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2 **Do facultative symbionts affect fitness of pea aphids in the sexual**
3 **generation?**

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14 **Running title:** *Facultative symbionts in sexual aphids*

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16 *Key words:* *Acyrtosiphon pisum*, male-killing, sexual selection, symbiosis, Aphididae,
17 Hemiptera, *Regiella insecticola*, *Hamiltonella defensa*

18 **Accepted: 23 October 2017**
19

Abstract

Many aphids carry one or more facultative symbiotic bacteria which can provide a variety of fitness benefits for their hosts. They have chiefly been investigated in asexually reproducing aphids, with studies of the sexual generation limited to investigation of transmission rates and the potential for sex ratio manipulation. The effects of two facultative symbionts on the mating success of pea aphids, *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae, Macrosiphini), were investigated in a no-choice experiment. We compared the fitness of aphids with natural infections of either *Regiella insecticola* Moran et al. or *Hamiltonella defensa* Moran et al. with that of aphids from genetically-identical cured lines. Female fecundity was unaffected by the presence of facultative symbionts. However, females mated to males cured of *H. defensa* laid fewer eggs on average than females mated to males carrying *H. defensa*; in one case the percentage of melanized eggs (eggs that were either not fertilized or in which early death occurred) was also smaller with cured compared to naturally infected males. In addition, males with *H. defensa* suffered higher mortality during the experiment than cured males. Four of the aphid lines used also hosted *Spiroplasma* infections, a symbiont previously reported to cause male-killing in pea aphids. Despite this, three of the *Spiroplasma*-infected lines produced males, two at high numbers. We conclude that removing a natural symbiont infection may have a negative fitness effect on male aphids in some aphid clones, whereas sexual females from the same clones are largely unaffected.

Abbreviated abstract

We investigated effects of facultative symbionts on the mating success of pea aphids, *Acyrtosiphon pisum*. Females mated to males cured of symbiont *Hamiltonella defensa* laid fewer eggs on average than females mated to males carrying *H. defensa*, whereas females were largely unaffected by their facultative symbiont status.

1 Introduction

2 Many insects harbour one or more species of symbiotic bacteria. For example, many phloem-
3 feeding insects, including aphids, engage in mutualistic and obligate relationships with
4 bacteria (primary symbionts) in order to supplement their otherwise nutritionally inadequate
5 diets (Moran, 2001). Alternatively or in addition to these primary symbionts, many insects
6 also host one or more species of facultative symbiont, which are not required for successful
7 development or reproduction. Several widespread insect symbionts act as reproductive
8 parasites, causing male-killing or cytoplasmic incompatibility in a phylogenetically broad
9 range of hosts (Duron et al., 2008). Males cannot usually transmit bacteria to their offspring,
10 and male-killing is presumed to favour their carrier sisters. However, other facultative
11 symbionts can provide various benefits including defence against natural enemies (Brownlie
12 & Johnson, 2009).

13 Insect-bacterial symbioses have been particularly well-studied in aphids. In these
14 insects, the primary symbiont, *Buchnera aphidicola* Munson et al., provides essential
15 nutrients absent from the phloem diet and is inherited maternally with complete fidelity
16 (Moran, 2001). Alongside their obligate primary symbiont, many aphids also possess one or
17 more facultative (secondary) bacterial species (Oliver et al., 2010), whose roles are not
18 primarily nutritional (Douglas et al., 2006). A variety of benefits to their hosts has been
19 described, including resistance to natural enemies (Oliver et al., 2003, 2005; Ferrari et al.,
20 2004; Scarborough et al., 2005; Łukasik et al., 2013; Heyworth & Ferrari, 2015) and to heat
21 shock (Chen et al., 2000; Montllor et al., 2002; Russell & Moran, 2006; Heyworth & Ferrari,
22 2015). In pea aphids, *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae), a male-killing
23 phenotype has been described for one symbiont species, *Spiroplasma* (Simon et al., 2011),
24 although this has not been found in any other aphid facultative symbionts (Moran & Dunbar,
25 2006; Simon et al., 2007).

26 Many aphids in northerly latitudes reproduce by cyclical parthenogenesis: there are
27 numerous asexual generations in the spring and summer months, during which female aphids
28 reproduce by apomictic parthenogenesis, but the onset of autumnal conditions (specifically
29 long nights) leads aphids to produce offspring (sexuparae) capable of giving birth to sexual
30 morphs (reviewed by Le Trionnaire et al., 2008). Following mating, the sexual females
31 (oviparae) lay eggs which overwinter and hatch the following spring to found the next year's
32 asexual lines. Aphid facultative symbionts can be transmitted with high fidelity both in the
33 viviparous asexual phase and from a sexual female to her eggs (Moran & Dunbar, 2006;
34 Koga et al., 2012). Unlike the majority of intracellular bacteria, certain facultative symbionts

(the gamma-proteobacteria *Regiella insecticola* Moran et al., *Hamiltonella defensa* Moran et al., and probably also *Serratia symbiotica* Moran et al.) may be paternally as well as maternally transmitted (Moran & Dunbar, 2006), although probably not at high rates under most circumstances (Peccoud et al., 2014).

