Variation and covariation of life-history traits in aphids is related to infection with the facultative symbiont *Hamiltonella defensa*

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Abstract

Host-symbiont associations play an important role in insects. In aphids, facultative symbionts affect host plant use and increase thermal tolerance and resistance to natural enemies. In spite of these beneficial effects on aphid fitness, the frequency of facultative symbionts in aphids ranges from low to intermediate. Tradeoffs induced by symbionts could prevent the fixation of symbionts in aphid populations. Therefore, we studied life-history traits and correlations between them in 21 clones of the black bean aphid, *Aphis fabae*, of which seven were infected with the facultative endosymbiont *Hamiltonella defensa*. We found that clones harbouring *H. defensa* exhibited significantly higher body mass at maturity and offspring production, and a marginally higher intrinsic rate of increase. However, development time and offspring body size did not differ between symbiont-free and infected clones. Additionally, body mass at maturity was positively correlated with offspring production, offspring body size and intrinsic rate of increase, whereas development time was negatively correlated with body mass at maturity, offspring production and offspring body size. Excluding infected clones had little effect on these correlations; only correlations between body mass at maturity and offspring production, and between development time and offspring body size became non-significant. Therefore, we did not find any evidence for tradeoffs between life-history traits induced by symbiont infection. In fact, infected clones had higher overall fitness than symbiont-free clones under the conditions of our experiment, suggesting that symbionts do not impose costs on aphids harbouring them.

Keywords: *Aphis fabae*; black-bean aphid; costs; fitness; secondary symbiont; tradeoff; symbiosis.

INTRODUCTION

Interactions between symbionts and insect hosts range from mutualistic to parasitic relationships (Montenegro *et al.*, 2006), which can yield positive or negative effects on host fitness (Wernegreen, 2004; Oliver *et al.*, 2005). Aphid-bacteria associations represent well-known cases of mutualistic symbiotic relationships, which play an important role in aphid nutrition, reproduction, development and defence (Oliver *et al.*, 2003; Leonardo & Mondor, 2005; Moran *et al.*, 2005). Almost all aphid species harbour *Buchnera aphidicola*, a vertically transmitted, obligate endosymbiont. This association was probably established 150 to 200 million years ago (Moran & Baumann, 1994). Aphids provide to *B. aphidicola* a stable niche and nutrients, while *B. aphidicola* produces essential amino acids for its host (Douglas, 1998).

Aphids can also harbor facultative symbionts, which are not essential to aphids but have important phenotypic effects on their hosts. For instance, *Hamiltonella defensa* and *Serratia symbiotica* confer resistance to parasitoid attack in the pea aphid, *Acyrtosiphon pisum* (Oliver *et al.* 2003), and *H. defensa* appears to have the same effect in the black bean aphid, *Aphis fabae* (Vorburger *et al.*, 2009). In the case of another facultative symbiont, *Regiella insecticola*, infection can influence the ability to use certain host plants (Tsuchida *et al.*, 2004), provide protection against a fungal pathogen (Scarborough *et al.*, 2005), or – at least in one known strain – increase resistance to parasitoids (Vorburger *et al.*, in press). Finally, it has been reported that both *H. defensa* and *S. symbiotica* confer heat tolerance in pea aphids (Montllor *et al.*, 2002; Russell & Moran, 2005). As all these effects seem beneficial to aphids because they increase fitness and/or survival, one would expect natural selection to favour and eventually fix these symbiotic relationships in natural populations. However, this is not the case. Facultative symbionts typically occur at low to intermediate frequencies in aphid populations (Montllor *et al.*, 2002; Oliver *et al.*, 2006; Simon *et al.*, 2007). Unless aphids frequently lose them, for which

there is no evidence at present (Darby & Douglas, 2003), this pattern suggests that there are also costs associated with harbouring facultative symbionts. Indeed, Russel & Moran (2005) reported that pea aphids infected with *R. insecticola* had reduced survival after a heat shock compared to uninfected aphids. Oliver *et al.* (2006) report reduced fecundity of infected aphids, albeit only when they were experimentally infected with more than one symbiont, which may not be common in natural populations. In addition, a population cage experiment suggested that aphids infected with *H. defensa* might be less competitive than those without this symbiont, despite a slight positive effect of *H. defensa* on aphid fecundity (Oliver *et al.*, 2008). However, the possibility that symbionts might affect correlations and potential tradeoffs among different traits that contribute to fitness has not received sufficient attention. Tradeoffs can constrain simultaneous benefits from two traits because both traits cannot be maximized at the same time (Stearns, 1989; Roff, 2001). Therefore, benefits or costs of harbouring symbionts could not only be related to increase or decrease in fecundity and/or survival, but also to changes in relationships between fitness-related traits in infected aphids.

