The defensive aphid symbiont *Hamiltonella defensa* affects host quality differently for *Aphelinus glycinis* versus *Aphelinus atriplicis*

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**Keywords:**
*Aphelinus atriplicis*
*Aphelinus glycinis*
*Aphis craccivora*
*Acyrthosiphon pisum*
*Hamiltonella defensa*
*APSE*
*Aphid endosymbionts*

**Abstract**

Endosymbiont interactions with hosts have important effects on fitness, including the fitness of many pest and beneficial species. Among these interactions, facultative endosymbiotic bacteria can protect aphids from parasitoids. *Aphis craccivora* and *Acyrthosiphon pisum* can harbor the symbiotic bacteria *Hamiltonella defensa* and its bacteriophage APSE. Infection by *H. defensa* defends these aphids against some but not all parasitoid species in the hymenopteran family Braconidae. Here, we report results on the effect of *H. defensa* on parasitism of aphids by species in the other major lineage of aphid parasitoids, *Aphelinus* species in the family Aphelinidae. Parasitism of aphids infected with *H. defensa/APSE* by two *Aphelinus* species did not differ from that of uninfected aphids. While *Aphelinus atriplicis* showed no difference in fitness components between infected and uninfected aphids, *Aphelinus glycinis* actually produced more adult progeny and larger female progeny on infected than on uninfected aphids. *Aphelinus glycinis* may increase host quality for itself by changing the titers of nutritional versus protective bacteria in such a way that aphids infected with *H. defensa* can be made more suitable for parasitoid development than uninfected aphids. Our results and reasoning suggest that these *Aphelinus* species may be less prone to harm by *H. defensa/APSE* that affect eggs because they have anhydropic, heavily chorionated eggs, which may not absorb toxins during embryogenesis.

**1. Introduction**

Insect-associated microbes can exert major effects on host fitness, often by enhancing resource acquisition or providing protection against biotic or abiotic threats (Moran et al., 2008; for reviews, see: O’Neill et al., 1997; Oliver and Martinez, 2014; Southamer et al., 1999). Aphid species have emerged as important models for the study of nutritional and protective symbioses (Baumann et al., 1995; Douglas, 1998; Moran, 2001; Oliver et al., 2010, 2014). Almost all aphid species harbor the obligate bacterial symbiont, *Buchnera aphidicola* Munson et al. (Gammaproteobacteria: Enterobacteriaceae), which supplies essential nutrients lacking in their phloem diet (Moran et al., 2008), and many are also infected with one or more maternally transmitted facultative symbionts (Oliver et al., 2010). Facultative symbionts are not generally required for aphid survival or reproduction, but often confer conditional costs and benefits (Oliver et al., 2010). From the perspective of the bacteria, the relationship with insect hosts is obligate because the bacteria are not found free-living in the environment. In some contexts, facultative symbionts impose constitutive costs on the aphids they infect (Oliver et al., 2008; Sakurai et al., 2005; Vorburger and Gouskov, 2011), or function as reproductive parasites (Simon et al., 2011), but in others they provide diverse benefits, including greater thermal tolerance (Montllor et al., 2002; Russell and Moran, 2006), wider host plant range (McLean et al., 2011; Tsuchida et al., 2004; Wagner et al., 2015), and defense against specialized natural enemies such as fungal pathogens (Lukasik et al., 2013; Parker et al., 2013; Scarborough et al., 2005) and parasitoid wasps (Oliver et al., 2003; Schmid et al., 2012; Vorburger et al., 2010). Given the importance of aphid-specialized parasitoid species in biological control programs (Heimpel et al., 2004; Hopper et al., 1998), the presence of defensive symbionts in either target or non-target species may affect the benefits and risks of biological control introductions (Ferrari et al., 2004; Vorburger, 2014). The best-developed cases for symbiont-mediated defense against parasitoids involve the pea aphid,
Acyrthosiphon pisum (Harris) (Hemiptera: Aphididae) and the black-bean aphid Aphis fabae (Scopoli) (Oliver et al., 2014; Vorburger, 2014). In the pea aphid, which harbors at least eight secondary symbionts (Russell et al., 2013), the symbiont Hamiltonella defensa Moran et al. (Gammaproteobacteria: Enterobacteriaceae) provides partial to complete protection against the braconid wasp Aphidius ervi Haliday (Martinez et al., 2014; Oliver et al., 2009, 2005, 2003) and likely its congener Aphidius eadyi Starý et al. (Hymenoptera: Braconidae) (Ferrari et al., 2004; Oliver et al., 2003). However, for the aphid to be protected, H. defensa must itself carry bacteriophages, named APSE (from the initials of Ac. pismum secondary endosymbionts), (Degnan and Moran, 2008; Moran et al., 2005; Oliver et al., 2009). Different APSE strain types (e.g. APSE-2, APSE-3, APSE-4) encode substantively different toxin-antagonists that are hypothesized to harm the internally developing wasps at different stages of development (Degnan and Moran, 2008). In the black bean aphid, H. defensa confers protection against attack by a different braconid, Lysiphlebus fabarum (Marshall) (Hymenoptera: Braconidae), although the level of defense varies with aphid age and the strains of bacterium and parasitoid, revealing the possibility of a co-evolutionary arms race (Schmid et al., 2012). Infection by H. defensa has been documented in 15–40% of aphid species screened (Henry et al., 2015), including the cornwea aphid, Aphis craccivora Koch (Hemiptera: Aphididae), which harbors H. defensa at varying frequencies in natural populations (Brady et al., 2014; Brady and White, 2013). Infection by H. defensa defends Ap. cracciola against some but not all parasitoid species in the family Braconidae (Asplien et al., 2014; Desneux et al., 2009). Infection by H. defensa completely eliminates parasitism by Binodoxys communis (Gahan) and Binodoxys koreanus Starý, but has no effect on parasitism by Lysiphlebus orientalis Starý and Rashkani and Aphidius colemani Viereck (Hymenoptera: Braconidae), which indicates at least genus-level specificity of protective effects by H. defensa (Asplien et al., 2014). Similarly, in pea and black bean aphids, H. defensa confers protection against some, but not all braconid parasitoids (Cayetano and Vorburger, 2015; Martinez et al., 2016).

