Only helpful when required: A longevity cost of harbouring defensive symbionts

C. VORBURGER[1*] & A. GOUSKOV[2,3]

[1]Institute of Integrative Biology, ETH Zürich, 8092 Zürich, Switzerland and EAWAG, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland (christoph.vorburger@eawag.ch)

[2]Institute of Evolutionary Biology and Environmental Studies, University of Zürich, 8057 Zürich, Switzerland

[3]present address: EAWAG, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland (alexandre.gouskov@eawag)

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***correspondence:** Christoph Vorburger
EAWAG - ECO
Überlandstrasse 133 Ph: +41 44 823 51 96
8600 Dübendorf Fax: +41 44 823 50 28
Switzerland christoph.vorburger@eawag.ch

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Abstract

Maternally transmitted symbionts can spread in host populations if they provide a fitness benefit to their hosts. *Hamiltonella defensa*, a bacterial endosymbiont of aphids, protects hosts against parasitoids but only occurs at moderate frequencies in most aphid populations. This suggests that harbouring this symbiont is also associated with costs, yet the nature of these costs has remained elusive. Here we demonstrate an important and clearly defined cost: reduced longevity. Experimental infections with six different isolates of *H. defensa* caused strongly reduced lifespans in two different clones of the black bean aphid, *Aphis fabae*, resulting in a significantly lower lifetime reproduction. However, the two aphid clones were unequally affected by the presence of *H. defensa*, and the magnitude of the longevity cost was further determined by genotype × genotype interactions between host and symbiont, which has important consequences for their coevolution.

keywords: cost of resistance, longevity, *Hamiltonella defensa*, parasitoid, symbiosis, trade-off

Introduction

Insects and other arthropods are frequently infected with heritable microbial endosymbionts. Such symbionts can increase in frequency in the host population by reproductive manipulations that favour their transmission (e.g. the induction of cytoplasmic incompatibility, feminization, male-killing or parthenogenesis by *Wolbachia*) (Stouthamer *et al.*, 1999), or by providing a net fitness benefit to their hosts (e.g. Jaenike *et al.*, 2010). Aphids harbour a wide variety of bacterial endosymbionts (Oliver *et al.*, 2010). The obligate endosymbiont *Buchnera aphidicola* is required for aphid survival and provides a nutritional benefit by synthesizing essential amino acids (Douglas, 1998). In addition to *B. aphidicola*, aphids commonly harbour facultative or secondary endosymbionts that may be beneficial but are not strictly required for aphid survival. One such symbiont belonging to the Enterobacteriaceae, *Hamiltonella defensa* (Moran *et al.*, 2005), has been shown to increase aphid resistance to parasitoids (Oliver *et al.*, 2003; Ferrari *et al.*, 2004; Oliver *et al.*, 2005; Vorburger *et al.*, 2009). Symbiont-conferred resistance provides a strong selective advantage in the presence of parasitoids (Herzog *et al.*, 2007; Oliver *et al.*, 2008), yet *H. defensa* only occurs at low to intermediate frequencies in natural population of aphids (Tsuchida *et al.*, 2002; Simon *et al.*, 2003; Oliver *et al.*, 2006; Vorburger *et al.*, 2009). This suggests that harbouring *H. defensa* also entails costs that select against infected aphids when selection by parasitoids is weak. Indeed, a study on pea aphids, *Acyrthosiphon pisum*, found that *H. defensa*-infected aphids declined in population cages when competing with uninfected aphids of the same clone in the absence of parasitoids (Oliver *et al.*, 2008). However, the reasons for this decline remained unclear because *H. defensa* had largely positive effects on aphid life-history traits: infected lines had significantly shorter generation times and a slightly higher fecundity (Oliver *et al.*, 2008). Comparisons of naturally infected and uninfected clones of the