In the present study, we evaluated the effects of natural infections with *H. defensa* on life-history traits of the black bean aphid, *Aphis fabae*. We compared development time, body mass at maturity, offspring production, offspring body size and intrinsic rate of increase between clonal lines with and without *H. defensa*. We also estimated correlations between life-history traits in all clones, only in clones harbouring *H. defensa* and only in uninfected clones. We decided to study life-history traits because they are closely associated with fitness and energetically related (Stearns, 1992; Roff, 1997). If individuals allocate more energy to growth, there will be less energy available for other functions such as reproduction (Reznick *et al.*, 2000). Therefore, two scenarios are plausible in aphids harbouring symbionts. First, facultative symbionts may increase the aphids' capacities to obtain energy, which can then be allocated to several functions

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389simultaneously (= no costs). This could be the case if - similar to the obligate endosymbiont *B.*

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590*aphidicola* - facultative symbionts provided nutritional benefits to the host. But, there is presently

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891no evidence that this is the case for *H. defensa* (Douglas *et al.*, 2006). Alternatively, symbionts

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1092may effect costs by increasing some reproductive traits at the expense of other correlated traits

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1293(e.g. by increasing offspring number to favour their own transmission, which might entail a

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1494reduction in offspring size).

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MATERIALS AND METHODS

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STUDY SPECIES

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2498The black bean aphid, *Aphis fabae*, is widespread in temperate regions of the northern

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2799hemisphere, where it is a major pest on several crop plants (Blackman & Eastop, 2000). It

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29100reproduces by cyclical parthenogenesis over most of its range, although obligate parthenogenesis

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31101occurs in southern Europe, where mild winters allow overwintering in the active stage (C.

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33102Sandrock, J. Razmjou & C. Vorburger, unpublished data). *Aphis fabae* is heteroecious (host-

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35103alternating), using European spindle (*Euonymus europaeus*) or the snowball tree (*Viburnum*

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37104*opulus*) as primary hosts where mating and egg-laying take place, and a wide range of secondary

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39105host plants during the parthenogenetic phase of the life cycle. Here we focus on *A. fabae fabae*,

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41106one of several described subspecies of *A. fabae* that differ in the secondary host plants they can

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43107colonize (Blackman & Eastop, 2000; Raymond *et al.*, 2001). *Aphis fabae fabae* mainly feeds on

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45108broad bean (*Vicia faba*), on which it can cause major damage, and on many Chenopodiaceae, e.g.

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47109sugar beet (*Beta vulgaris*) or the common weed *Chenopodium album*.

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TEST CLONES

The 21 clones of *A. f. fabae* used in this study were collected in Switzerland in June and July 2006. Samples were taken from secondary hosts by screening suitable plants for the presence of *A. f. fabae* and clipping infested leaves or shoots. A single parthenogenetic female from each sample was used to establish clonal lines that were maintained on caged seedlings of broad bean (*Vicia faba*, var. 'Scirocco') grown in 0.07-l plastic pots. Clonal lines were kept in growth chambers under conditions that ensure continuous apomictic parthenogenesis (16 h photoperiod at 20° C), and propagated every 10-11 days by transferring 3 – 5 young adults on new seedlings as parents of the next generation.