The vast majority of experimental work on aphid symbiont function has been carried out in asexual aphids, but the presence of facultative symbionts has been shown in some instances to alter the number of sexual offspring produced and to affect the critical dark period at which the production of sexual forms is induced (Leonardo & Mondor, 2006). Simon et al. (2011) also found that sexuparae with symbionts had lower fecundity as well as a reduced reproductive lifespan. However, little else is known about the function of bacterial symbionts in the aphid sexual generation. Symbionts in aphids spend the majority of their time in individuals that do not reproduce sexually, meaning that this group provides an interesting contrast to many of the other insects in which the effects of symbionts have been studied. For example, the importance of reproductive manipulation may be diminished relative to the importance of providing benefits to the host. In this study, we explored whether facultative symbionts affect pre-copulatory mate choice in pea aphids, and whether reproductive fitness was impacted by the facultative symbiont infection status.

Understanding the effects of symbionts in the pea aphid sexual generation is complicated by the fact that the pea aphid taxon consists of a number of genetically distinct host-associated populations or ‘biotypes’ (Ferrari et al., 2008; Peccoud et al., 2009). At least seven facultative symbiont species have been identified from pea aphids (Chen et al., 1996; Fukatsu et al., 2001; Moran et al., 2005; Guay et al., 2009) and usually one or sometimes two facultative symbiont species are common in a particular biotype, with other bacterial species occurring more rarely (Frantz et al., 2009; Ferrari et al., 2012; Peccoud et al., 2015). The reasons for this symbiont-plant association remain unclear (Ferrari et al., 2004; Leonardo, 2004; Tsuchida et al., 2004; McLean et al., 2011) but the correlation implies that some advantage may accrue to possessing particular symbiont species or strains on particular plants (Henry et al., 2013), and that there is therefore a potential selection pressure for aphids to select mates on the basis of their facultative symbiont complement. Some aphid species appear to exhibit mate choice, both pre-copulation (Raymond et al., 2001) and post-copulation (Raymond et al., 2001; Hales, 2005), and female pea aphids display a degree of inbreeding avoidance by reducing sperm transfer from closely related males (Huang & Caillaud, 2012), so such discrimination seems feasible.

We used five clonal lines of pea aphids from a single biotype (adapted to alfalfa,

Medicago sativa L., Fabaceae), infected with one or both of the two facultative symbionts most commonly associated with this biotype, *H. defensa* or *R. insecticola*. Of pea aphids sampled from *M. sativa* in southern England (UK), 68% carry *H. defensa* and 13% *R. insecticola* (Ferrari et al., 2012). Sexual morphs were induced and these were combined in no-choice crosses with individuals from other clones. Pre-mating behavioural parameters were recorded in timed observation sessions, whereas post-mating reproductive fitness and compatibility was assessed by counting both the total numbers of eggs laid and the numbers of eggs which were melanized, an indication of fertilization and early development of the embryo. In this way, we were able to show, first, whether artificial elimination of facultative symbiont infections affected pre-copulatory mate choice and, second, whether removing facultative symbionts affected the reproductive fitness of either sexual female or male aphids.

Materials and methods

Study organisms

In summer (long daylight period) conditions, pea aphids reproduce by apomictic parthenogenesis, allowing lines of genetically identical aphids ('clones') to be maintained. All pea aphid clones used in our experiments were derived from single individuals originally collected from *M. sativa* plants in Berkshire and Oxfordshire (southern England) during June and July 2003 and July and August 2008. It was confirmed that these aphid clones were specialized on alfalfa through performance tests and microsatellite typing was used to confirm that the aphid clones were genetically distinct from one another (Ferrari et al., 2008). Prior to the experiments, the aphid clones were maintained on leaves of *Vicia faba* L. (Fabaceae), a plant on which almost all pea aphids perform well (Sandström & Pettersson, 1994; Ferrari et al., 2006, 2008), with a L16:D8 photoperiod, at 20°C and >60% r.h.

The facultative symbiont complement of the different aphid clones was assessed using diagnostic PCR using primers specific to the 16S ribosomal RNA genes of known pea aphid facultative symbionts [see McLean et al. (2011) for details of primers used]. Of the five aphid clones used in this study, three were infected with *H. defensa* (clones 161, 328, 330), one only with *R. insecticola* (333), and one originally with both *H. defensa* and *R. insecticola* but cured of *H. defensa* prior to this study (222). Clones 161, 222, 330, and 333 also harboured *Spiroplasma* spec. infections.