black bean aphid, *Aphis fabae*, also indicated additional benefits rather than costs of possessing *H. defensa*. In a sample of 24 different clones collected in Switzerland, nine were found to harbour *H. defensa*, and they exhibited a higher daily fecundity on average than the 15 clones without *H. defensa* (Vorburger *et al.*, 2009). Similarly, a comparison of life-history traits including a somewhat reduced set of 21 clones of *A. fabae* (seven harbouring *H. defensa*) revealed that adult size and offspring production were higher on average in the *H. defensa*-infected clones (Castañeda *et al.*, 2010). However, these studies only focused on young adults and the correlative evidence from natural infections does not prove a causal link between *H. defensa* infection and aphid fecundity. Thus, identifying the elusive costs of harbouring this defensive symbionts will require the experimental separation of symbiont-conferred effects from genetic variation of the hosts, and a comprehensive assessment of fitness-relevant traits at all life stages. Both is readily possible in aphids. Their clonal mode of reproduction and the possibility to experimentally infect clones with facultative symbionts by microinjection permits the production of sublines with and without symbionts in the same genetic background. We used this approach to introduce six different isolates of *H. defensa* into each of two naturally uninfected clones of *A. fabae*. A life-table experiment using these lines revealed that infection with *H. defensa* strongly decreased aphid lifespan, resulting in lower lifetime reproduction, and that the magnitude of this longevity cost was determined by genotype × genotype interactions between host and symbiont.

Material and methods

Aphid lines

Aphis fabae is an important pest aphid that is widely distributed in temperate regions of the northern hemisphere. It reproduces by cyclical parthenogenesis, with one sexual, oviparous

generation over winter followed by many asexual, viviparous generations between spring and autumn. The two clones used in this study, A06-405 and A06-407, were collected during the asexual phase in summer 2006 from the same site in Switzerland. These clones possess different multilocus genotypes based on eight microsatellite loci (Coeur d'Acier *et al.*, 2004), and they were diagnosed as uninfected with facultative endosymbionts by diagnostic PCR (Sandström *et al.*, 2001; Russell *et al.*, 2003; Tsuchida *et al.*, 2006; Vorburger *et al.*, 2009; McLean *et al.*, 2011). Since their collection, they were maintained in the laboratory on broad beans (*Vicia faba*) under environmental conditions that ensure continued reproduction by apomictic parthenogenesis (16 h photoperiod at 20 °C). We generated *H. defensa*-infected sublines of these clones using a microinjection protocol as described in Vorburger et al. (2010), transferring symbiont-containing hemolymph from six different clones of *A. fabae* that were naturally infected with *H. defensa*. Collection details and microsatellite genotypes of the six donor clones as well as the two recipient clones are provided in Table 1. All of the donor clones exhibit complete or partial resistance to *Lysiphlebus fabarum*, the most important parasitoid of *A. fabae* (Vorburger *et al.*, 2009; R. Rouchet & C. Vorburger, unpublished data).

Based on a combination of diagnostic PCR and of sequencing the amplicons of PCR reactions using the general bacterial primers 10F and 35R for the 16S ribosomal RNA gene (Sandström *et al.*, 2001; Russell & Moran, 2005), *H. defensa* was the only facultative endosymbiont present in the donor clones. The six *H. defensa* isolates are labelled H 9, H 30; H 76, H 323, H 402 and H Af6, in reference to their clone of origin. Although the different donor clones were collected from as far apart as southern France and Switzerland (Table 1), they should not be regarded as coming from different, isolated populations. Aphids have a high dispersal ability (Llewellyn *et al.*, 2003), and a population genetic survey using microsatellites found very low levels of genetic differentiation in *A. fabae* across Europe (Sandrock *et al.*,

93 submitted). We have no genetic information about the relatedness among the *H. defensa*
94 isolates used here and their relatedness to known defensive isolates in other aphid species (e.g.
95 Oliver *et al.*, 2005), but phylogenetic analyses suggest that horizontal transmission among
96 species occurs at least occasionally (Sandström *et al.*, 2001; Russell *et al.*, 2003).
97 Successful transmission of *H. defensa* by microinjection normally results in stable infections
98 of clonal lines, since vertical transmission under laboratory conditions is virtually perfect. We
99 confirmed the presence of *H. defensa* in the recipient lines by diagnostic PCR for the first
100 three generations after transfection as well as immediately before use in the experiment. For
101 one of the two recipient clones (A06-407) we also verified that protection against parasitoids
102 by *H. defensa* is still expressed in the new genetic background (R. Rouchet & C. Vorburger,
103 unpublished data). The tranfected lines carried their *H. defensa* infections for between 20 and
104 40 generations prior to the experiment described below.
105
106 **Life-table experiment**
107 To estimate potential effects of the infection with *H. defensa* on aphid life-history traits we
108 carried out a life-table experiment similar to the one described in Vorburger (2005). The
109 experiment took place in a climatised room under fluorescent light with a 16 h photoperiod at
110 20°C. All 14 aphid lines (two uninfected and six infected from each clone) were split into
111 eight replicates that were maintained on caged *V. faba* seedlings growing in plastic pots of
112 0.07 l volume. One replicate per line was assigned to a random position in eight different
113 plastic trays (randomised complete blocks). To avoid the potential inflation of among-line
114 variation by maternal or grand-maternal environmental effects carried over from the stock
115 culture, we maintained the replicates for two generations (each generation on a fresh plant)
116 before we assayed the life-history traits in the third generation after the split. The test
117 generation was initiated by placing four adult females from the second generation on a new