All clonal lines were genotyped at eight microsatellite loci (Coeur d'Acier *et al.*, 2004), and screened for the presence of facultative endosymbiotic bacteria as described in Vorburger *et al.* (2009). Briefly, we amplified part of the bacterial 16S ribosomalRNA gene using universal bacterial primers (10F, 35R; Sandström *et al.*, 2001; Russell and Moran, 2005) and ran the PCR reactions on 2% agarose gels. When a product was present, it was sequenced for comparison with sequences of known endosymbionts of aphids. We confirmed the sequencing results by diagnostic PCR, using specific primer pairs for the most common symbionts *H. defensa*, *R. insecticola* and *S. symbiotica* (Sandström *et al.*, 2001; Russell *et al.*, 2003; Tsuchida *et al.*, 2006). The 21 clones all exhibited different multilocus microsatellite genotypes. Seven clones were infected with *H. defensa*, whereas the remaining 14 clones were not infected with any known facultative symbionts. The clones' microsatellite genotypes and infection status are listed in Table 1, together with additional collection information. Please note that this set of clones includes 19 clones also used in a published study of *A. f. fabae*'s resistance to parasitoids (Vorburger *et al.*, 2009).

EXPERIMENTAL PROCEDURES

To start the experiment, each clone was split into seven sublines, and each subline was assigned to a random position in seven different plastic trays (randomized complete blocks – one subline of each clone per tray). Trays within the plant growth chamber and sublines within trays were regularly rotated. To eliminate environmental maternal and grandmaternal effects that could be carried over from the stock culture, sublines were maintained for two generations before traits were assayed in the third generation. Adults from the second subline generation were separately enclosed in clip cages on leaves of broad bean seedlings. After one day, adults were removed from plants, and the newborn nymphs were left on the plants for six days. Afterwards, we removed all but one randomly selected nymph, which was checked every 24 h to record the first reproduction (= development time). On the day of first reproduction, adult females were weighed (= body mass at maturity) to the nearest μg on a Mettler MX5 microbalance (Mettler-Toledo GmbH, Greifensee, Switzerland) and placed back on the same seedling. On a daily basis, newborn nymphs produced by each aphid female were counted and removed from the clip cage for a period equal to development time (= offspring production), following Wyatt & White (1977). All newborn nymphs from the first reproductive day of each aphid were collected into 70% ethanol. The nymphs were mounted on slides and photographed with a digital camera attached to a microscope. Length of the hind tibia (= offspring body size) was measured from each digital picture using the Image J software (National Institutes of Health, USA).

Additionally, we estimated the intrinsic rate of increase (r_m) for each aphid, which relates the fecundity of an aphid to its development time (Wyatt & White, 1977). It was estimated using the follow equation: $r_m = 0.738 * (\ln M_d) / T_d$, where $\ln M_d$ is the natural logarithm of M_d (= offspring production), T_d is development time and 0.738 is a correction factor (regression slope

between intrinsic rate obtained from life-table and Wyatt and White's methods). This simplified method of estimating r_m has been shown to come remarkably close to the classical estimator of Birch (1948), which would require a complete life-table and more tedious calculations.

STATISTICAL ANALYSIS

Life-history traits were analysed by performing nested ANOVAs with block and infection status as fixed factors, and clone as a random factor nested in infection status. We also investigated the relationship between traits by calculating correlations of clone means (r_{cm} , see Via, 1991), which are an approximation of the genetic correlations. These correlations were calculated using Pearson product-moment correlations among the clones' mean trait values. However, correlations between intrinsic rate of increase and development time, and between intrinsic rate of increase and offspring production were not estimated because these traits are algebraically related (see equation 1). Clone-mean correlations were calculated for all clones, only for clones without facultative symbionts, and only for clones harbouring *H. defensa*. To compare the correlations between the two groups of clones with and without *H. defensa*, Fisher's z -transformation with Hotelling's approximation for small sample sizes was used as detailed in Sokal and Rohlf (1995, p. 574-583). All analyses were performed using Statistica 6.0 software (Statsoft, OK, USA).

RESULTS

We observed a significant difference between clones of *A. fabae* harbouring *H. defensa* and uninfected clones for body mass at maturity and offspring production (Table 2). Adults of infected clones were 18% heavier and produced 16% more offspring than adults of clones without *H. defensa* (Fig. 1). Thus, aphids harbouring *H. defensa* were larger and more fecund on average (Fig. 1). Accordingly, infected clones had a higher intrinsic rate of increase than

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uninfected clones (Fig. 1C), although this effect was marginally non-significant (Table 2). Development time and offspring body size did not differ significantly between clones with and without *H. defensa* (Table 2). The intrinsic rate of increase also showed statistically significant variation among clones with the same infection status (Table 2, Fig. 1C), indicating a strong genetic component in the variation for this trait.

