

1 **Symbiont-conferred protection against Hymenopteran parasitoids in**
2 **aphids: how general is it?**

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14

15 **Abstract**

16 1. Hosts are often targeted by multiple species of parasites, leading to a confluence of
17 selective pressures on them. In response, hosts may either evolve defences that act very
18 generally, or specific defenses against particular parasites. Aphids are attacked by multiple
19 species of endoparasitoid wasps, and there is clear evidence that heritable endosymbionts
20 can confer resistance against some of these wasps. Less clear is how symbiont-conferred
21 resistance in a single host acts against multiple parasitoid species.

22 2. We addressed this question in the black bean aphid, *Aphis fabae* (Scopoli). Unprotected
23 aphids and aphids protected by three different strains of the defensive endosymbiont
24 *Hamiltonella defensa* were exposed to four species of parasitic wasps: the parthenogenetic
25 species *Lysiphlebus fabarum* (Marshall), which was represented by three different asexual
26 lines, and the sexual species *Aphidius colemani* (Viereck), *Binodoxys angelicae* (Halliday) and
27 *Aphelinus chaonia* (Walker).

28 3. *Hamiltonella defensa* provided strong protection against *L. fabarum* and *Aphidius colemani*,
29 but there was no evidence that *H. defensa*-infected aphids were more resistant to the other
30 parasitoid species. While *Aphidius colemani* was virtually unable to parasitize any aphids
31 harbouring *H. defensa*, there was variation among the three asexual lines of *L. fabarum* in how
32 susceptible they were to the defense provided by the different symbiont strains, resulting in a
33 significant genotype-by-genotype interaction.

34 4. Our results suggest that symbiosis with *H. defensa* does not provide aphids with a general
35 defense against parasitoid wasps, possibly because some species have evolved specific
36 counter-adaptations or because biological differences preclude the symbiont's effectiveness
37 against these species.

38

39 Introduction

40 Particular host species are often targeted by multiple species of parasites (Schmid-Hempel,
41 2011; Starý, 2006). This can lead to complex coevolutionary outcomes in which a confluence
42 of selective pressures acts upon hosts and parasites (Caldarelli *et al.*, 1998; Hackett-Jones *et*
43 *al.*, 2009; Osnas & Dobson, 2012). Depending upon the frequency of encounter between these
44 organisms and the degree of competition between parasites, hosts can come to adopt a broad
45 defense against multiple parasites, or towards stronger defense against particular parasites
46 and weaker defense against others (Britton, 2009; Nuismer & Thompson, 2006; Pires &
47 Guimaraes, 2013; Poitrineau *et al.*, 2003; Takasu, 1998; Taraschewski, 2006), while parasites
48 can continue to specialize on particular hosts or to target multiple hosts, albeit often less
49 effectively (Johnson *et al.*, 2009; Krasnov *et al.*, 2004; Poisot *et al.*, 2011; Sato, 1995). The
50 degree of specificity in the host's defenses thus exerts an important influence on the evolution
51 of parasite host ranges.

52 Aphids are an important and well-studied example of hosts that must deal with attacks from
53 multiple species of parasites, most notably Hymenopteran parasitoids. Many aphid species are
54 known to be targeted by at least two and often very many species of parasitic wasps (e.g.
55 Hackett-Jones *et al.*, 2009; Müller *et al.*, 1999; Starý, 2006)The aphid considered in this
56 study, *Aphis fabae* (Hemiptera: Aphididae), is an important crop pest that is widely distributed
57 across temperate regions of the northern hemisphere (Blackman & Eastop, 2007). Based on
58 lists of aphid-parasitoid associations found in the field (Kavallieratos *et al.*, 2004; Star, 2006),
59 it can be parasitised by more than a dozen species of parasitoid wasps from the subfamily
60 Aphidiinae (Hymenoptera, Braconidae). However, one species is clearly the most important
61 parasitoid of *A. fabae*: *Lysiphlebus fabarum*. In two field-based investigations in Europe this
62 species comprised 81% and 92% of primary parasitoids that emerged from *A. fabae* hosts,
63 respectively (R. Rouchet & C. Vorburger, unpublished data, 2009; Rothacher, 2014),. Like
64 other members of the Aphidiinae, *L. fabarum* is a koinobiont parasitoid, i.e. it does not arrest
65 host development. The larva hatching from the egg injected by the female wasp develops

66 inside the still active aphid and only kills it at the end of its larval period. Notably, most
67 populations of *L. fabarum* are asexual, producing diploid daughters without fertilization
68 (thelytoky) (Belshaw *et al.*, 1999). Apart from two very closely related taxa from the same
69 genus (*L. cardui* and *L. confusus*) that are likely to be conspecific with *L. fabarum* (Sandrock *et*
70 *al.*, 2011b), the second most common aphidiine parasitoid of *A. fabae* in central Europe is
71 *Binodoxys angelicae*. It comprised 14% of aphidiines emerging from *A. fabae* in Swiss and
72 French populations (R. Rouchet & C. Vorburger, unpublished data, 2009). This species is
73 notable in that it uses special abdominal structures to facilitate more precise oviposition into
74 the host (Völkl & Mackauer, 2000). A third important parasitoid wasp of *A. fabae* is the
75 diminutive *Aphelinus choania* (Hymenoptera: Chalcidoidea: Aphelinidae). In the field
76 experiment of Rothacher (2014), this wasp comprised 2.3 % of all primary parasitoids
77 emerging from *A. fabae*, that is nearly a third of the parasitoids that did not belong to *L.*
78 *fabarum*.