seedling, allowing them to reproduce for 4 h, and then removing the adults and all but one newborn nymph from the plant. These individuals represented the experimental cohort, which was checked daily for survival. After six days, we started monitoring the animals every 8 h to determine the time of their final moult (adult ecdysis), from which we calculated development time (duration from birth to adult ecdysis). We weighed all newly moulted adults to the nearest μg on a Mettler MX5 microbalance (Mettler Toledo GmbH, Greifensee, Switzerland) to determine their fresh mass as an estimate of body size and then replaced them on their plants. After that, their offspring were removed and counted daily until they died. To ensure that the aphids developed under favourable conditions and that they remained easy to find every day, we transferred the adults to new seedlings every 5 days. From the number of offspring produced over the first 7 days of reproduction we calculated the daily fecundity (mean number of offspring produced per day) as an estimate of reproductive performance of young adults. This estimate could not be obtained for individuals that survived for less than 7 days after adult ecdysis, which was then treated as missing data. We also determined the lifetime reproductive output (total number of offspring produced from adult ecdysis until death) and the age at death. Finally, to obtain an overall fitness estimate for each individual, we used the complete life-table data to calculate F'_i, following Service & Lenski (1982):

$$F'_i = \sum_{x=0}^{\infty} F_N{}^{-x} S_{xi} B_{xi} , \qquad (1)$$

where S_{xi} is the survival of individual i to age class x (one or zero), B_{xi} is the number of daughters produced by individual i in age class x, and F_N is the finite rate of increase of the entire experimental cohort over the duration of one age class (i.e. 1 day in this experiment). F_N is obtainable from the stable-age equation (Lenski & Service, 1982, equ. 4), which we solved iteratively. We found F_N to be 1.37, which corresponds to the mean of the F'_i (Lenski

& Service, 1982). F'_i is generally interpreted as the lifetime contribution of individual *i* to population growth, which is a useful measure of individual fitness (Lenski & Service, 1982). Two individuals were accidentally killed in a transfer during the experiment and had to be excluded from all analyses.

Statistical analyses

Aphid life-history traits were analysed with general linear models using the open source statistical software R 2.9.2 (R Development Core Team, 2009). We tested for the effects of experimental block, aphid clone, subline and the clone × subline interaction. The variance among sublines and the variance explained by the clone × subline interaction was further partitioned into contributions from the variance between uninfected (H-) and infected (H+) sublines and the variance within H+ sublines (i.e. among different *H. defensa* isolates), using linear orthogonal contrasts. Because the block effect was far from significant in all analyses, we pooled the variance among blocks into the residual term. Survival data were analysed with a Cox proportional hazards regression, testing for the effects of aphid clone, subline and their interaction.

Results

Infection with *H. defensa* had no detectable effect on aphid development time, but development was significantly slower in clone A06-407 than in clone A06-405 (Table 2, Fig. 1a). Aphid body size measured as adult fresh mass did not differ significantly between clones, nor was there a significant difference among sublines, but the contrast between the means of uninfected (H-) and infected (H+) sublines indicated a slight but significant reduction of body size in the presence of *H. defensa* (Table 2, Fig. 1b). The fecundity of young adult aphids was

similar for both clones but exhibited variation among sublines (Table 2, Fig. 1c). The marginally significant contrast between H- and H+ suggests that this was at least partly due to a slight reduction in fecundity of infected aphids. However, this effect differed between the two aphid clones as indicated by the significant aphid clone × subline interaction (Table 2), which largely reflected the inconsistent effects of the different isolates of *H. defensa* on the two clones (Table 2, Fig. 1c).