Curing facultative symbiont infections

1 Natural facultative symbiont infections were eliminated from lines of the study aphid clones
2 using oral administration of antibiotics following McLean et al. (2011); aphid lines were
3 deemed cured if there was a complete lack of amplification of the specific 16S primer of their
4 original symbiont infection for at least six generations following the antibiotic treatment. The
5 symbiont infection statuses of the experimental clones are shown in Table 1. Briefly, four
6 clones (161, 328, 330, and 333) had a natural facultative symbiont infection (*Regiella* or
7 *Hamiltonella*) removed. Aphid clonal lines from which these facultative symbionts had been
8 removed are hereafter referred to as ‘cured’ clones, designated by a prefix ‘C’. In all cases, at
9 least eight generations elapsed between antibiotic curing and initiating sexual generation
10 induction (see below). The presence of *Spiroplasma* was not affected by the antibiotics we
11 administered, so ‘cured’ clones were not free from all facultative symbiont species. A fifth
12 clone (222) had previously been cured of *Hamiltonella* but retained a co-infection of *Regiella*
13 and *Spiroplasma*.

15 **Induction of sexual aphids**

16 Pea aphids produce sexual offspring if they and their mothers experience a long dark period
17 (Via, 1992). To produce males, adult females were placed on leaves of *V. faba* for 24 h and
18 their offspring then put in an incubator at 17°C, with an initial L14:D10 photocycle. One
19 week later (when the aphids were third instar) the day length was reduced to a light period of
20 13 h and 10 min. This was gradually reduced over the next 5 days to a light period of 12 h
21 and 50 min. The aphids by then had started to reproduce and the offspring, the sexuparae,
22 were kept until they became adults. The later offspring of the sexuparae (produced >5 days
23 after first reproduction) were examined when fourth instar to identify males.

24 The procedure for producing females was similar and carried out using a separate
25 incubator. Five days after the male lines had been initiated, adult asexual females were
26 allowed to reproduce for 24 h and their offspring placed in an incubator. One week later, the
27 critical light photoperiod of 12 h and 30 min was applied, reduced over the next 5 days to 12
28 h and 10 min. At this point, the females had begun to reproduce and the resultant sexuparae
29 were retained in the incubator. Their earliest offspring (produced in the first 5 days of
30 reproduction) were the sexual females. The offspring were examined while fourth instars to
31 check that no males (produced later in the reproductive life of the same females) were
32 included.

33 Sexual aphids were produced in two separate temporal blocks; in the second block,
34 the time between initiation of male and female lines was lengthened to 10 days to ensure that

1 maximum numbers of both were available simultaneously as receptive adults. One clone
2 (161) failed to produce any males and one clone (222) produced only very few males and
3 hence no data are available for male mating performance in these two clones.

4 Wing polymorphism is genetically controlled in pea aphid males (Caillaud et al.,
5 2002); all males produced by our experimental clones were wingless. Males were used in the
6 experiment when they had been adult for 2-7 days whereas females had been adult for 3-6
7 days, the time at which they are thought to be most sexually receptive (M Caillaud, pers.
8 comm.). During experiments, aphids were not matched with mates from the same clone to
9 prevent any inbreeding effects complicating interpretation of results.

11 **Effects of facultative symbionts on sexual selection: pre-mating**

12 Effects of facultative symbionts on pre-mating behaviour were assessed using timed
13 observations of aphids in no-choice situations and were carried out for males from
14 *Hamiltonella*-infected and *Hamiltonella*-free lines of clones 328 and 330. Males of each
15 clone were paired with female aphids of four different clones (avoiding intra-clone pairings);
16 all females carried natural symbiont infections (their symbionts had not been removed using
17 antibiotics) (Table 1). Two female aphids were placed in a Petri dish containing a layer of
18 agar gel and a leaf of *V. faba*, placed on the gel before it set, so that it was impossible for the
19 aphids to move underneath the leaf. One male aphid was then added to the dish and the
20 aphids observed for a period of 2 h, beginning at the 4th hour of the light period, the time at
21 which activity is likely to be greatest (M Caillaud, pers. comm.). Three elements of behaviour
22 were observed: the number of contacts made between the male aphid and either female before
23 copulation occurred, the time that elapsed before copulation, and the duration of copulation
24 when it took place. Observations were carried out on eight separate days and five different
25 observers participated in recording the results, each of whom watched no more than nine
26 dishes at any one time. At least four trials were carried out for each male/female and
27 cured/infected combination, with an average number of seven replicates. However, mating
28 only occurred during the 2-h observation period in 45 of the 149 instances, resulting in 1-4
29 replicates for each of the male lines used (clone 328 infected and cured males, clone 330
30 infected and cured males) with each infected female clone. The experimental aphids were
31 then retained for the post-mating element of the assay.