79 Black bean aphids are often strongly protected against *L. fabarum* by the secondary
80 endosymbiont *Hamiltonella defensa* (Enterobacteriales: Enterobacteriaceae) (Schmid *et al.*,
81 2012; Vorburger *et al.*, 2009), which is also found in numerous other aphids (Degnan & Moran,
82 2008; Oliver *et al.*, 2010). It also confers resistance to parasitoids in pea aphids
83 (*Acyrtosiphon pisum* Harris) (Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Oliver *et al.*, 2003), but
84 apparently not in grain aphids (*Sitobion avenae*) (Łukasik *et al.*, 2013). This bacterium is
85 transmitted vertically with high fidelity (Darby & Douglas, 2003; Peccoud *et al.*, 2014). With
86 much less frequency, it can also be transmitted horizontally, either via the contaminated
87 ovipositors of parasitoid wasps (Gehrer & Vorburger, 2012) or through sex (Moran & Dunbar,
88 2006). Despite the strong benefits conferred by *H. defensa* and its multiple means of
89 transmission, it does not typically reach fixation in natural populations of aphids and tends to
90 be found at moderate frequencies only (Chen & Purcell, 1997; Oliver *et al.*, 2006; Oliver *et al.*,
91 2014; Russell *et al.*, 2013; Tsuchida *et al.*, 2002). In a collection comprising over 400
92 individuals from different locations in France and Switzerland (R. Rouchet, J. Herzog & C.
93 Vorburger, unpublished data), the symbiont was found in just over half of *A. fabae* individuals.

This suggests that, in contexts in

94 which parasitoids are absent or rare, harbouring *H. defensa* imposes substantial life history
95 costs (Oliver *et al.*, 2008; Simon *et al.*, 2011; Vorburger & Gouskov, 2011). It is likely that a
96 variety of factors, some of which may play into this cost-benefit equation, result in the
97 moderate frequency of these symbionts, including interactions with host genotype (Vorburger
98 & Gouskov, 2011) and temperature (Bensadia *et al.*, 2006; Russell & Moran, 2006), or co-
99 infections with other bacteria (Guay *et al.*, 2009).

100 While it is known that *H. defensa* provides protection against *L. fabarum* in *A. fabae* and
101 against *Aphidius ervi* Haliday and *Aphidius aedyi* (Hymenoptera: Braconidae: Aphidiinae) in
102 the pea aphid (Hemiptera: Aphididae) (Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Oliver *et al.*,
103 2003; Vorburger *et al.*, 2009), no investigations have heretofore explicitly tested the defense
104 conferred by multiple variants of a particular symbiont against multiple species of parasitoid to
105 assess the generality of protection. Here we report such a test in which we exposed black
106 bean aphids harbouring one of three different strains of *H. defensa* to four species of
107 parasitoids. In addition to the aforementioned species, we included the aphidiine *Aphidius*
108 *colemanni*, which is a less important natural enemy of *A. fabae* in the field populations we
109 study (R. Rouchet & C. Vorburger, unpublished data), but is an important biocontrol agent
110 used for greenhouse releases (Fernandez & Nentwig, 1997) that readily develops in *A. fabae*
111 (Vorburger *et al.*, 2010).

112 Determining whether *H. defensa* provides a general defense against various parasitoid species
113 or a more targeted defense against particular species in this important aphid/symbiont system
114 will contribute to a more holistic view of the selective pressures acting on *H. defensa* and *A.*
115 *fabae*, and perhaps offer insights into how these forces are driving the frequency of the
116 bacterium in the populations of this aphid.

117

118

119 **Methods**

120 ***Experimental lines***

121 We utilized asexual lines of *L. fabarum*, sexual stocks of the other parasitoid species *B.*
122 *angelicae*, *Aphidius colemani* and *Aphelinus chaonia*, and asexual lines of the host, *A. fabae*.
123 With a uniform host genetic background, the effects of different endosymbiont strains can be
124 measured unconfounded by host genetic variation; for those lines of parasitoids that are also
125 asexual, the exact same combinations of hosts and parasitoids can be replicated, providing the
126 necessary power to test for *G* x *G* interactions.

127 Our experiment used four sublines of a single clone of *A. fabae fabae*, the nominal subspecies
128 of *A. fabae*. This clone (nr. 407) was collected in July 2006 in St. Margrethen, Switzerland, and
129 has been confirmed to be free of any known facultative symbionts of aphids (Vorburger *et al.*,
130 2009). It has since been maintained on broad bean plants (*Vicia faba*) under summer-like
131 conditions (20°C with a 16h photoperiod) that ensure continuous parthenogenetic reproduction
132 in black bean aphids. Clone 407 was chosen for the current investigation because it is known
133 to be highly susceptible to parasitoids when it is symbiont-free (Vorburger *et al.*, 2009). The
134 other three sublines used here were each infected with a different isolate of *H. defensa*,
135 acquired from the hemolymph of donor clones via microinjections as explained in Vorburger *et*
136 *al.* (2010). The injections were performed at least 30 generations before the sublines' use in
137 the current study. These sublines comprised stable, heritable symbiotic associations that were
138 corroborated by diagnostic PCR just prior to the experiment. The infected sublines were
139 designated 407^{H15}, 407^{H76} and 407^{H402} (with superscripts denoting the respective symbiont
140 isolates). The three *H. defensa* isolates represent different strains based on sequence
141 variation at three housekeeping genes (L. Cayetano, J.-C. Simon & C. Vorburger, unpublished
142 data) and they were chosen on the basis of their known range of effectiveness in conferring
143 resistance against different lines of *L. fabarum* (Cayetano & Vorburger, 2013). H15 was
144 originally obtained from a clone that was collected in Toscana, Italy, on 8 May 2006, the clone
145 from which H76 was sourced was collected in La Grande Motte, France, on 5 May 2006, and