We detected significant correlations between some life-history traits in *A. fabae*, especially related to development time and body mass at maturity (Fig. 2). Considering all clones, body mass at maturity was positively correlated with offspring production, offspring body size and intrinsic rate of increase (Fig. 2A-C, Table 3). At the same time, development time was negatively correlated with body mass at maturity, offspring production and offspring body size (Fig. 2D-F, Table 3). Hence, aphid clones with shorter development times reached larger body mass and produced more and larger offspring than aphids with longer development times (Fig. 2). Additionally, “fast” clones had a higher intrinsic rate of increase than “slow” clones.

When clones with *H. defensa* were excluded, all correlations remained very similar in sign and magnitude (Table 3), and four of the six significant correlations were still significant, namely those between body mass at maturity and offspring body size and intrinsic rate of increase, as well as the negative correlations between development time and body mass at maturity and offspring production (Table 3). Thus, also when only *H. defensa*-free aphids were considered, fast-maturing clones produced larger adults that had more and larger offspring than slow-maturing clones.

When only infected clones were analyzed, all correlations were non-significant, mainly reflecting limited power with only seven clones harboring *H. defensa*. Many correlation coefficients were similar to those in non-infected clones, although two correlations differed in sign (Table 3). However, in none of the cases was the comparison of correlations between the

206 two groups significant. Thus, there is no firm evidence that genetic correlations among life-
207 history traits in *A. fabae* are altered by infection with *H. defensa*.

DISCUSSION

210 The present study compared variation and covariation for life-history traits between clones of the
211 black bean aphid infected with *H. defensa* and uninfected clones. The results suggest that in
212 addition to known benefits such as thermal tolerance and resistance to natural enemies (Montllor
213 *et al.*, 2002; Oliver *et al.*, 2003), this symbiont may also have positive effects on aphid life-
214 history traits; infected clones exhibited larger body mass and a higher fecundity than clones
215 without *H. defensa*. This is correlative evidence, however, because we worked with naturally
216 infected clones and did not manipulate infections.

217 Our findings agree with those from a study measuring susceptibility to parasitoids and
218 fecundity in a very similar set of clones, which also found *H. defensa*-bearing clones to be more
219 fecund on average (Vorburger *et al.*, 2009). In contrast to the present study, where aphids were
220 confined to leaves with clip cages, the study by Vorburger *et al.* (2009) allowed aphids to forage
221 freely on the stems of broad bean plants. Those conditions were much more benign for the aphids
222 and resulted in a mean fecundity about twice as high as that observed here. That clones
223 harbouring *H. defensa* were more fecund under both conditions suggests that this is a general
224 phenomenon and not contingent on a specific environment.

225 Most studies have evaluated benefits and costs in the aphid-symbiont relationship
226 exploring differences in mean trait values (e.g. Russell & Moran 2005; Oliver *et al.*, 2006), but
227 few studies have addressed changes in the relationships between different traits. According to
228 life-history theory, individuals that mature late have more time to grow and hence achieve a
229 larger body size compared to individuals with a short development time. Such a tradeoff between