The only really striking effect of harbouring *H. defensa* we observed was a reduction in longevity. An inspection of the survivorship curves (Fig. 2) shows clearly that in both clones, mortality rates differed among sublines, with uninfected aphids living longer on average than aphids harbouring *H. defensa*. This resulted in a significant subline effect in a Cox proportional hazards regression (LR $\chi^2 = 78.1$, df = 6, $P < 0.001$). Interestingly, the two clones were unequally affected by the presence of *H. defensa* (clone × subline interaction, LR $\chi^2 = 38.3$, df = 6, $P < 0.001$). The reduction in longevity by the different isolates of *H. defensa* was much more severe in clone A06-407 than in A06-405 (Fig. 2). This is also evident when survivorship is analysed as age at death (Fig. 1d). Individuals of both clones died younger when they were infected with *H. defensa*. This seems to be a rather general effect of this symbiont because the contrast analysis showed that the significant subline effect was largely due to the difference between H- and H+ (Table 2). However, clone A06-407, which produced longer-lived individuals when uninfected (44.0 days ± 1.3 SE vs. 32.3 ± 3.9 days in A06-405), suffered a reduction by almost two thirds to 16.5 ± 0.9 days average lifespan, whereas A06-405 only suffered a reduction of about one fourth to 23.6 ± 1.0 days average lifespan, reversing the order of their performance in the presence of *H. defensa* (Fig. 1d). This was reflected in the significant clone × subline interaction on the age at death, to which the contrast between H- and H+ contributed most of the variation. Yet the contrast analysis also

showed that the effects of the different isolates of *H. defensa* on longevity depended on the aphid clone (Table 2).

The marked differences in longevity we observed translated directly into differences in the most inclusive fitness estimates we obtained, namely the lifetime reproductive output and F_i', a life-table based measure of an individual's contribution to population growth, of which the means can be interpreted as an estimate of the finite rate of increase for each subline (Lenski & Service, 1982; Service & Lenski, 1982). Both measures varied significantly among sublines (Table 2, Figs. 1e, f), and the contrast between H- and H+ explained much of this variation (Table 2). In accordance with the stronger reduction of longevity, the negative effect on fitness was more pronounced in clone A06-407. However, different isolates of *H. defensa* contributed unequally to this fitness reduction: clone A06-407 suffered most from the presence of isolates H 30 and H 76, for example. This was supported by a significant clone × subline interaction for both traits, much of which is explained by the interaction of the different isolates within the H+ group and the two aphid clones (Table 2).

Discussion

By demonstrating substantial fitness costs of harbouring *H. defensa*, this experiment supports the notion that infected aphids are competitively inferior in the absence of parasitoids (Oliver *et al.*, 2008), thus preventing the fixation of this facultative symbiont in natural populations. Our study is the first to provide a mechanistic understanding of these costs: infection with *H. defensa* shortens an aphid's life. The shorter lifespan was the main reason for the reduced lifetime reproduction of infected aphids in our experiment, because the observed reductions of fecundity were small. Under the benign conditions of our laboratory experiment, the costs of harbouring *H. defensa* in terms of lifetime reproductive output were

quite substantial (Fig. 1e), yet this result should be interpreted with caution. In the field, aphids are unlikely to live their full potential lifespans due to extrinsic sources of mortality such as predation, and in periods of populations growth (e.g. the exponential growth phase of aphid populations in spring), early reproduction contributes more to fitness than late reproduction (Stearns, 1992). Therefore, the fitness costs of harbournig *H. defensa* may be less pronounced under field conditions.

It is tempting to conclude that the reduction of longevity caused by *H. defensa* is mechanistically linked to the protection it provides against parasitoids. The protection results from the presence of toxin-encoding bacteriophages within *H. defensa*'s genome (Degnan & Moran, 2008a, b; Oliver *et al.*, 2009). These toxins appear to kill the eggs or early larval stages of parasitoids, but they may also have negative effects on the aphids themselves, thus reducing their longevity. This hypothesis is yet to be tested. It will be particularly important to know whether the toxin genes are expressed constitutively or only upon attack by parasitoids.