146 the clone from which H402 was sourced was collected in St. Margrethen, Switzerland, on 1
147 June 2006. Despite their geographically widespread origin, the clones from which these
148 isolates were obtained should not be assumed to originate from truly independent populations.
149 Many aphid species have a high propensity for dispersal (Llewellyn *et al.*, 2003; Loxdale *et al.*,
150 1993), including our study species *A. fabae*, for which a population genetic survey found very
151 low levels of genetic differentiation across Europe (Sandrock *et al.*, 2011a).

152 *Lysiphlebus fabarum* was represented in this study as three asexual isofemale lines,
153 designated 07-64, 06-242, and 06-658. These were selected for their range of effectiveness in
154 successfully parasitizing different sublines of *A. fabae* (Cayetano & Vorburger, 2013; Rouchet
155 & Vorburger, 2012; Schmid *et al.*, 2012). They were originally obtained from *A. f. fabae* in the
156 wild, making them appropriate for this study because of their evident adaptation to these
157 aphids in a natural setting. 07-64 was obtained in Wildberg, Switzerland in 2007, 06-242 was
158 obtained from Quartino, Ticino, Switzerland in 2006, and 06-658 was obtained from Madingley,
159 Cambridgeshire, UK in 2006. Thelytokous lines of *L. fabarum* are able to restore diploidy using
160 central fusion automixis, thereby losing heterozygosity (Belshaw & Quicke, 2003). Our wasps
161 are thus largely genetically constant within lines, though they lack true clonality. Adult wasps
162 emerge at about two weeks after oviposition.

163 *Aphelinus chaonia* were collected in Steinmaur, Switzerland on August 2012, *B. angelicae*
164 were collected from aphid colonies in Oerlikon, Zurich, Switzerland in June 2013, and *Aphidius*
165 *colemani* was kindly provided by Nickolas Kavallieratos (Benaki Phytopathological Institute,
166 Kifissia, Greece) from a large sample obtained in Athens, Greece in April 2013. After being
167 acquired, the sexual species in this study were maintained as well-mixed mass populations in
168 order to avoid inbreeding.

169 While the sex of individual *Aphidius colemani* and *B. angelicae* can be clearly determined by
170 unaided visual inspection (with female abdomens being more bulbous and pointed than those
171 of males), such discrimination could not be reliably performed for the diminutive *Aphelinus*
172 *chaonia*, which had to be inspected carefully under a microscope. They were lightly

173 anaesthetized with ethyl acetate vapour and segregated by sex according to the distinct genital
174 structures of females. They were placed into separate Eppendorf tubes and allowed to recover
175 until they were visibly active again (judged by rapid movement) before use in the experiment.

176

177 ***Experimental design and procedures***

178 This experiment quantified the resistance provided by different isolates of *H. defensa* against
179 six different lines of parasitoids, comprising three lines encompassing one asexual species (*L.*
180 *fabarum*), and three sexual lines corresponding to one sexual species each (*Aphidius*
181 *colemeni*, *B. angelicae*, *Aphelinus chaonia*). The uninfected subline and the three *H. defensa*-
182 infected sublines of aphid clone 407 were exposed to each of the six parasitoid lines for a full
183 factorial design with 24 different combinations (4 aphid lines x 6 parasitoid lines) that were
184 replicated ten times. One replicate per combination was placed at random positions in each of
185 ten different plastic trays that represented experimental blocks (randomized complete blocks
186 design). Five blocks were processed on each of two consecutive days and all blocks were kept
187 in the same climate chamber at a constant 20°C.

188 The 240 aphid colonies from which replicates were derived were maintained for two
189 generations prior to experimental treatments in order to prevent between-line variation arising
190 from environmental maternal and/or grand-maternal effects carried over from the original stock
191 culture. Fresh seedlings of broad beans grown in 0.07 litre plastic pots were used in each
192 generation. For the test generation, three adult females were placed on new plants contained
193 in 0.07 litre pots (encased in cellophane bags) and removed again after they had reproduced
194 for 24 h. Aphid nymphs on each plant were counted 48 hours after the mothers had been
195 removed, that is when they were 48-72 h old. These experimental colonies had a mean size of
196 18.2 ± 5.4 (S.D.) individuals. The juveniles were then exposed to a single wasp per cage for 24
197 h. Nine days after aphids were exposed to wasps, colonies were checked for 'mummies'
198 (successfully parasitized individuals containing a cocoon with the parasitoid pupa). Mummies
199 produced by *Aphidius colemeni*, *B. angelicae* and *L. fabarum* are easily discernable due to

200 their brown colouration. The mummies were counted along with the surviving individuals from
201 the initial batch of juveniles (now adults). *Aphelinus choania* develops more slowly than the
202 aphidiine wasps and forms black and inconspicuous mummies that are difficult to recognize in
203 a colony of black aphids. Therefore, we checked the number of aphids still present after nine
204 days, i.e. when we scored all other replicates as well, but then counted the number of wasps
205 produced in each replicate when they emerged approximately three weeks after exposure to
206 the parasitoids. However, only two of the 40 replicates exposed to this parasitoid produced any
207 wasps at all, precluding a meaningful analysis of this treatment. We suspected that females
208 may require stimulation by the presence of males to oviposit more readily, so we repeated the
209 exposures to *Aphelinus chaonia* in a follow-up experiment involving only this parasitoid
210 species, using the same procedures but adding one female and one male wasp per cage. This
211 produced a modest but appreciable increase in parasitization by this species (see Results).