Here, we report results on the effect of H. defensa on parasitism of Ap. cracciola and Ac. pismum by species in the other major lineage of aphid parasitoids, species of Aphelinus (Aphelinidae), for which the reported effects of H. defensa/APSE are mixed. Recent research has shown that Aphis fabae Scopoli (Hemiptera: Aphididae) infected with H. defensa is not protected from parasitism by Aphelinus chamaon (Hymenoptera: Aphelinidae), although the APSE strain was not identified (Cayetano and Vorburger, 2015). However, other research has shown that Ac. cracciola infected with H. defensa were protected from parasitism by Aphelinus abdominalis (Dahlman) (Hymenoptera: Aphelinidae) when the aphids came from one host plant species (Lotus pedunculatus Cavanilles; Fabaceae), but not when they came from two other host plant species (Ononis spinosa L. and Medicago sativa L.; Fabaceae) (McLean and Godfray, 2015).

In the present research, we compared parasitism of aphids infected with H. defensa/APSE with parasitism of uninfected aphids sharing a common aphid genotype. We measured parasitism of Ap. cracciola by Aphelinus arificlis Kurdjumov and Aphis cracciai Hopper and Woolley (Hymenoptera: Aphelinidae), species that differ strongly in host range. We measured parasitism of Ac. pismum by Aphelinus arificlis only because Aphelinus cracciai does not parasitize this aphid species. Aphelinus arificlis is native to countries around the Black Sea, including the Ukraine and the Republic of Georgia, but was introduced into the USA to control the Russian wheat aphid, Diuraphis noxia (Kurdjumov) (Hemiptera: Aphididae) in the early 1990s (Heraty et al., 2007; Hopper et al., 1998). Aphis cracciai is a recently described species native to Asia (Hopper et al., 2012), which is being released in the USA to control the soybean aphid, Aphis glycines Matsumura (Hemiptera: Aphididae). Aphetes glycines has a very narrow host range, being limited to some species in the genus Aphis, whereas Aphelinus arificlis has a very broad host range, parasitizing aphid species in at least six genera distributed across the phylogeny of aphids (KRH, unpublished data).