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5 231 early maturity is of chief importance for fitness (Roff, 1981; Via & Shaw, 1996), which one
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8 232 would expect in predominantly r-selected animals like aphids. However, we found for *A. fabae*
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10 233 that clones with a short development time tended to have larger body mass and leave a higher
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12 234 number of larger offspring compared to clones with longer development time (Fig. 2). These
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14 235 associations were equally evident when just clones without *H. defensa* or when all clones were
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16 236 considered, but the clones infected with *H. defensa* tended to exhibit higher mean trait values
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18 237 (Fig. 1), resulting in their clustering toward the 'fitter' end of bivariate trait distributions (Fig. 2).
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20 238 Therefore, it can be expected that aphid clones harbouring this symbiont being positively
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22 239 selected, increasing symbiont frequency. Then why do facultative symbionts only occur at low to
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24 240 intermediate frequencies in aphid populations? Possibly, differences between field and laboratory
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26 241 approaches are responsible for this discrepancy. For instance, Darby *et al.* (2003) reported that
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28 242 the fecundity of pea aphids harbouring *H. defensa* did not differ from that of symbiont-free
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30 243 aphids under natural conditions, suggesting that the symbionts may have negligible effects in the
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32 244 field. Clearly, more effort is needed to establish symbiont effects on ecologically relevant traits in
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41 246 Another interesting finding of this study was that we did not find any tradeoffs between
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43 247 life-history traits in *A. fabae*. Similar results were found in the peach-potato aphid, *Myzus*
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45 248 *persicae* (Vorburger, 2005). It almost seems as if all components of fitness could be maximized
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47 249 at the same time in these aphids, independent of their infection status. But generally, tradeoffs
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49 250 may only be expressed under certain (often stressful) environmental conditions (Sgrò &
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51 251 Hoffmann, 2004), or they may be expressed across environments. For instance, fitness trade-offs
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53 252 among host plants have been reported in the pea aphid, *A. pisum* (Via, 1991), and in the black
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55 253 bean aphid, *A. fabae* (Mackenzie, 1996), yet these studies seem to have included representatives
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of more than one host race or subspecies, which we tried to avoid here by focusing on only one subspecies of *A. fabae*. In the polyphagous aphid *M. persicae*, fitness hierarchies among clonal genotypes were surprisingly consistent across different, unrelated host plants (Vorburger & Ramsauer, 2008). Further, Nespolo *et al.* (2008) found little effect of temperature on the correlation structure of different life-history traits in the aphid *Rhopalosiphum padi*. Also, no evidence of tradeoffs was found between reproductive traits and defence against parasitoids in the pea aphid (Ferrari *et al.*, 2001; but see Gwynn *et al.*, 2005), the peach-potato aphid (von Burg *et al.*, 2008), and the black bean aphid (Vorburger *et al.*, 2009). Thus, it appears that variation in 'general vigor' (Fry 1993) among aphid clones is often large enough to mask expected trade-offs, and that infection with symbionts such as *H. defensa* may contribute to this variation by improving the overall condition of their hosts. Just looking for negative correlations between traits might therefore be of limited value to understand why *H. defensa* does not go to fixation in aphid populations. A promising result from a different approach is reported by Oliver *et al.* (2008), who found that endosymbionts did not negatively affect aphid life-history traits, yet infected lines were nevertheless outcompeted by uninfected lines in population cages.

To summarize, we found that clones of *A. f. fabae* harbouring *H. defensa* perform significantly better than uninfected clones for important life-history traits, and that correlations between these traits provide no evidence for tradeoffs. These findings suggest that infected clones have an overall fitness higher than symbiont-free clones. Therefore, these results do not give an explanation why defensive symbionts such as *H. defensa* are not completely fixed in aphid populations. Additional studies are needed to evaluate if these finding can be generalised to other aphid-symbiont interactions and why beneficial symbionts are not more widespread in aphid populations.

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Figure Captions

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Figure 1. Clonal means of (A) body mass at maturity, (B) offspring production and (C) intrinsic rate of increase (mean $\pm$ SE) in clones of *Aphis fabae* harbouring the facultative symbiont *Hamiltonella defensa* (white circles, $n = 7$) and symbiont-free clones (black circles, $n = 14$). Continuous lines represent means of clones harbouring *H. defensa* and broken lines represent means of clones without symbionts. Body mass at maturity (A) and offspring production (B) were significant higher for clones with *H. defensa* than for clones without symbionts at $P < 0.05$, whereas intrinsic rate of increase (C) was marginally higher ($P = 0.054$) for clones with *H. defensa* than for clones without symbionts.