212

213 **Statistics**

214 Two measures were used to quantify susceptibility to parasitoids: the proportion of all aphids
215 exposed to parasitoids that became mummified, and the proportion of mummies among all
216 individuals still present on the day of counting (minus the individuals that died in the 9 days
217 between wasp exposure and mummy counting and were thus unaccounted for). With the
218 exception of *B. angelicae* (see Figure S1 in Supplementary Material), the results for both
219 measures were qualitatively the same, so we report only the results using proportion of
220 mummies among all aphids exposed to wasps. The analysis of the alternative measure is
221 provided as Supplementary material. Proportions were arcsin-square root transformed and
222 analysed with linear models, testing for the effects of block, aphid subline, parasitoid line
223 (comprising the three asexual lines of *L. fabarum* in addition to *Aphidius colemani* and *B.*
224 *angelicae*) or parasitoid species (in which the asexual lines of *L. fabarum* were pooled
225 together), and their interactions.

226 In 8 replicates, no mummies were formed and all aphids had died before scoring. These were
227 excluded from the analysis of the proportion of mummies among individuals still present on
228 day 9 after exposure (Table S1).

229 During the counting of mummies and surviving aphids, it became apparent that there was
230 variation for the mortality incurred by aphids among colonies exposed to different parasitoid
231 species. While comparing such mortality was not a motivation for the design of the experiment,
232 it was appreciated that such differences conceivably have implications for biocontrol (see
233 Discussion). Therefore, we decided to also analyse variation in mortality that was not due to
234 mummification. This was estimated as the proportion of individuals that did neither survive nor
235 get mummified, i.e. that must have died soon after exposure to parasitoids. For this colony size
236 was used as a covariate, while block was excluded as its effect was far from significant ($P =$
237 0.956) and its variance was therefore pooled into the residual term of the analysis. A simple
238 linear model is presented to capture the most striking and pertinent overall result, namely of
239 wasp species (see Results).

240 Replicates exposed to *Aphelinus choania* could not be included in the overall analyses
241 because this treatment had to be repeated. They were thus analysed separately. We also ran
242 separate linear model analyses for all other parasitoid lines, followed by post hoc comparisons
243 (Tukey's honest significant difference, or HSD) among aphid sublines to test which infected
244 sublines differed significantly from the uninfected control (to gauge whether there is significant
245 protection) and whether there were differences between infected sublines.

246 Data was entered and analysed with SPSS/PASW 18.

247

248

249 **Results**250 *Susceptibility to parasitism*

251 There was significant variation among aphid sublines in the susceptibility to parasitism (Table
252 1a, left), largely due to *Hamiltonella*-free sublines experiencing higher rates of parasitism
253 overall than infected sublines ($F_{1,181} = 49.549$, $P < 0.001$; Fig. 1). Parasitoid lines also differed
254 significantly in the rates of parasitism they achieved and importantly, there was a significant
255 aphid subline x parasitoid line interaction (Table 1a, left), indicating that the relative
256 susceptibilities of the different aphid sublines depended on the parasitoid line they were
257 attacked by. This is clearly evident in Fig. 1, which also reports the post-hoc comparisons
258 among aphid sublines for each line of the parasitoids. *Aphidius colemani*, for example,
259 successfully parasitized approximately 40% of the aphids uninfected with *Hamiltonella* but
260 almost none of the infected aphids, indicating strong protection by the symbiont. *Binodoxys*
261 *angelicae* and *Aphelinus chaonia*, on the other hand, achieved lower rates of parasitism on
262 *Hamiltonella*-free aphids, but they also parasitized similar proportions of the aphids harbouring
263 *Hamiltonella*, hence there was no evidence for protection against these two parasitoids.
264 *Lysiphlebus fabarum* achieved similar or slightly higher rates of parasitism on symbiont-free
265 aphids as *Aphidius colemani*, and there was – as expected – clear evidence for protection by
266 *Hamiltonella*. However, the level of protection provided by the different isolates of the symbiont
267 depended on which asexual line of *L. fabarum* was considered. All isolates of *Hamiltonella*
268 increased the resistance against line 06-658, but against each of the other two lines, one
269 isolate did not provide significant protection (H402 against line 07-64 and H15 against line 06-
270 242, respectively; Fig. 1). This is the reason why the effects of aphid subline and the aphid
271 subline x parasitoid line interaction remained significant when the analysis was restricted to
272 *Hamiltonella*-infected sublines only (Table 1a, right). The interaction is driven by a strong
273 genotype-by-genotype interaction between the *Hamiltonella* isolates and the asexual lines of *L.*
274 *fabarum* ($F_{4,72} = 6.486$, $P < 0.001$ for the aphid subline x parasitoid line interaction in an
275 analysis restricted to the three *L. fabarum* lines and aphids harbouring *Hamiltonella*).