33 **Effects of facultative symbionts on sexual selection: post-mating**

34 Post-mating effects of facultative symbionts were observed by placing aphids in no-choice

combinations and counting the eggs laid. The clones used for these experiments are shown in Table 1. To study the effects of male symbiont status on egg laying and fertilization rates we considered only matings between naturally-infected or cured males and symbiont-infected females, whereas to study the effects of female symbiont status we considered only matings between naturally-infected or cured females and symbiont-infected males. Where relevant, aphids in the first block of the post-mating experiment were maintained in the triplets used for the pre-mating observations; in the second block, all triplets had not been observed beforehand. In both blocks, triplets were kept on Petri dishes containing a cut leaf of *V. faba* with the petiole only inserted into agar gel. The dishes were kept in an incubator maintained at 14 °C with a 12 h and 10 min light period. The number of eggs and surviving aphids in each dish was recorded after the first 7 days (when the aphids were transferred to a fresh Petri dish) and then after an additional 7 days. Viable eggs change from their initial pale colour to completely dark within a few days; unfertilized eggs, or fertilized eggs which die at an early stage, fail to melanize. One week after the aphids were removed from a dish, the egg colours were recorded so that the total number and proportion of melanized eggs for different aphid crosses could be analysed.

Statistical analysis

Statistical analysis was carried out in R v.3.0.2 (R Development Core Team, 2013). For the behaviour data, the effects were analysed of both male clone (i.e., clones 328 or 330) and male symbiont status (i.e., *Hamiltonella* present or absent) on three behavioural responses ('time before first contact', 'time before copulation', and 'copulation duration'). For all analyses of pre-mating observations, data from different female clones were pooled to ensure sufficient replication. The data on time to first contact and time to mating is right censored, because not all pairs contacted or copulated during the 2-h observation period, and were analysed using Cox's proportional hazards models. Mating duration was assessed using generalised linear modelling (GLM) assuming a quasipoisson error distribution (to account for overdispersion).

For the post-mating data, survival of males and females 1 week after introduction was compared for all symbiont-infected and all cured lines with a two-sample binomial test for equality of proportions (excluding data for clone 222, which had no cured counterpart). The post-mating data on number of eggs laid and proportion of eggs melanized were analysed using GLM with quasipoisson and quasibinomial error distributions, respectively. All crosses where no eggs were laid were included in the analysis of the total number of eggs but

removed from the dataset before analysis of fertilization rates. We carried out separate analyses to examine the effects of symbionts on males and effects on females. For each, we examined the effects of aphid clone and symbiont status, and the interaction between the two, after incorporating survival data (female survival in week 1, female survival in week 2, and male survival in week 1) in the models (because survival could affect these measures of egg number independently of any symbiont-related effects). Male survival in week 2 was extremely low and was omitted from the analysis. Symbiont identity was not included in the main analyses because replication was insufficient to distinguish between symbiont and clone effects; instead, a separate analysis was carried out using only data from infected aphids to determine whether egg number or melanization rates were influenced by the parental clones harbouring the same symbiont species rather than different symbionts.

Results

Production of males by aphids infected with *Spiroplasma* spp.

Five aphid clones were used in our experiments, four of which were naturally infected with the facultative symbiont *Spiroplasma*, an infection which remained in the lines subject to antibiotic treatment. This study was not intended to quantify male-killing, and so precise numbers of males produced were not recorded. However, we observed that of the four clones infected with *Spiroplasma*, one produced no adult male individuals at all (clone 161), one produced a low number of males relative to sexual females (222), and two (330 and 333) produced a similar number of males to the *Spiroplasma*-free clone 328.

Effects of facultative symbionts on sexual selection: pre-mating

Mating was observed to occur in approximately one third of the trials observed (45 out of 149 observations). There was no evidence that presence of symbionts in male aphids affected the time before first contact ($\chi^2 = 0.842$, d.f. = 1, $P = 0.36$), time before first copulation ($\chi^2 = 0.15$, d.f. = 1, $P = 0.69$), or duration of copulation when it occurred ($F_{1,42} = 2.638$, $P = 0.011$). Likewise, although males of clone 328 were quicker to contact and mate than those of clone 330 (first contact: $\chi^2 = 3.955$, d.f. = 1, $P = 0.05$; first copulation: $\chi^2 = 5.43$, d.f. = 1, $P = 0.02$), the clones did not differ in copulation duration ($F_{1,43} = 1.636$, $P = 0.21$), nor were there any interactions between male clone and male symbiont status (first contact: $\chi^2 = 0.0002$, d.f. = 1, $P = 0.99$; first copulation: $\chi^2 = 0.196$, d.f. = 1, $P = 0.66$; copulation duration: $F_{1,41} = 0.001$, $P = 0.97$).