Our experiment not only identified clear costs of harbouring *H. defensa* resulting from early mortality, it also showed that the magnitude of these costs depends on the host's genetic background. One aphid clone was much more affected than the other. Furthermore, the costs depended on the exact combination of host clone and symbiont isolate, reflecting a genotype × genotype interaction between *A. fabae* and *H. defensa*. This may have important consequences for the frequencies of *H. defensa* in natural aphid populations as well as for the dynamics of host-symbiont coevolution. It suggests that the cost-benefit ratio of possessing *H. defensa* would differ among aphid genotypes. For certain host-symbiont combinations (e.g. aphid clone A06-407 with *H. defensa* isolate H 30 in the present experiment; Figs. 1 & 2), the net effect on aphid fitness resulting from the symbiosis is likely to be negative, despite increased resistance to parasitoids. Such combinations are unlikely to be encountered in the field. In other combinations (e.g. clone A06-405 with H76), the benefits of increased

resistance are likely to exceed the longevity cost. Obviously, the cost-benefit ratio will also be affected by the risk of attack by parasitoids, which will vary in space as well as in time. We can thus expect that the *H. defensa*-infected aphids we observe in the field do not represent random combinations of host and symbiont genotypes, but rather well-matching combinations that were favoured by natural selection because the protective effect of *H. defensa* comes at comparatively low costs. A potential test of this hypothesis would include a similar experiment using lines from which natural infections with *H. defensa* were removed by antibiotic curing (e.g. McLean *et al.*, 2011), the prediction being that the gain in longevity would then be relatively modest. If this was indeed the case, it would help explain why comparisons of naturally infected and uninfected clones of *A. fabae* did not reveal any evidence for costs (Vorburger *et al.*, 2009; Castañeda *et al.*, 2010). Another explanation could be that just like other vertically transmitted symbionts, *H. defensa* relies on host reproduction for its own transmission. That is why its ability to protect aphids against parasitoids evolved in the first place, but the same would apply to the symbiont's own effects on host survival. Upon successful infection of a host lineage, *H. defensa* should evolve to be less 'virulent' in that it shows reduced effects on host survival. However, evolution of reduced virulence towards the host could be constrained if it entailed reduced protection against parasitoids. Whether such a trade-off exists remains to be investigated.

To conclude, we show that in the black bean aphid, *A. fabae*, the defensive symbiont *H. defensa* is only helpful when required, i.e. when aphids are under strong selection by parasitoids. In the absence of parasitoids, harbouring *H. defensa* is associated with costs which are mostly due to a reduction of host longevity. The magnitude of the negative effect on host survival is to a large extent determined by genotype × genotype interactions between hosts and symbionts, which has important consequences for their coevolution.

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357 **Figure captions**

358

359 **Fig. 1** Life-history traits of infected and uninfected sublines of two clones of *Aphis fabae*:
360 development time from birth to adult ecdysis (a), adult mass measured as fresh weight of
361 newly ecdysed adults (b), daily fecundity averaged over the first 7 days of reproduction (c),
362 age at death (d), lifetime number of offspring (e), and overall fitness measured as F_i', an
363 estimate of individual contribution to population growth (f). The bars left of the gap contrast
364 the means (+ 1 SE) of the uninfected and all infected sublines, the bars right of the gap depict
365 the means of infected sublines separately for each isolate of *Hamiltonella defensa*.

366

367 **Fig. 2** Survivorship curves of sublines of *Aphis fabae* clone A06-405 (a) and A06-407 (b),
368 that are either uninfected (black lines) or experimentally infected with one of six different
369 isolates of the defensive endosymbiont *Hamiltonella defensa* (coloured lines).

370

Table 1 Collection information and genotypes at eight microsatellite loci (Coeur d'Acier *et al.*, 2004) for the eight clones of *Aphis fabae* used in this study. The two recipient clones used in the life-table experiment were experimentally infected with *Hamiltonella defensa* by microinjection of hemolymph from each of the six donor clones.