276

277 <Table 1 goes here>

278 <Figure 1 goes here>

279

280 When the analysis of parasitism rates was carried out at the level of parasitoid species by
281 pooling the three asexual lines of *L. fabarum* (Table 1b, left), there was a significant effect of
282 parasitoid species as well as significant variation among aphid sublines and a significant aphid
283 subline x parasitoid species interaction. The latter two effects reflect that the aphids harbouring
284 *Hamiltonella* were more resistant, but only against two of the four parasitoid species tested.

285 When the analysis at the level of parasitoid species is restricted to aphids with *Hamiltonella*,
286 these two effects are no longer significant (Table 1b, right).

287 *Variation in mortality not due to mummification*

288 Parasitoids differed in the mortality they inflicted on aphids that were not mummified (Table 2;
289 Fig. 2). *Binodoxys angelicae* was clearly the most harmful species, leaving relatively few
290 survivors to count in colonies exposed to the wasp. The mortality apparently inflicted by *B.*
291 *angelicae* did not differ among aphid sublines ($F_{3,35} = 1.181$, $P = 0.331$). *Aphidius colemani* and
292 *L. fabarum* imposed relatively little, if any, harm on non-mummified aphids and did not
293 significantly differ from one another (Fig. 2).

294

295 <Table 2 goes here>

296 <Figure 2 goes here>

297

298 It cannot be ruled out that the overall pattern was partially driven by colony size (Table 2),
299 which may have introduced competition for resources on these small plants. Indeed there was

300 a weak but significant positive correlation between number of aphids and death rate ($r = 0.186$,
301 $P = 0.004$). However, colony size did not differ significantly among colonies exposed to
302 different parasitoids ($F_{2,197} = 1.240$, $P = 0.292$), lending credence to a genuine effect accruing
303 from wasp species itself.

304 *Aphelinus choania*, not included in the main analysis, seemed to be no more harmful than
305 either *Aphidius colemani* or *L. fabarum* (Fig. 2).

306

307 Discussion

308 Here we asked how generally the defense conferred by *H. defensa* acts under a realistic
309 scenario that any given host species is exploited by a community of multiple parasitoid
310 species. Does symbiont-conferred protection to *A. fabae* extend to parasitoid species other
311 than the most important one, *L. fabarum*? The answer is not straightforward. Our results
312 showed that the bacterium provides *A. fabae* with very strong protection also against *Aphidius*
313 *colemani*, a frequently used biocontrol agent, but evidently not against the more distantly
314 related species *B. angelicae* and *Aphelinus choania*, which commonly attack *A. fabae* as well.

315 To find protection against *Aphidius colemani* was not surprising given that this species is
316 known to be susceptible to another protective symbiont of aphids, *Regiella insecticola*
317 (Vorburger *et al.*, 2010), and that its congeners *A. ervi* and *A. eadyi* are known to be
318 susceptible to *H. defensa*-conferred protection in the pea aphid (Ferrari *et al.*, 2004; Oliver *et*
319 *al.*, 2005; Oliver *et al.*, 2003). *Aphidius colemani* was also the species most closely related and
320 biologically similar to *L. fabarum* (Kambhampati *et al.*, 2000; Shi & Chen, 2005). The observed
321 strength of protection against *Aphidius colemani* was nevertheless striking. We do not have a
322 mechanistic explanation as to why the three isolates of *H. defensa* we tested did not protect
323 against *B. angelicae* and *Aphelinus chaonia*. In pea aphids, *H. defensa*-conferred protection
324 has been shown to be mediated by phage-encoded toxins that are released by the symbiont
325 and appear to kill the parasitoid's egg or early larva (Degnan & Moran, 2008; Moran *et al.*,

326 2005; Oliver *et al.*, 2009). It is possible that the relatively large eggs produced by parasitoids of
327 the genus *Aphelinus* (Le Ralec, 1991) are less susceptible to these toxins than the minute
328 eggs of the aphidiines. On the other hand, *B. angelicae* also belongs to the Aphidiinae, but its
329 more elaborate oviposition method might result in a more precise choice of oviposition site
330 such that its eggs may be less accessible to the host's defenses, including the symbiont-
331 produced toxins. It is known that parasitoids of *Drosophila* selected for increased infectivity
332 avoid host resistance by embedding their eggs in host tissue rather than injecting it into the
333 hemolymph (Kraaijeveld *et al.*, 2002). Notably, *B. angelicae* was also the parasitoid that
334 inflicted the highest level of harm on aphids that was due to reasons other than mummification,
335 as gauged by the high death rates of non-mummified aphids – possibly also a consequence of
336 its peculiar oviposition behaviour. From a biocontrol standpoint, the fact that this parasitoid left
337 few survivors is obviously significant, especially given that it inflicted high mortality irrespective
338 of symbiont presence or variant.