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Figure 2. Significant correlations ($P < 0.05$) between (A) body mass at maturity and offspring production, (B) body mass at maturity and offspring body size, (C) body mass at maturity and intrinsic rate of increase, (D) development time and body mass at maturity, (E) development time and offspring production, and (F) development time and offspring body size in clones (mean $\pm$ SE) of *Aphis fabae* harbouring the facultative symbiont *Hamiltonella defensa* (white circles, $n = 7$) and symbiont-free clones (black circles, $n = 14$). Correlations showed in B, C, D and E continued being significant at $P < 0.05$ when clones harbouring *H. defensa* were excluded. Any correlation was significant ($P > 0.05$) between clones harbouring *H. defensa*.

Table 2. Nested ANOVAs testing for the effects of block (random factor), infection with *Hamiltonella defensa* (fixed factor) and clone (random factor nested in infection status) for development time, body mass at maturity, fecundity, intrinsic rate of increase and offspring body size. Asterisks show significant effects at $P < 0.05$.

Effect	df	MS	F	P
Development time				
Block	6	0.38	0.79	0.566
Infection	1	0.93	1.16	0.294
Clone(infection)	19	0.80	1.67	0.056
Residuals	88	0.48		
Body mass at maturity				
Block	6	39817	1.15	0.339
Infection	1	398667	7.79	0.012*
Clone(infection)	19	51145	1.48	0.112
Residuals	90	34543		
Offspring production				
Block	6	38	0.67	0.677
Infection	1	503	7.62	0.012*
Clone(infection)	19	66	1.16	0.314
Residuals	81	57		
Intrinsic rate of increase				
Block	6	0.0005	0.83	0.548
Infection	1	0.0063	4.20	0.054
Clone(infection)	19	0.0015	2.50	0.002*
Residuals	81	0.0006		
Offspring body size				
Block	6	202	0.66	0.684
Infection	1	355	0.85	0.369
Clone(infection)	19	419	1.36	0.168
Residuals	84	307		

Table 3. Clonal mean correlations ($r_{cm} \pm SE$) between life-history traits of the black bean aphid (*Aphis fabae*), considering all clones (n = 21), symbiont-free clones (n = 14) and clones harbouring *Hamiltonella defensa* (n = 7), as well as a statistical comparison of correlations between symbiont-free and infected clones. Abbreviations of traits: development time (Td), body mass at maturity (Bs), offspring production (Md), intrinsic rate of increase (r_m) and offspring body size (Os). Significant correlations are in bold ($P < 0.05$).

Traits	All clones		Symbiont-free clones		<i>H. defensa</i> infected clones		Comparison of correlations	
	r_{cm}	<i>P</i>	r_{cm}	<i>P</i>	r_{cm}	<i>P</i>	Fisher's <i>z</i>	<i>P</i>
Td-Bs	-0.59 ± 0.19	0.005	-0.56 ± 0.24	0.038	-0.40 ± 0.41	0.380	-0.47	0.639
Td-Md	-0.58 ± 0.19	0.006	-0.61 ± 0.23	0.021	-0.11 ± 0.45	0.820	-1.14	0.253
Td-Os	-0.48 ± 0.20	0.029	-0.42 ± 0.26	0.131	-0.58 ± 0.37	0.174	0.29	0.774
Bs-Md	0.51 ± 0.20	0.019	0.52 ± 0.25	0.059	-0.33 ± 0.42	0.475	1.66	0.097
Bs-Os	0.65 ± 0.17	0.001	0.66 ± 0.22	0.010	0.68 ± 0.33	0.093	0.08	0.940
Bs- r_m	0.58 ± 0.19	0.006	0.55 ± 0.24	0.040	0.15 ± 0.44	0.756	0.90	0.366
Md-Os	0.26 ± 0.22	0.247	0.34 ± 0.27	0.229	-0.56 ± 0.37	0.187	1.74	0.082
Os- r_m	0.42 ± 0.21	0.059	0.41 ± 0.26	0.151	0.24 ± 0.43	0.605	0.40	0.688

Appendix 1. Clonal means ($\pm$ SE) for the five life-history traits assayed in *Aphis fabae*. HTL stands for hind tibia length means on newly born first instar nymphs as an estimate of offspring body size.