					Microsatellite locus							
Sample ID	Collection site	Collection date	Host plant	Facultative symbiont	AF-48	AF-50	AF-82	AF-85	AF-86	AF-181	AF-beta	AF-F
Recipient clones												
A06-405	St. Margrethen, Switzerland	01.07.2006	*Chenopodium album*	-	315 317	257 257	167 177	220 220	217 219	311 311	280 282	127 127
A06-407	St. Margrethen, Switzerland	01.07.2006	*Chenopodium album*	-	315 315	272 272	177 177	218 220	215 215	309 309	280 282	127 127
Donor clones												
A06-9	La Spezia, Italy	08.05.2006	*Vicia faba*	*Hamiltonella defensa*	315 321	257 272	171 177	220 222	219 219	309 309	280 282	127 127
A06-30	Sarzana, Italy	08.05.2006	*Vicia faba*	*Hamiltonella defensa*	315 315	257 257	177 177	220 220	219 219	311 311	266 282	132 136
A06-76	La Grande Motte, France	17.05.2006	*Chenopodium album*	*Hamiltonella defensa*	315 315	257 272	192 204	220 222	217 219	311 313	280 280	127 127
A06-323	Aesch, Switzerland	27.06.2006	*Vicia faba*	*Hamiltonella defensa*	315 315	257 272	177 177	220 222	215 215	309 309	280 280	134 136
A06-402	St. Margrethen, Switzerland	01.07.2006	*Chenopodium album*	*Hamiltonella defensa*	315 315	257 257	177 177	220 220	219 219	309 313	280 280	127 127
Af6	Zürich, Switzerland	25.05.2004	*Euonymus europaeus*	*Hamiltonella defensa*	315 315	257 257	177 177	218 222	219 219	311 317	280 282	127 134

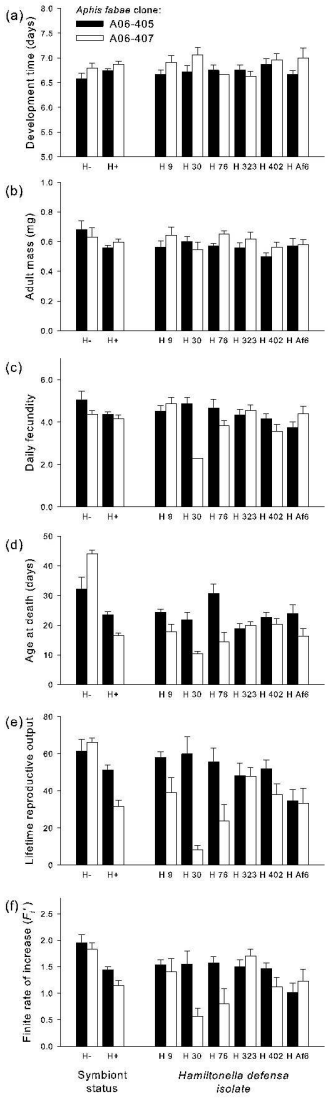
Table 2 General linear model results for the six life-history traits measured.

Source of variation	df	MS	*F*	*P*
Development time				
Aphid clone	1	0.509	4.796	0.031
Subline	6	0.126	1.190	0.319
between H- and H+	1	0.188	1.777	0.186
among H+	5	0.114	1.076	0.379
Aphid clone × subline	6	1.272	1.200	0.314
between H- and H+	1	0.022	0.204	0.653
among H+	5	0.148	1.395	0.234
Residual	89	0.106		
Adult mass				
Aphid clone	1	0.017	1.194	0.278
Subline	6	0.022	1.561	0.168
between H- and H+	1	0.080	5.639	0.020
among H+	5	0.011	0.763	0.579
Aphid clone × subline	6	0.013	0.903	0.496
between H- and H+	1	0.028	1.977	0.163
among H+	5	0.010	0.668	0.649
Residual	89	0.014		
Daily fecundity				
Aphid clone	1	1.441	2.409	0.125
Subline	6	1.378	2.304	0.043
between H- and H+	1	2.425	4.055	0.048
among H+	5	1.185	1.982	0.092
Aphid clone × subline	6	1.772	2.964	0.012
between H- and H+	1	0.608	1.017	0.317
among H+	5	1.988	3.324	0.009
Residual	71	0.598		