All Aphelinus species are koinobiont endoparasitoids of aphids (Hagen and VanDrenBosch, 1968; Viggiani, 1984), and their hosts continue to develop after oviposition for a week at 20°C, at which point Aphelinus larvae kill their hosts but leave the host exoskeleton intact, causing it to harden and turn black in a process called mummiication (Christiansen-Weniger, 1994). Aphelinus females prefer 2–4th instar aphids for oviposition, but will oviposit in all stages, including alate adults (Rohne, 2002). Because both Ac. pismum and Ap. cracciola develop from the beginning of the first instar to adult in 7 days at 20C (Lu and Kuo, 2008; Soffan and Aldawood, 2014), the stage in which female parasitoids oviposit has little effect on the aphid stage and size that is mummiified. Adult Aphelinus eat nectar and honeydew, but adult females also feed on aphid hemolymph to get amino acids and fats for egg production and survival (Hagen and VanDrenBosch, 1968; Wu and Heimpel, 2007).

Females of Aphelinus glycinis carry a mean of 8 (7–9) eggs or 12 (12–13) eggs one day after emergence, depending on whether they had four or six ovarioles (KRH, unpublished data). However, like other Aphelinus species (e.g., Perng and Liu, 2002; Wu and Heimpel, 2007), they produce far more eggs throughout their lives. In the laboratory, Aphelinus arificlisis can parasitize ~ 100 Diuraphis noxia during a lifetime with a median longevity of one week (KRH, unpublished data). Aphelinus eggs are heavily chorionated with substantial yolk, and these eggs absorb little or nothing from their hosts during embryogenesis, i.e. before hatching as 1st instars. Indeed, our criterion for egg maturity is lack of uptake of neutral red dye because mature eggs of Aphelinus have a chorion that is impervious to this relatively small molecule (molecular weight = 289), whereas immature eggs stain red (Hopper et al., 2013). Given the yolky and heavily chorionated eggs of Aphelinus species, we hypothesized that they might not be affected by H. defensa and its APSE phage. Furthermore, we hypothesized that H. defensa/APSE toxins affected later stages, Aphelinus arificlisis might be more susceptible to the effects of Hamiltonella/APSE than Aphelinus glycinis, given that the latter species specializes on aphids in the genus Aphis whereas Aphelinus arificlisis is a broad generalist that may not have evolved resistance to H. defensa/APSE.

2. Materials and methods

2.1. Insect sources and rearing conditions

Aphelinus glycinis was collected in the Peoples Republic of China under a Memorandum of Understanding between their Ministry of Agriculture and the United States Department of Agriculture (USDA). Aphelinus arificlisis was collected by employees of the USDA, Agricultural Research Service (ARS), in the Republic of Georgia with the permission of that government. The parasitoids were imported into the USDA, ARS, Beneficial Insect Introductions Research Unit containment facility in Newark, Delaware, under permits from the USDA, Animal and Plant Health Inspection Service (Permit Numbers PS26P-08-02142 and PS26P-09-01929). No specific permissions were required to collect Ap. cracciola or Ac. pismum because these are cosmopolitan aphids that occur in the field throughout North America. None of the species collected or studied are endangered or protected.