339 An important caveat for the interpretation of our results is indicated by the differences among
340 the three thelytokous lines of *L. fabarum* in how they were affected by the presence of the
341 different isolates of *H. defensa* in their host. There is clearly substantial genetic variation in this
342 species in the ability to overcome different strains of the defensive symbiont, as already
343 indicated by earlier work (Cayetano & Vorburger, 2013; Rouchet & Vorburger, 2012; Schmid *et al.*,
344 2012; Vorburger *et al.*, 2009). It is entirely plausible that such genetic variation is also
345 present in the other parasitoids we tested. Although we worked with sexual mass cultures of
346 stocks that were founded by many individuals and should therefore have comprised some
347 genetic variation, the original collections were made at a single location only. We cannot be
348 sure, therefore, whether the strong protective effect of *H. defensa* against *Aphidius colemani*
349 and the apparent lack of protection against *B. angelicae* and *Aphelinus chaonia* are
350 representative of these parasitoid species as a whole. A second caveat that applies particularly
351 to parasitism by *Aphelinus chaonia* is that a moderate level of protection by *H. defensa* would
352 have been difficult to detect due to the very low rates of parasitism overall, even in *H. defensa*-
353 free aphids. The reason for their poor success is unknown. The fact that we counted emerging

354 wasps rather than mummies in this species may have led to a slight underestimation of the
355 true rate of parasitism, e.g. if some wasps died at the mummy stage or were overlooked due
356 to their tiny size, but this alone cannot explain the low rates. It is possible that the laboratory
357 conditions under which the experiment took place, or the aphid life stage offered, may simply
358 have been suboptimal for this parasitoid.

359 These caveats notwithstanding, a clear verdict of our simple experiment remains that
360 symbionts do not protect equally well against all parasitoids. The defense by *H. defensa*
361 exhibits specificity with respect to parasitoid species and even genotypes within species.
362 Similar specificity is observed in the bacterial endosymbiont *Regiella insecticola* that protects
363 pea aphids against some but not all species of fungal pathogens (Parker *et al.*, 2013;
364 Scarborough *et al.*, 2005). The benefit a symbiont provides will thus be conditional on the local
365 community of parasitoids and pathogens. As discussed by Oliver *et al.* (2014) and Vorburger
366 (2014), this may have important consequences for the frequency and the dynamics of infection
367 with defensive symbionts in natural aphid populations. On one hand, the observed specificity
368 provides the ingredients for negative frequency-dependent selection driven by natural
369 enemies, and such symbiont-mediated coevolution can contribute to the maintenance of
370 symbiont diversity (Kwiatkowski *et al.*, 2012). On the other hand, the possession of *H. defensa*
371 is also associated with costs to the host, such as a reduction in lifespan or lower competitive
372 ability in the absence of parasitoids (Oliver *et al.*, 2008; Vorburger & Gouskov, 2011).
373 Depending on the relative frequencies of different parasitoids, this cost-benefit equation may
374 look different in different places, selecting for or against infection with particular strains of the
375 symbiont. The symbiont composition of aphid populations may thus be highly dynamic,
376 mediated by variation in local selection. Inversely, the symbionts in the host have the potential
377 to influence the parasitoid community that establishes on it. Depending on the degree of
378 symbiont-conferred protection of the aphid hosts, the relative fitness of different parasitoid
379 species or even different genotypes of the same species may change (see the variable
380 performance of *L. fabarum* lines when faced with different strains of the symbiont). There is
381 indeed evidence from a field experiment that symbiont-protected aphid populations are not

382 only less parasitized, they also differ in the parasitoid community they support (Rothacher,
383 2014).

384 Finally, returning to the biocontrol aspect, it is somewhat discouraging that protection by *H.*
385 *defensa* was most effective against the commonly used biocontrol agent *Aphidius colemani*.
386 Possibly, the exceptionally wide host range of this species (Starý, 2006) - certainly a desirable
387 trait when many pest aphids are to be targeted – may also entail that it can frequently rely on
388 unprotected hosts in the field and thus experience weaker selection for counter-adaptations to
389 overcome symbiont-conferred protection. It would be worth investigating whether the use of
390 more specialized parasitoids could be more effective for the control of aphids that are
391 frequently infected with defensive symbionts.

392

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400

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403 Assays and experimental procedures: Luis Cayetano, Christoph Vorburger

404 Data collation: Luis Cayetano

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408

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Figure legends

Figure 1. Rates of successful parasitism by parasitoid lines/species across multiple symbiont isolates. '-' denotes symbiont-free aphid subline. Same letters above error bars denote no statistically significant differences using Tukey honest significant difference (HSD) post-hoc tests after separate linear model analyses on each parasitoid line. Raw values shown.

Figure 2. Death rates of non-mummified aphids inflicted by different parasitoid species, averaged across aphid sublines. Same letters above error bars denote no statistically significant differences using Tukey honest significant difference (HSD) post-hoc tests after separate linear model analyses on each parasitoid line. *Aphelinus choania* was excluded from the main analysis but is shown for comparison.

Tables

Table 1. General linear model results for rates of parasitism achieved by parasitoids (*Lysiphlebus fabarum*, *Binodoxys angelicae* and *Aphidius colemani*) using parasitism rate calculated as the proportion of individuals mummified among the initial number of aphid nymphs exposed to parasitoids. Results for all parasitoid lines (a) and at the level of parasitoid species, with the three asexual lines of *L. fabarum* pooled together (b) are shown. The analyses were performed once with all aphid sublines, and once with only symbiont-infected sublines, because only in the latter case do the interactions between sublines and parasitoid lines truly reflect $G \times G$ interactions between symbionts and parasitoids. 'MS' denotes mean square.

