20	0.095	3	0.07	0.36	0.782	3	0.34	0.51	0.678
Residual	81	130.34			78	0.21			76	0.67		

Fig. 1. A winged adult and several nymphs of the black bean aphid, Aphis fabae, under attack by two individuals of the aphid parasitoid Lysiphlebus fabarum. Photograph by Christoph Vorburger.
88x62mm (300 x 300 DPI)

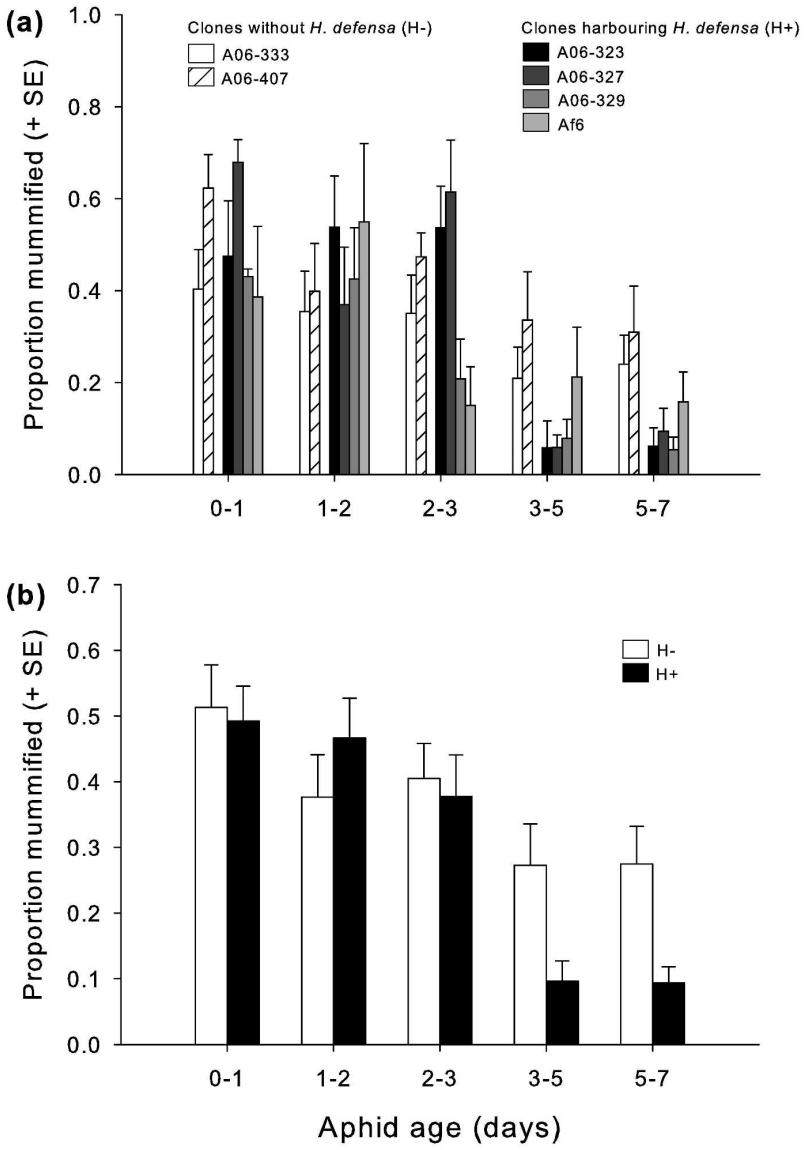

Fig. 2. Susceptibility of five age classes of the black bean aphid, Aphis fabae, to the parasitoid Lysiphlebus fabarum. (a) Means plotted separately for all six clones used in experiment 1, and (b) averaged across clones that do (H+) or do not (H-) harbour the defensive endosymbiont Hamiltonella defensa.
234x352mm (300 x 300 DPI)

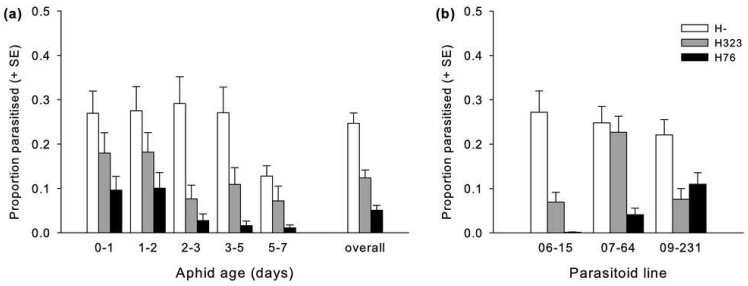

Fig. 3. Susceptibility to the parasitoid Lysiphlebus fabarum of one uninfected (H-) and two experimentally infected sublines of Aphis fabae clone A06-407, harbouring two different isolates of the defensive endosymbiont Hamiltonella defensa (H76 and H323). (a) Means plotted separately for the five age classes and overall, averaged across parasitoid lines. (b) Means plotted separately for each parasitoid line used in experiment 2, averaged across age classes, to illustrate the strong host subline × parasitoid line interaction.
111x34mm (300 x 300 DPI)