Aphelinus cracciola is a cosmopolitan pest of legumes and is anholocyclic, i.e. reproduces asexually, throughout most of its range (Blackman and Eastop, 2006). Aphyrsicosiphon pisum is an almost cosmopolitan pest of legumes and is holocyclic, i.e. alternates by asexual and sexual reproduction in temperature regions (Blackman and Eastop, 2006). Aphelinus arificlisis was collected originally in Kentucky, USA, in 2009, and Ac. pismum were collected in Utah, USA, in 2007. The aphids were cultured on fava bean, Vicia faba L., in 12 cm pots in plant growth chambers at 20 °C and a 16L:8D h photoperiod. Isofemale lines were initiated from single female aphids on individual V. faba plants. Lines from the original populations tested positive for
infection with only *H. defensa* using Denaturing Gradient Gel Electrophoresis (DGGE) analysis of PCR-amplified fragments of 16S rRNA and confirmed with diagnostic PCR (Russell et al., 2013). APSE type 3 was found in *H. defensa* in *Ac. pismum* and APSE type 4 was found in *H. defensa* in *Ap. craccivora*. Aphid lines without *H. defensa* were generated by a selective curing technique in which aphids were fed an artificial diet that was treated with equal parts of the antibiotics gentamycin, defatoxiamine and ampicillin for 3 days, after which the aphids were placed on *V. faba* plants (Dykstra et al., 2014). Clearing of *H. defensa* from offspring of aphids from these lines was confirmed by PCR (Russell et al., 2013). Cured (AC1H-) and uncured lines (AC1H+) from *Ap. craccivora* and cured (ASHH-) and uncured lines (ASHH+) from *Ac. craccivora* were used for the experiments reported here. They were shipped to Newark, Delaware, USA, in spring 2012, where they were maintained for 40–50 generations prior to these experiments. Both cured and uncured lines were tested using diagnostic PCR for the presence of *H. defensa* and APSE prior to assays.

*Apelinus atriplicis* was collected as mummified *Diaraphis noxia* on wheat near Tbilisi, Republic of Georgia, in 2000; *A. glycins* was collected as mummified *Aphis glycines* Matsumura (Hemiptera: Aphididae) on soybean near Xiuyan, Liaoqing Province, Peoples Republic of China, in 2007. *Aphelinus glycins* was described from the culture used in this study (Hopper et al., 2012); *A. atriplicis* was described by Kurdjumov (1913), and the culture used in this study was included in a molecular phylogeny of species in the *Aphelinus variipes* complex (Heraty et al., 2007). Cultures were established in the quarantine facility at the USDA-ARS, Beneficial Insect Introduction Research Unit, Newark, Delaware, USA. The experiments on parasitism of each aphid species by each wasp species were designed as randomized complete-blocks blocks: location within rearing chamber was the blocking factor and each cage with a female parasitoid, aphids, and plants was an experimental unit. There were 48 experimental units each for *Apelinus glycins* on *Ap. craccivora* and *Apelinus atriplicis* on *Ap. craccivora* and *Ac. pismum* (24 each for *Hamiltonella*-infected versus uninfected aphids). Where the female was missing or dead after 7 days, the experimental unit was dropped from the analysis. For each analysis, block was included in the initial model but was not significant and so was removed from the final model. For each parasitoid and aphid species combination, we tested the effects of *H. defensa* infection on the number of aphids parasitized (mummified), the number of adult parasitoid progeny, progeny sex ratio (proportion males), and progeny size (µg dry mass) of males and females. Because the dependent variables often had non-normal distributions with variances proportional to means, we used generalized linear models with appropriate distributions (e.g. binomial, normal, gamma, Poisson, negative binomial) for the dependent variables to test for effects of model factors using glm or glm.nb in R version 3.3.3 (R Core Team, 2014). The negative binomial distribution gave the best fit for the number of parasitized aphids and the number of adult progeny. The normal distribution fit well for sex ratio, and the gamma distribution fit well for weights of females and males. For each of the chosen distributions, the residual deviance divided by error degrees of freedom did not differ significantly from 1, which should be the case for distributions that fit well (Littell et al., 1996). For the figures, least square means and confidence intervals were converted back to the original distributions, using the appropriate inverse link functions. Because the confidence intervals were often asymmetrical, where appropriate in the text, we report means and asymptotic 95% confidence levels using the following format: mean [lower confidence level – upper confidence level]. Data are archived on the Ag Data Commons website (data.nal.usda.gov; DOI 10.15482/USDA.ADC/1356635).