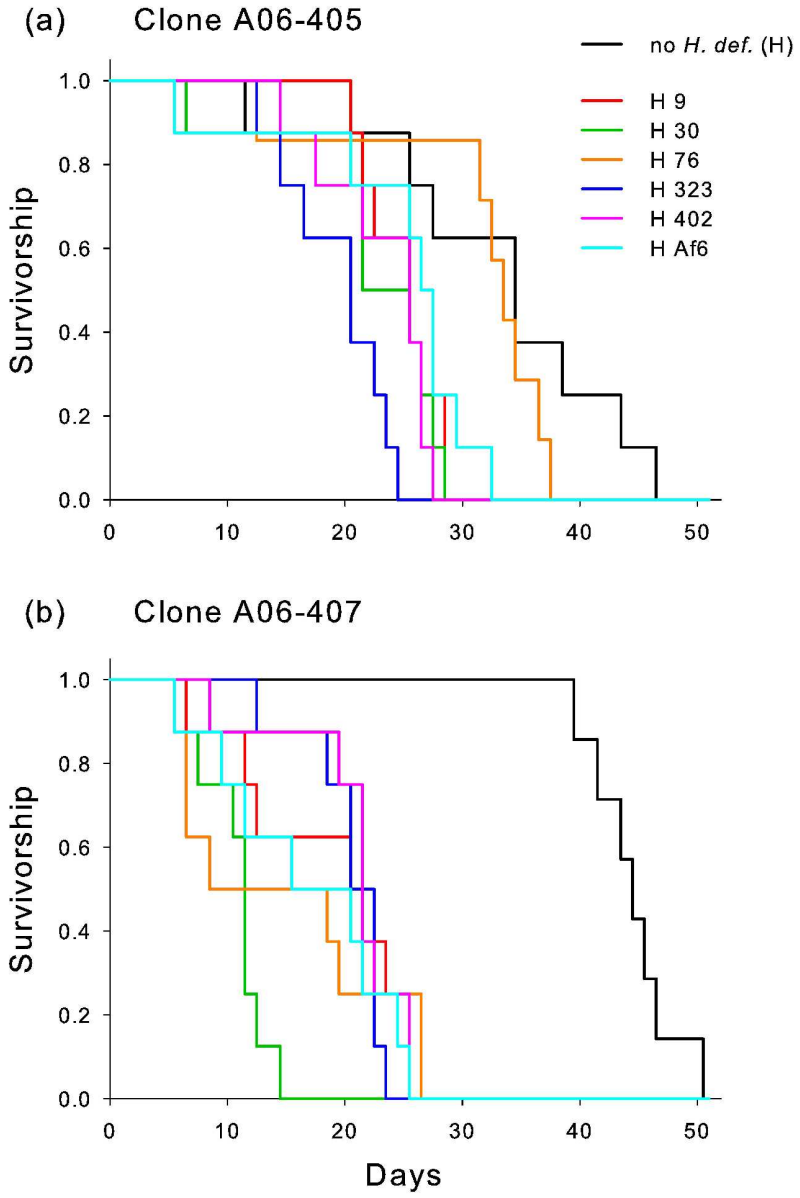
Table 1 continues on next page

Table 1 continued

Age at death				
Aphid clone	1	643.2	15.050	< 0.001
Subline	6	725.9	16.984	< 0.001
between H- and H+	1	3981.7	93.248	< 0.001
among H+	5	75.86	1.777	0.125
Aphid clone × subline	6	315.2	7.375	< 0.001
between H- and H+	1	1143.3	26.775	< 0.001
among H+	5	148.49	3.478	0.006
Residual	96	42.7		
Lifetime reproduction				
Aphid clone	1	7677.8	23.663	< 0.001
Subline	6	1578.5	4.865	< 0.001
between H- and H+	1	6004.0	18.502	< 0.001
among H+	5	692.1	2.133	0.068
Aphid clone × subline	6	1591.4	4.905	< 0.001
between H- and H+	1	1895.0	5.840	0.018
among H+	5	1532.2	4.722	<0.001
Residual	96	324.5		
F_i' *(finite rate of increase)*				
Aphid clone	1	2.220	8.594	0.004
Subline	6	1.373	5.314	< 0.001
between H- and H+	1	4.597	17.790	< 0.001
among H+	5	0.728	2.816	0.020
Aphid clone × subline	6	0.825	3.194	0.007
between H- and H+	1	0.095	0.368	0.546
among H+	5	0.972	3.761	0.004
Residual	96	0.258		



168x567mm (600 x 600 DPI)



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