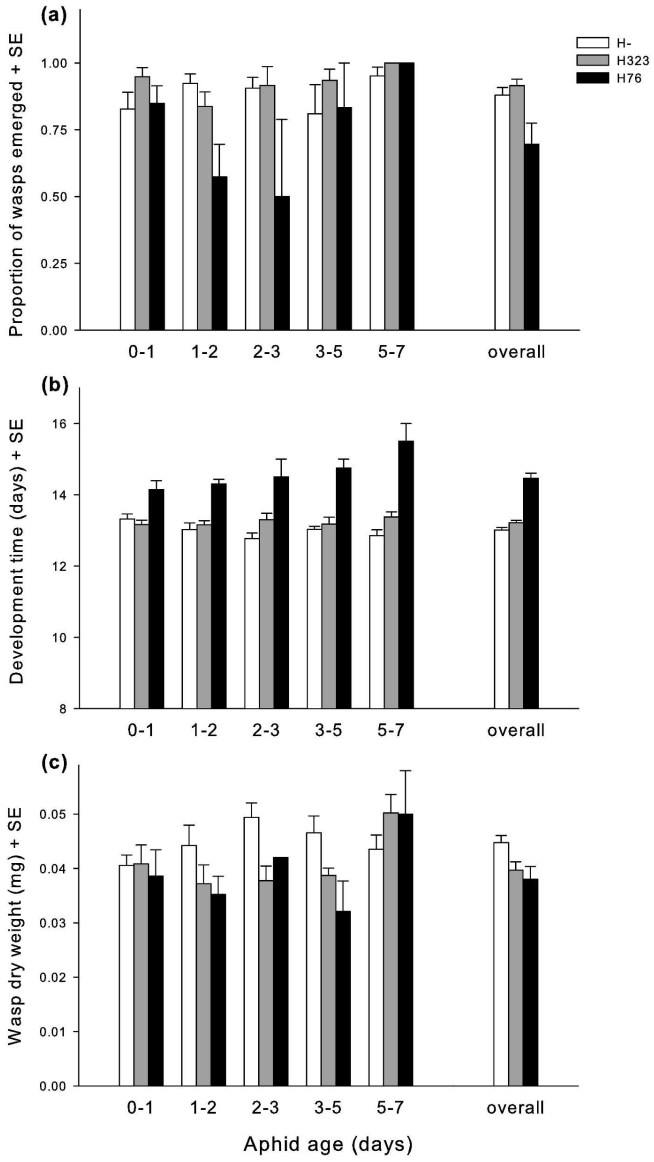

Fig. 4. Effects of host infection with Hamiltonella defensa on (a) parasitoid emergence (mean proportions of mummies hatching), (b) mean parasitoid development time (time from oviposition to emergence), and (c) mean parasitoid size (dry weight).
299x432mm (300 x 300 DPI)

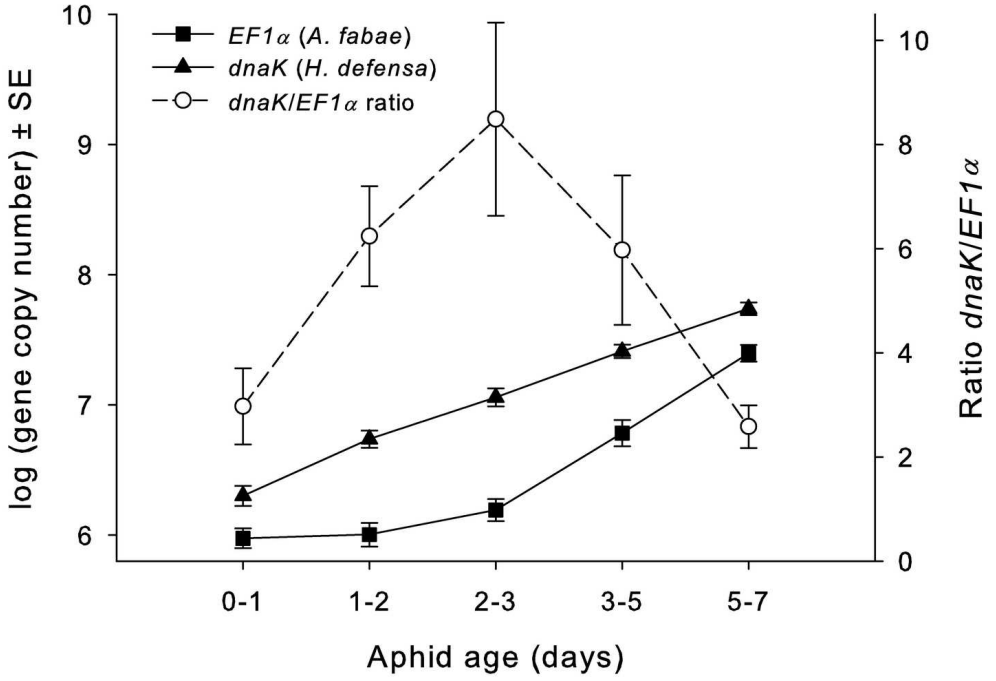

Fig. 5. Development with age of aphid cell numbers and endosymbiont populations, quantified as copy numbers of the Aphis fabae EF1α gene and the Hamiltonella defensa dnaK gene, respectively, using qPCR.
123x91mm (300 x 300 DPI)