### 3. Results

As hypothesized, *H. defensa* infection of *Ap. craccivora* did not affect the number of aphids parasitized by *Apelinus atriplicis* or *Apelinus glycins* (Fig. 1a, Table 1). However, *H. defensa* infection of *Ap. craccivora* increased the fitness of *Apelinus glycins*, which produced 60 limited (Bai and Mackauer, 1990; Hagen and VanDenBosch, 1968). Their lifetime fecundities are 100–300, and given that we exposed them to 100 aphids at the beginning of the experiments, and the aphids would produce > 2000 progeny during the 7 day exposure, there were far more aphids than they could parasitize during this period so the likelihood of superparasitism is quite low.

The density of aphids, amount of plant material, and cage size meant that parasitoids were not limited by search rate. Aphids parasitized by these *Apelinus* species mummify about 7 days after being parasitized and adult parasitoids emerge 10 days after mummification. To ensure that aphids parasitized throughout the exposure period had time to mummify but would not yet have emerged as adults, we collected mummified aphids 7 days after the end of the exposure of aphids to parasitoids and held them for adult parasitoid emergence. After the adults emerged, we recorded the number of mummified aphids, the number of mummies from which adults emerged, and the number and sex of adult parasitoids. We dried the adult progeny of each female at 50 °C for one hour and weighed the sexes separately on a microbalance. Because we scored parasitism after the larval parasitoids killed and mummified their hosts, which occurs during the parasitoid third instar, this measure of parasitism is a combination of acceptance of aphids for oviposition and suitability of aphids for parasitoid survival to third instar.

### 2.3. Design structure and statistical analysis

The experiments on parasitism of each aphid species by each wasp species were designed as randomized complete-blocks blocks: location within rearing chamber was the blocking factor and each cage with a female parasitoid, aphids, and plants was an experimental unit. There were 48 experimental units each for *Apelinus glycins* on *Ap. craccivora* and *Apelinus atriplicis* on *Ap. craccivora* and *Ac. pismum* (24 each for *Hamiltonella*-infected versus uninfected aphids). Where the female was missing or dead after 7 days, the experimental unit was dropped from the analysis. For each analysis, block was included in the initial model but was not significant and so was removed from the final model. For each parasitoid and aphid species combination, we tested the effects of *H. defensa* infection on the number of aphids parasitized (mummified), the number of adult parasitoid progeny, progeny sex ratio (proportion males), and progeny size (µg dry mass) of males and females. Because the dependent variables often had non-normal distributions with variances proportional to means, we used generalized linear models with appropriate distributions (e.g. binomial, normal, gamma, Poisson, negative binomial) for the dependent variables to test for effects of model factors using glm or glm.nb in R version 3.3.3 (R Core Team, 2014). The negative binomial distribution gave the best fit for the number of parasitized aphids and the number of adult progeny. The normal distribution fit well for sex ratio, and the gamma distribution fit well for weights of females and males. For each of the chosen distributions, the residual deviance divided by error degrees of freedom did not differ significantly from 1, which should be the case for distributions that fit well (Littell et al., 1996). For the figures, least square means and confidence intervals were converted back to the original distributions, using the appropriate inverse link functions. Because the confidence intervals were often asymmetrical, where appropriate in the text, we report means and asymptotic 95% confidence levels using the following format: mean [lower confidence level – upper confidence level]. Data are archived on the Ag Data Commons website (data.nal.usda.gov; DOI 10.15482/USDA.ADC/1356635).
Aphelinus atriplicis

only 9 out of 24 females were recovered on infected aphids so that twice as many aphids (47 [32 on *A. pisum* longevity of one week (KRH, unpublished data). However, for these survival rates exceed expectations from experiments on longevity


mummi of *Aphelinus atriplicis* and *Aphelinus glycinis*. Asterisks between means indicate significant difference (** *P* = 0.01). Error bars are asymptotic 95% confidence intervals.

percent more adult progeny on infected aphids than on uninfected aphids (Fig. 1, Table 1). Infection of *A. pisum* by *H. defensa* did not affect the number of mummies or adult progeny produced by *Aphelinus atriplicis* (Fig. 1b, Table 1c).