Source	all aphid sublines				<i>Hamiltonella</i> -infected sublines only			
	d.f.	MS	F	P	d.f.	MS	F	P
(a) All parasitoid lines								
Block	9	0.202	2.630	0.070	9	0.113	2.094	0.035
Aphid subline	3	1.577	20.557	<0.001	2	0.193	3.583	0.031
Parasitoid line	4	0.997	12.998	<0.001	4	1.049	19.511	<0.001
A. subline x parasitoid line	12	0.328	4.278	<0.001	8	0.297	5.517	<0.001
Residual	171	0.077	-	-	126	0.054	-	-
(b) Parasitoid species								
Block	9	0.202	2.004	0.041	9	0.113	1.326	0.229
Aphid subline	3	1.006	9.987	<0.001	2	0.026	0.308	0.736
Parasitoid species	2	0.784	7.784	0.001	2	0.883	9.820	<0.001
A. subline x parasitoid species	6	0.242	2.399	0.030	4	0.118	1.389	0.241
Residual	179	0.101	-	-	132	0.085	-	-

Table 2. General linear model results for mortality that was not due to mummification. 'MS' denotes mean square.

Source	d.f.	MS	F	P
Colony size	1	0.275	4.704	0.031
Aphid subline	3	0.060	1.019	0.385
Parasitoid species	2	2.631	44.982	<0.001
A. subline x parasitoid species	6	0.035	0.596	0.733
Residual	187	0.058	-	-

SUPPLEMENTARY ONLINE APPENDIX

Table S1. General linear model results for rates of parasitism achieved by different parasitoids using parasitism rate calculated as the proportion of mummies among mummies and surviving aphids, excluding individuals that died for reasons other than parasitism. Results for all parasitoid lines (a) and at the level of parasitoid species, with the three lines of *L. fabarum* pooled together (b) are shown. The analyses were performed once with all aphid sublines, and once with only symbiont-infected sublines, because only in the latter case do the interactions between sublines and parasitoid lines strictly reflect *G x G* interactions between symbionts and parasitoids. 'MS' denotes mean square.

Source	all aphid sublines				<i>Hamiltonella</i> -infected sublines only			
	d.f.	MS	<i>F</i>	<i>P</i>	d.f.	MS	<i>F</i>	<i>P</i>
(a) All parasitoid lines								
Block	9	0.561	2.798	0.004	9	0.296	1.798	0.076
Isolate	3	2.433	12.123	<0.001	2	0.114	0.696	0.501
Parasitoid line	4	3.396	16.922	<0.001	4	4.289	26.077	<0.001
Isolate x parasitoid line	12	0.750	3.736	<0.001	8	0.460	2.798	0.007
Residual	163	0.201	-	-	118	0.164	-	-
(b) Parasitoid species								
Block	9	0.560	2.366	0.015	9	0.298	1.394	0.198
Isolate	3	1.447	6.107	0.001	2	0.183	0.853	0.428
Parasitoid species	2	4.599	19.415	<0.001	2	6.217	29.050	<0.001
Isolate x parasitoid species	6	0.930	3.924	0.001	4	0.343	1.605	0.177
Residual	171	0.237	-	-	124	0.214	-	-

<Figure S1 goes here>

Figure S1. Rates of parasitism achieved by different parasitoid lines/species, calculated as proportion of mummies to mummies + surviving aphids. The result shown here for *Binodoxys angelicae* is clearly driven by the considerable mortality imposed by this parasitoid that was not due to mummification (see Fig. 2 in text), leaving few surviving aphids at the time parasitism was scored.

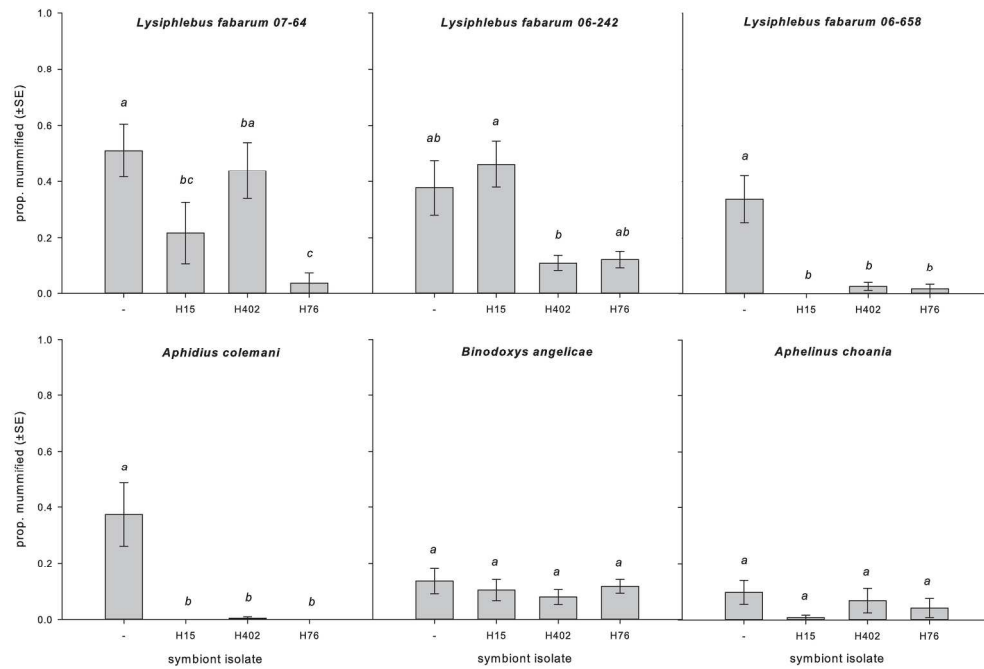


Figure 1. Rates of successful parasitism by parasitoid lines/species across multiple symbiont isolates. '-' denotes symbiont-free aphid subline. Same letters above error bars denote no statistically significant differences using Tukey honest significant difference (HSD) post-hoc tests after separate linear model analyses on each parasitoid line. Raw values shown.