Effects of facultative symbionts on sexual selection: post-mating

Survival

There were no effects of facultative symbiont presence on female survival in the 1st week after they were introduced to males (proportion test: $\chi^2 = 0.327$, d.f. = 1, $P = 0.57$; Figure 1A). However, symbiont presence did impact male survival in the 1st week: survival was lower for males infected with *H. defensa* or *R. insecticola* than for cured males (proportion test: $\chi^2 = 9.808$, d.f. = 1, $P = 0.002$; Figure 1B).

Effects of symbiont infection status on males

Male survival did not affect the number of eggs laid by their mates ($F_{1,209} = 2.708$, $P = 0.10$), nor the proportion melanized ($F_{1,170} = 0.192$, $P = 0.66$). Females paired with cured males laid fewer eggs than females paired with symbiont-infected males ($F_{1,206} = 5.323$, $P = 0.022$; Figure 2A). There was no effect of interaction between male clone and male symbiont status on the number of eggs laid ($F_{1,204} = 0.9121$, $P = 0.40$). We found that male clones varied in the effect of symbiont presence on the proportions of eggs melanized (clone effect: $F_{2,168} = 8.146$, $P < 0.001$; symbiont status effect: $F_{1,167} = 7.077$, $P = 0.009$; clone*symbiont status: $F_{2,165} = 5.207$, $P = 0.006$). Females mated to males from clone 330 had a lower proportion of melanized eggs in the absence of their symbiont, whereas fertilization rates for the other two clones were largely unaffected (Figure 2B).

Effects of symbiont infection status on sexual females

There was no evidence of differences in the number of eggs produced by aphids from infected and cured lines of the same aphid clone (symbiont status effect: $F_{1,115} = 0.717$, $P = 0.40$; clone*symbiont status: $F_{3,112} = 0.858$, $P = 0.47$; Figure 3A), nor of clonal differences in the total number of eggs laid by individual aphids ($F_{3,116} = 0.278$, $P = 0.84$). There were no differences between aphid clones in the proportions of eggs fertilized ($F_{3,93} = 2.356$, $P = 0.077$) and symbiont infection status did not affect egg fertilization rates in any clone (symbiont status effect: $F_{1,92} = 0.042$, $P = 0.83$; clone*symbiont status: $F_{3,89} = 0.742$, $P = 0.53$; Figure 3B). As expected, female survival had a strong positive effect on the total number of eggs laid, although this was only significant for survival in the 1st week (survival week 1: $F_{1,121} = 24.726$, $P < 0.001$; survival week 2: $F_{1,120} = 1.461$, $P = 0.23$); the pattern is the same in the 2nd week, but there were very few aphids surviving, and the analysis therefore has low power to detect an effect.

Interactions between symbiont species

We had limited statistical power to detect differences between crosses involving different symbiont species. Nevertheless, we examined the data to see whether crosses between aphids which both harboured *H. defensa* differed from those where one aphid hosted *H. defensa* and the other *R. insecticola*. There was no indication that *H. defensa* × *H. defensa* crosses differed from *H. defensa* × *R. insecticola* crosses in the total numbers of eggs laid (male symbiont*female symbiont: $F_{1,206} = 0.805$, $P = 0.37$), nor in the proportions of eggs that were melanized (81% in both crosses).

Discussion

We found that facultative symbionts can have a significant effect on the reproductive fitness of male aphids: a female aphid mated to a male with a natural facultative symbiont infection tends to lay more eggs than a female aphid mated to a male from a cured line. In addition, for males of one clone (330), a greater proportion of eggs was melanized when the father was infected with *H. defensa* than when the father was cured. Sexual female aphids did not show any effect of symbiont infection: female survival was not consistently affected by symbiont infection status, and there is no evidence that female reproductive performance was affected by symbiont status once differences in the probability of survival had been taken into account.