Although these experiments were not designed to test the effects of Hamiltonella for *Aphelinus glycinis*, 42 females were recovered out of 48 tested (2 were not recovered on uninfected aphids and 4 were not recovered on infected aphids). For *Aphelinus atriplicis* on *A. craccivora*, 38 females were recovered out of 48 tested (6 were not recovered on uninfected aphids and 4 were not recovered on infected aphids). Given that the females were 1–5 days old at the beginning of the experiments, these survival rates exceed expectations from experiments on longevity of *Aphelinus atriplicis*, in which adults of this parasitoid had a median longevity of one week (KRH, unpublished data). However, for *A. atriplicis* on *A. pisum*, only 29 females were recovered out of 48 tested, and only 9 out of 24 females were recovered on infected aphids so that *Aphelinus atriplicis* adults appeared to have survived significantly less well on infected *A. pisum* (*χ^2^ = 8.7, *P* = 0.003).

These parasitoid species differed in numbers of *A. craccivora* mummiified (model deviance = 9.7, df = 1; residual deviance = 89.1, df = 78; *P* = 0.002), with *Aphelinus glycinis* parasitizing more than twice as many aphids (47 [32–68]) than *Aphelinus atriplicis* (19 [13–28]) and producing twice as many adult progeny on this aphid species (32 [23–45]) than *Aphelinus atriplicis* (19 [13–27]). Given that *Aphelinus glycinis* is a specialist on aphid species in the genus *Aphis* and *Aphelinus atriplicis* has a very broad host range, this result is consistent with the pattern that specialists often do better on their hosts than generalists on the same hosts.

The numbers of mummiified *A. craccivora* and adult progeny varied among females of both parasitoid species (Fig. 2), but the variation was the same magnitude for infected and uninfected aphids so it is unlikely to result from genetic variation in parasitoid susceptibility to *H. defensa* or APSE. Thus this variation cannot result from genetic variation in susceptibility to *H. defensa* and its APSE phage, as has been found in other systems (Rouchet and Vorburger, 2012). The coefficient of variation (standard deviation/mean) in number of mummiified *A. craccivora* was over twice as large for *Aphelinus atriplicis* than for *Aphelinus glycinis* (1.2 versus 0.5, respectively), as was the coefficient for variation in number of adult progeny (1.3 versus 0.6, respectively). This was in large part because *Aphelinus atriplicis* variances were significantly larger than *Aphelinus glycinis* variances (for number of mummiified aphids: *t* = 2.5, *P* < 0.007; for number of adult progeny: *t* = 2.5, *P* < 0.01). About half of *Aphelinus atriplicis* females produced < 10 mummiified aphids (53%) and adult progeny (58%), whereas other females produced the number of mummies and adults expected if they had used their full egg loads. This suggests segregating variation in *Aphelinus atriplicis* for parasitism of *A. craccivora*, independent of infection with *H. defensa*. Such variation has been observed in parasitoids with broad host ranges (Hopper et al., 1993).

Sex ratios were not affected by *H. defensa* infection (Fig. 3a; Table 1). However, sex ratios of *Aphelinus atriplicis* on *A. pisum* and *A. craccivora* and *A. glycinis* on *A. craccivora* were female biased (*χ^2^ = 33.7, *P* < 0.0001; *χ^2^ = 25.2, *P* < 0.0001; *χ^2^ = 49.3, *P* < 0.0001, respectively). Female biased sex ratios are common in *Aphelinus*, perhaps because they attack gregarious hosts so that local mate competition leads to fewer males per aphid colony. In any case, females of both species made the same allocation of fertilized (female) versus unfertilized (male) eggs, regardless of *H. defensa* infection.

Neither female nor male masses of *Aphelinus atriplicis* were affected by *H. defensa* infection (Fig. 3B,C; Table 1). For *Aphelinus glycinis* the pattern was more complex: male mass was not affected by *H. defensa* infection, but female mass was 10 percent greater on *A. craccivora* infected with *H. defensa* than on uninfected aphids (Fig. 3B,C; Table 1A1A).

### Table 1

Analysis of deviance for the effect of infection of aphids by Hamiltonella defensa on number of parasitized (mummiified) and parasitoid fitness (see text for details of statistical analyses).