222x154mm (300 x 300 DPI)

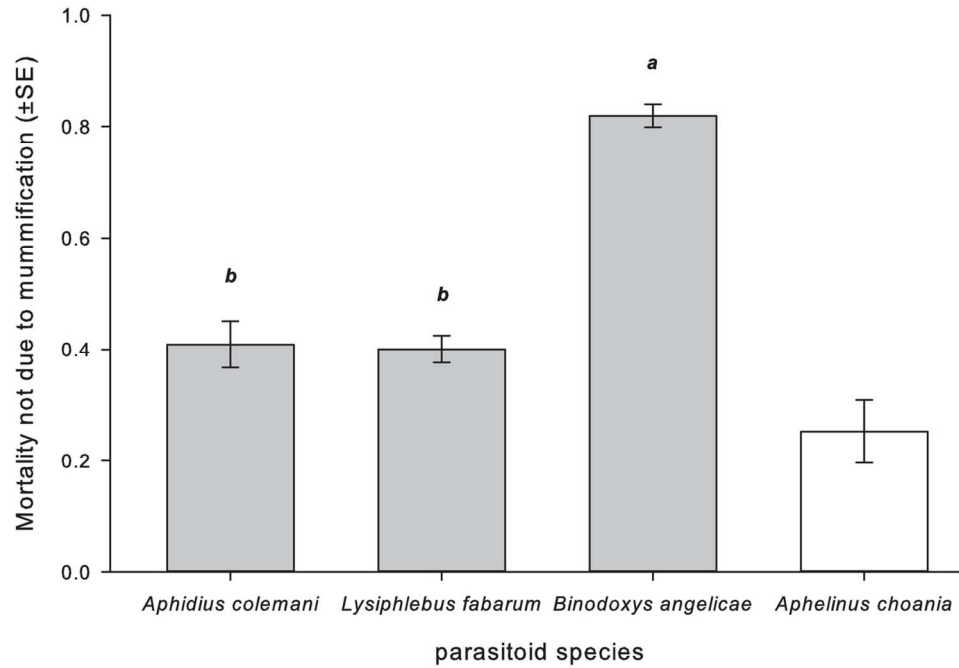


Figure 2. Death rates of non-mummified aphids inflicted by different parasitoid species, averaged across aphid sublines. Same letters above error bars denote no statistically significant differences using Tukey honest significant difference (HSD) post-hoc tests after separate linear model analyses on each parasitoid line. *Aphelinus choania* was excluded from the main analysis but is shown for comparison.

118x86mm (300 x 300 DPI)

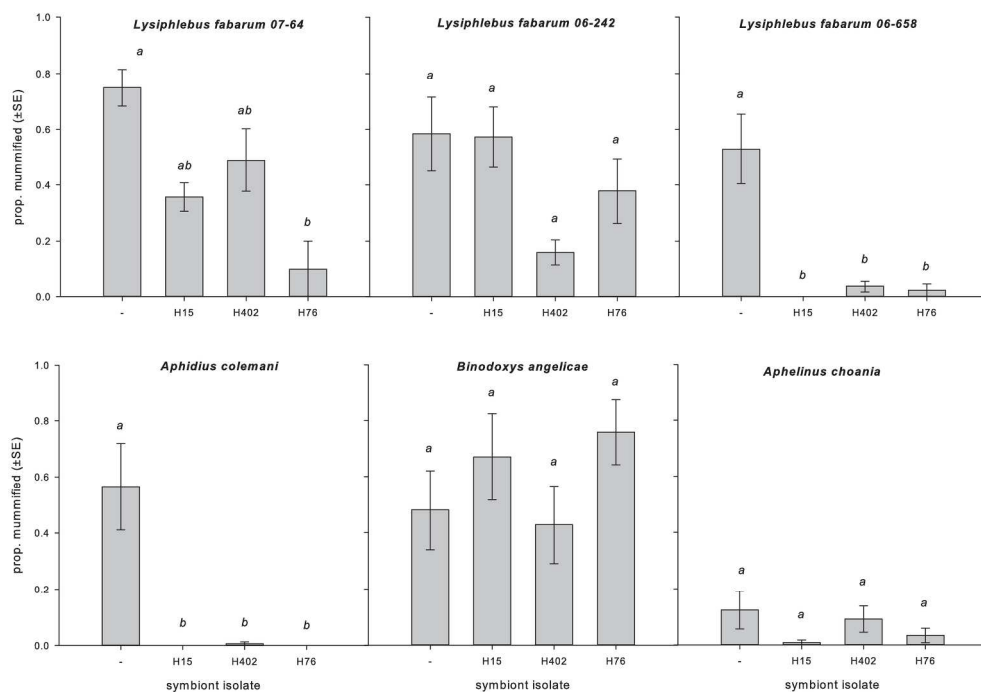


Figure S1. Rates of parasitism achieved by different parasitoid lines/species, calculated as proportion of mummies to mummies + surviving aphids. The result shown here for *Binodoxys angelicae* is clearly driven by the considerable mortality imposed by this parasitoid that was not due to mummification (see Fig. 2 in text), leaving few surviving aphids at the time parasitism was scored.

229x164mm (300 x 300 DPI)