Costs and benefits of facultative symbionts in aphids are not straightforward to measure. For example, *H. defensa* is costly to aphids in competition assays (Oliver et al., 2008) and can reduce longevity (Simon et al., 2011; Vorburger & Gouskov, 2011). However, both observational (Castañeda et al., 2010; Nyabuga et al., 2010) and experimental (McLean et al., 2011) work has suggested that symbionts tend to enhance rather than reduce fecundity. We suggest that male aphids in our experiments experienced a net reduction in fitness when facultative symbionts were absent, as their partners laid fewer eggs and/or a lower proportion of those eggs were melanized. In our experiments, the effects of symbiont species and male clonal line cannot be separated, because clone 333 was the only line with *R. insecticola*. The effects of symbiont status that we observed in males were driven largely by the two clones that naturally carried *H. defensa*, with clone 333 appearing largely unaffected by symbiont removal, except for experiencing higher mortality. We note that previous studies in aphids have established that virgin males live longer than males exposed to sexual females (Dixon &

1 Kundu, 1997). If mating generally tends to reduce male aphid longevity, the lower survival
2 we observed in infected males could reflect their higher frequency of mating rather than a
3 cost of symbiont carriage per se.

4 Why should male aphid fitness be more affected than female fitness by the presence
5 of facultative symbionts in our experimental lines? In pea aphids, the interests of the
6 facultative symbionts and their host are largely aligned because vertical transfer is more
7 important than horizontal transfer (Henry et al., 2013). Transmission through the aphid sexual
8 generation is therefore of vital importance to facultative symbionts, but recent work suggests
9 that rates of paternal transmission are in general very low (Peccoud et al., 2014; Vorburger et
10 al., 2017), even if it can occur at relatively high rates for some aphid clones (Moran &
11 Dunbar, 2006). This reduces the likelihood that there is strong selection for symbionts
12 specifically to benefit males since the infection is unlikely to be inherited paternally. It is
13 possible that facultative symbiont phenotypes that have evolved to benefit aphids in the
14 asexual generations may have the side-effect of providing benefits to males.

15 Given that our experiments involved only a very small number of aphid clonal lines,
16 drawn from a single biotype, we are cautious about drawing general conclusions as to
17 symbiont influence across all pea aphids or aphids more broadly. However, our results
18 demonstrate that facultative symbionts which are not reproductive parasites are relevant in
19 the pea aphid sexual generation. It would be interesting in further work to test experimentally
20 whether facultative symbionts can play a role in creating barriers between pea aphid biotypes,
21 and whether in biotypes that naturally tend to lack facultative symbionts, there is an
22 advantage or cost to their presence in the sexual generation.

23 Of the five aphid clones used in this experiment, only one (clone 328) was free from
24 *Spiroplasma* infection. We therefore cannot rule out the role of an interaction between
25 *Spiroplasma* and *R. insecticola* or *H. defensa* in producing the phenotype of the uncured
26 clones, nor the possibility that in cured clones, the effect of removing one facultative
27 symbiont was to allow the remaining *Spiroplasma* to proliferate to the detriment of the host.
28 The reduced performance of cured males from clone 330 could therefore result from a single
29 infection of *Spiroplasma*, rather than the removal of *H. defensa*. We think that this is unlikely
30 to be the whole explanation for the differences we observed because we saw stronger effects
31 in clone 328 (which lacked *Spiroplasma*). Nevertheless, we cannot exclude the possibility
32 that our results reflect the interaction between symbionts, rather than the straightforward
33 effects of presence or absence of the *H. defensa* or *R. insecticola* symbionts.

34 *Spiroplasma* is known to cause male-killing in aphids (Simon et al., 2011) and a

diverse range of other insects (Hurst et al., 1999; Williamson et al., 1999; Jiggins et al., 2000). Our observations demonstrate that in pea aphids this phenotype is not produced by all symbiotic strains: one of our *Spiroplasma*-infected clones produced no adult male individuals at all in our experiments (clone 161), but clones 330 and 333 produced many males. Clone 222 produced comparatively few males, perhaps a consequence of a ‘partial male-killing’ phenotype. Our use of natural infections means that we cannot distinguish whether variation in *Spiroplasma* effects is due to the symbiont strain (Anbutsu & Fukatsu, 2003), or to the host background (Kageyama et al., 2009). If it is the symbiont that is responsible, it may be that other phenotypic effects of *Spiroplasma*, such as protection against fungal pathogens (Łukasik et al., 2013), are important for maintaining *Spiroplasma* strains which lack male-killing in pea aphid populations. Alternatively or in addition, some aphids may have evolved resistance to the male-killing effects of *Spiroplasma*.

Facultative symbionts are widespread in insects, and many studies have highlighted negative effects on host reproduction, such as male-killing. In this study, we found that presence of the facultative symbionts we investigated (*H. defensa* and *R. insecticola*) appears to be advantageous or neutral in the pea aphid sexual generation. We identified a negative effect of removing natural infections on male reproductive fitness, but no clear effects of removing symbionts on sexual female fitness over and above that determined by the infection status of her mate, or on aphid mating behaviour. In addition, we observed that some aphids carrying *Spiroplasma*, a symbiont previously described as male-killing in pea aphids (Simon et al., 2011), were able to produce males, demonstrating phenotypic variation in this trait among aphids and/or bacterial strains.