<table>
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<th>Model</th>
<th>Residual</th>
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<tr>
<td>Number of parasitized aphids</td>
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<td>Number of adult parasitoids</td>
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<td>6.2</td>
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<tr>
<td>Male mass</td>
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<td>0.2</td>
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<td>(b) <em>Aphelinus atriplicis</em> on <em>Aphelinus craccivora</em></td>
<td></td>
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<tr>
<td>Number of parasitized aphids</td>
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<td>0.6</td>
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<tr>
<td>Number of adult parasitoids</td>
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<td>6.6</td>
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<tr>
<td>Sex ratio</td>
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</tr>
<tr>
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<tr>
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<td>(c) <em>Aphelinus atriplicis</em> on <em>Acythosiphon pisum</em></td>
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<td>Number of parasitized aphids</td>
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![Fig. 1. Effects of infection of aphids by Hamiltonella defensa and its phage APSE on parasitism. (a) numbers of mummiified aphids and (b) numbers of adult parasitoid progeny of the parasitoids *Aphelinus atriplicis* and *Aphelinus glycinis*. Asterisks between means indicate significant difference (** *P* = 0.01). Error bars are asymptotic 95% confidence intervals.](image)
4. Discussion

The heritable bacterial symbiont *H. defensa* is known to confer resistance in several aphid species to certain braconid and aphelinid parasitoids of aphids (Asplen et al., 2014; Cayetano and Vorburger, 2015; Martinez et al., 2016). For *Aphelinus atriplicis*, we found no significant effects of *H. defensa* infection on the numbers of parasitized aphids or the numbers of adult progeny. However, poor recovery of *Aphelinus atriplicis* females at the end of experiment from the treatment with infected *Ac. pisum* suggests that exposure to *H. defensa* or APSE while host feeding may cause adult mortality. Unexpectedly, in the second species examined, *Aphelinus glycinis*, infection with *H. defensa* actually increased the number of adult progeny and the size of female progeny. Thus, defense against *Aphelinus glycinis* was compromised by the presence of *H. defensa* at least to the extent that parasitoid fitness was increased. While there is an increasing awareness of the roles of heritable symbionts in conferring protection against natural enemies (Oliver et al., 2014), underappreciated is the notion that symbiont infection can also decrease host performance in particular antagonistic interactions. In another recent example of this phenomenon, *Wolbachia*, which is now known to confer protection against a range of viral pathogens (Hamilton and Perlman, 2013), also increases the susceptibility of the African armyworm, *Spodoptera exempta* (Walker) to infection by baculoviruses (Graham et al., 2012).

At least two hypotheses could explain why *H. defensa* has not evolved to provide resistance against parasitism by these *Aphelinus* species. First, these *Aphelinus* species may rarely parasitize these aphids so that there has been little selection for *H. defensa*/APSE to protect against these parasitoids. There are no field data on parasitism rates of *Ap. craccivora* or *Ac. pisum* by *Aphelinus glycinis* or *Aphelinus atriplicis*. However, field surveys tend to show lower levels of parasitism of aphids by aphelinids than by braconids (Pons et al., 2011). Second, the barrier of heavily chorionated eggs in *Aphelinus* may be difficult to overcome so that even if selection were intense, there may be no segregating variation in *H. defensa* to provide a response. APSE-4, including the specific haplotype harbored by *H. defensa* in *Ap. craccivora* in the experiments reported here, has shiga-like toxin homologs (including a possible functional correlate of stxA, the cytotoxic alpha unit) which can destroy cells (Degnan and Moran, 2008). APSE-3 found in the *Ac. craccivora* line used here has a gene coding for a homology of the YD-repeat toxin gene (Degnan and Moran, 2008). However, toxins must contact the cells, which may not happen in *Aphelinus* before egg hatch. These toxins are > 300 amino acids long (MW > 33,000) and thus may not pass through the *Aphelinus* chorion given that neutral red dye (MW = 289) does not pass. Given that all *Aphelinus* have anhydropic, heavily chorionated eggs that take a long time to hatch and may not absorb toxins during embryogenesis, these *Aphelinus* species may not be affected by *H. defensa*/APSE that we tested, if the mechanism of aphid defense involves toxins that affect parasitoid eggs. This has been suggested for *Praon poquodorum* (Hymenoptera: Braconidae) which is not affected by *H. defensa*/APSE (Martinez et al., 2016). One caveat is that we only examined two *H. defensa*/APSE strains in two aphid species, and it is possible that other strains, using different protective mechanisms, can provide protection against *Aphelinus* parasitoids. *H. defensa* strain diversity appears to be very limited in North American *A. craccivora* (Dykstra et al., 2014), suggesting widespread susceptibility to *Aphelinus* in this aphid. *Ap. craccivora* shows low levels of genetic variation (Angelella, 2015), and although aphids from locust versus alfalfa differ in performance on these plants, the differences are associated with infection by *Arsenophonus* (Wagner et al., 2015). However, in *Ac. pisum*, there are numerous *H. defensa*/APSE strains that confer varying levels of protection.