Sample ID	Development time (days)	Body mass at maturity (micrograms)	Offspring production	Offspring body size (HTL, micrometers)	Intrinsic rate of increase (days ⁻¹)
203	10.33 $\pm$ 0.80	598.50 $\pm$ 72.00	24.71 $\pm$ 2.55	249.77 $\pm$ 6.16	0.230 $\pm$ 0.017
204	9.86 $\pm$ 0.13	799.57 $\pm$ 41.44	40.71 $\pm$ 1.90	252.39 $\pm$ 3.21	0.277 $\pm$ 0.004
205	10.33 $\pm$ 0.42	655.50 $\pm$ 54.56	35.67 $\pm$ 1.52	245.94 $\pm$ 9.34	0.257 $\pm$ 0.009
206	10.17 $\pm$ 0.17	814.83 $\pm$ 56.90	27.20 $\pm$ 4.63	255.34 $\pm$ 7.25	0.233 $\pm$ 0.017
208	10.00 $\pm$ 0.45	811.17 $\pm$ 111.25	36.50 $\pm$ 5.00	256.67 $\pm$ 8.39	0.263 $\pm$ 0.012
252	11.00 $\pm$ 0.58	528.33 $\pm$ 85.76	29.00 $\pm$ 6.11	245.67 $\pm$ 7.51	0.222 $\pm$ 0.010
253	10.60 $\pm$ 0.40	836.80 $\pm$ 60.97	31.60 $\pm$ 2.54	263.21 $\pm$ 8.22	0.240 $\pm$ 0.006
255	10.00 $\pm$ 0.00	688.00 $\pm$ 65.89	34.00 $\pm$ 3.11	238.33 $\pm$ 3.23	0.259 $\pm$ 0.006
257	10.33 $\pm$ 0.34	700.83 $\pm$ 108.05	31.00 $\pm$ 4.27	250.25 $\pm$ 8.08	0.239 $\pm$ 0.004
267	9.50 $\pm$ 0.29	813.50 $\pm$ 73.75	37.00 $\pm$ 3.81	269.50 $\pm$ 10.97	0.280 $\pm$ 0.008
323	9.29 $\pm$ 0.18	876.43 $\pm$ 46.00	37.86 $\pm$ 1.62	256.88 $\pm$ 3.90	0.289 $\pm$ 0.005
327	9.57 $\pm$ 0.20	803.30 $\pm$ 63.53	36.57 $\pm$ 1.39	256.80 $\pm$ 4.42	0.278 $\pm$ 0.006
329	10.33 $\pm$ 0.42	774.17 $\pm$ 65.23	37.60 $\pm$ 5.27	244.61 $\pm$ 14.64	0.252 $\pm$ 0.016
336	10.00 $\pm$ 0.32	689.20 $\pm$ 90.08	34.00 $\pm$ 3.89	253.33 $\pm$ 6.71	0.259 $\pm$ 0.010
401	10.00 $\pm$ 0.00	622.33 $\pm$ 83.27	30.33 $\pm$ 1.67	252.22 $\pm$ 13.73	0.252 $\pm$ 0.004
402	9.86 $\pm$ 0.14	969.86 $\pm$ 122.77	31.33 $\pm$ 3.24	262.99 $\pm$ 10.40	0.256 $\pm$ 0.005
404	9.57 $\pm$ 0.20	836.57 $\pm$ 64.60	37.00 $\pm$ 2.78	263.17 $\pm$ 3.65	0.283 $\pm$ 0.008
405	9.67 $\pm$ 0.21	712.17 $\pm$ 69.15	31.33 $\pm$ 3.00	237.78 $\pm$ 3.91	0.264 $\pm$ 0.009
407	10.83 $\pm$ 0.65	586.33 $\pm$ 89.70	29.27 $\pm$ 1.92	234.71 $\pm$ 6.40	0.233 $\pm$ 0.014
409	9.60 $\pm$ 0.24	766.60 $\pm$ 35.57	37.00 $\pm$ 1.00	257.85 $\pm$ 10.16	0.276 $\pm$ 0.011
445	10.00 $\pm$ 0.32	593.00 $\pm$ 64.35	30.20 $\pm$ 3.65	253.50 $\pm$ 4.77	0.251 $\pm$ 0.015

Figure 2. Castañeda et al.