Acknowledgements

The authors are grateful to Marina Caillaud for her useful advice and to Margriet van Asch, Lee Henry, Piotr Łukasik, Asya Naish, and Hannah Halford for their assistance with practical work. The manuscript benefited considerably from the thorough and constructive contributions of its reviewers. This work was supported by BBSRC (BB/E010857/1) and a NERC studentship to AHCM.

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Figure captions

Figure 1 Effects of facultative symbionts on mean (\pm SEM) proportion of survival of (A) female and (B) male pea aphids of various clones for the 1st week after pairing. Aphids were

either cured (white bars) or with facultative symbionts (grey bars).

Figure 2 Effects of facultative symbionts on three clones of male pea aphid fitness: mean (\pm SEM) (A) number of eggs laid by two females over 2 weeks after introducing the male, and (B) proportion of eggs laid which were melanized. Aphids were either cured (white bars) or with facultative symbionts (grey bars).

Figure 3 Effects of facultative symbionts on four clones of sexual female pea aphids: mean (\pm SEM) (A) number of eggs laid by two females over 2 weeks after introducing a male, and (B) proportion of eggs laid which were melanized. Aphids were either cured (white bars) or with facultative symbionts (grey bars).

Table 1 Symbiont status of pea aphid clones used in experiments

Aphid clone code ¹	Symbiont status	Males present?	Experiments		
			Behaviour	Female symbiont effects	Male symbiont effects
161	<i>Hamiltonella</i> + <i>Spiroplasma</i>	No	F	F	F
C161	<i>Spiroplasma</i>	No		F	
328	<i>Hamiltonella</i>	Yes	F, M	F, M	F, M
C328	No symbiont	Yes	M	F	M
330	<i>Hamiltonella</i> + <i>Spiroplasma</i>	Yes	F, M	F, M	F, M
C330	<i>Spiroplasma</i>	Yes	M	F	M
333	<i>Regiella</i> + <i>Spiroplasma</i>	Yes	F	F, M	F
C333	<i>Spiroplasma</i>	Yes		F	
222	<i>Regiella</i> + <i>Spiroplasma</i>	Few	F		F

M, males from clone used in experiment; F, females used in experiment.

¹The prefix 'C' is used to designate cured clones.