![Distributions of numbers of mummified aphids and numbers of adult parasitoid progeny. (a) *Aphelinus atriplicis* and (b) *Aphelinus glycinis* with and without infection of *Aphis craccivora* by *Hamiltonella defensa* and its phage APSE-4.](image-url)
Neither of the above hypotheses can explain why *Aphelinus glycinis* produced more adult progeny and larger female progeny on aphids infected with *H. defensa* than on uninfected aphids. Dykstra et al. (2014) showed no difference in weight between infected versus uninfected aphids, using the same *A. craccivora*, the same strain of *H. defensa*, and the same APSE type used in the experiments reported here. Thus the difference in *Aphelinus glycinis* size on infected and uninfected aphids cannot result simply from a difference in the size of infected versus uninfected aphids. However, infection with endosymbionts, including *H. defensa*, can influence the pool of available metabolites circulating in the aphid hemocoel (Burke et al., 2010), thus one possible explanation is that the aphid nutritional profile is altered in a way that specifically benefits *A. glycinis*. Alternatively, many aphid secondary symbionts (although not specifically *H. defensa*) confer protection against microbial pathogens (Lukasik et al., 2013; Oliver et al., 2010), thus *H. defensa* may eliminate an *Aphelinus glycinis* competitor. A third explanation may be found in the complex interactions between parasitoids, aphid nutritional and protective bacterial symbionts, and bacteriophages. Parasitism by *Aphidius ervi* increased the titer of *Buchnera aphidicola* and APSE but decreased the titer of *H. defensa* in *Acythosiphon pism* lines susceptible to parasitism (Martinez et al., 2014). *Aphelinus glycinis* may increase host quality by changing the titer of nutritional versus protective bacteria in such a way that aphids infected with *H. defensa* can be made more suitable for parasitoid development than uninfected aphids.

Heritable symbionts, which are widespread among insects, are now associated with a wide range of protective roles, including protection against parasitoids in several aphid species and *Drosophila* (Oliver et al., 2014). Building on these initial reports are findings that protective symbioses are often dynamic, with symbiont or strain specificity to particular natural enemy species or genotypes (Asplen et al., 2014; Rouchet and Vorburger, 2014). Adding to this complexity, we found that not only does *H. defensa* not confer protection against two aphelinid parasitoids, but it actually increases the fitness of one aphelinid species. Although negative effects on host fitness in the presence of natural enemies can constrain the spread of heritable symbionts with host populations (Dykstra et al., 2014; Oliver et al., 2014), this increase in parasitoid fitness may not affect the frequency of defensive symbionts because the parasitoids appear to attack infected and uninfected aphids with equal frequency. Finding symbiont specificity to particular natural enemies informs the sustainable management of pests in agricultural systems. While braconid susceptibility to aphid secondary symbionts and their phages has led to suggestions for arti
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**Acknowledgments**

This research was funded by the United States Department of Agriculture, Agriculture Research Service and by Award 2009-02237 from the United States Department of Agriculture, Agriculture and Food Research Initiative to KRH KMO JAW MKA GEH.

**References**