Figure 1

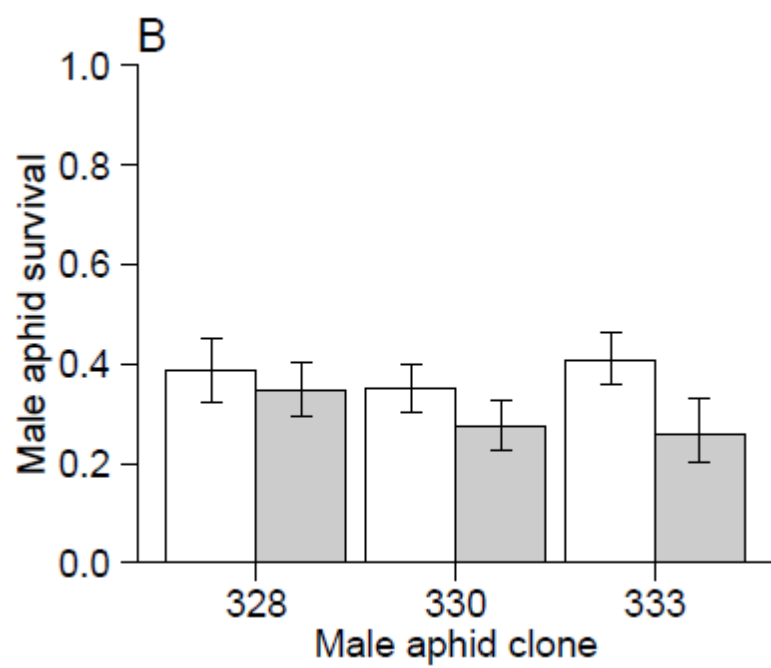
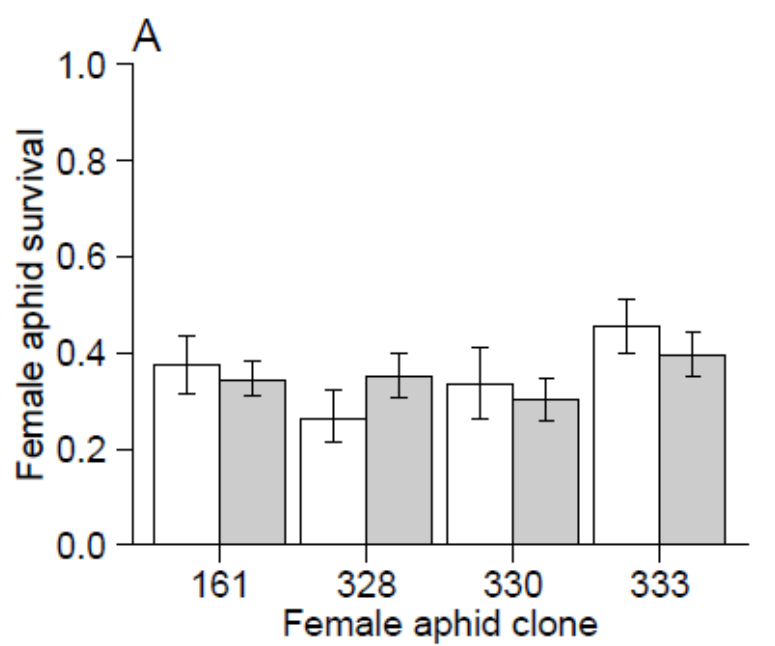
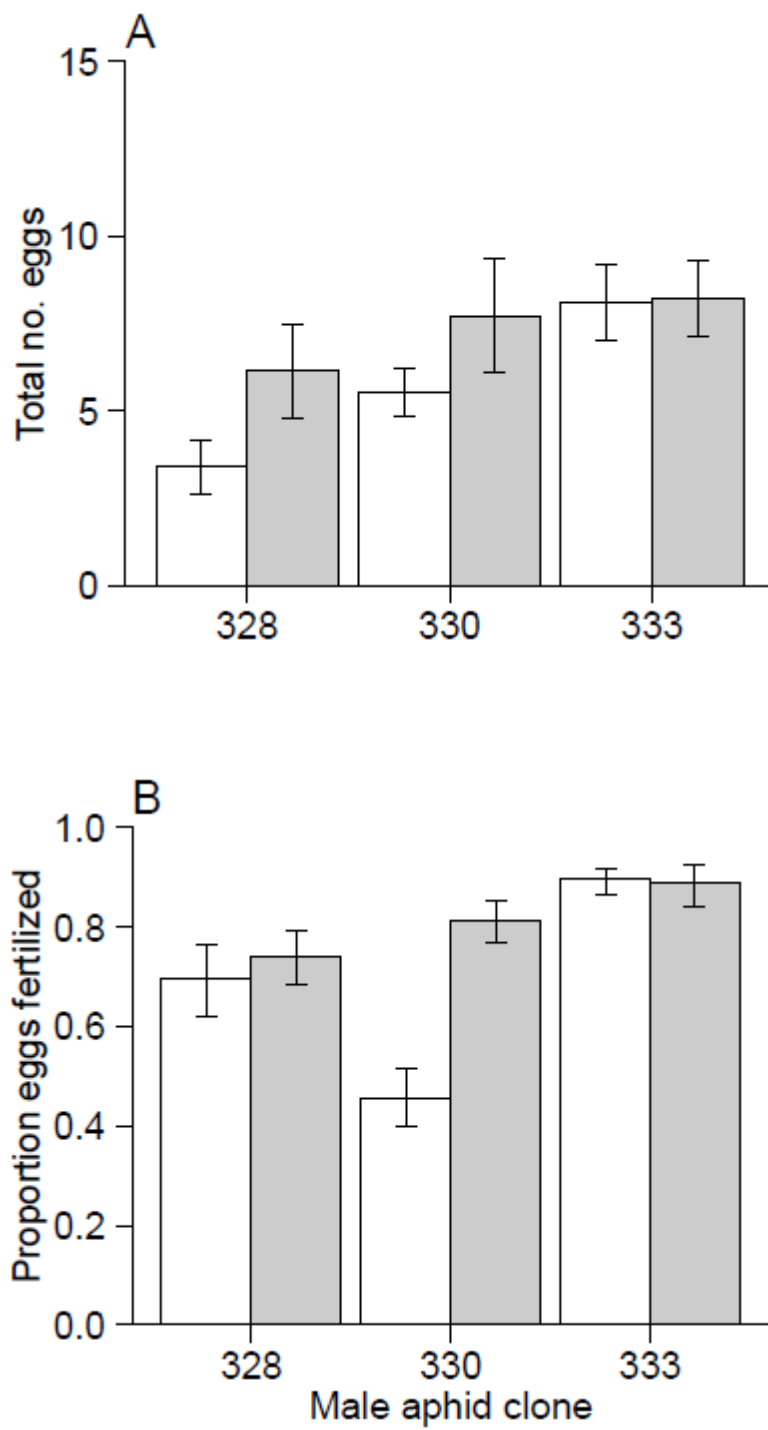


Figure 2



1 Figure 3

