TITLE:

Multiple phenotypes conferred by a single insect symbiont are independent

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ABSTRACT:

Many microbial symbionts have multiple phenotypic consequences for their animal hosts. However, the ways in which different symbiont-mediated phenotypes combine to affect fitness are not well understood. We investigated whether there are correlations between different symbiont-mediated phenotypes. We used the symbiont *Spiroplasma*, a striking example of a bacterial symbiont conferring diverse phenotypes on insect hosts. We took 11 strains of *Spiroplasma* infecting pea aphids (*Acyrthosiphon pisum*) and assessed their ability to provide protection against the fungal pathogen *Pandora neoaphidis* and the parasitoids *Aphidius ervi* and *Praon volucre*. We also assessed effects on male offspring production for five of the *Spiroplasma* strains. All but one of the *Spiroplasma* strains provided very strong protection against the parasitoid *P. volucre*. As previously reported, variable protection against *P. neoaphidis* and *A. ervi* was also present; male-killing was likewise a variable phenotype. We find no evidence of any correlation, positive or negative, between the different phenotypes, nor was there any evidence of an effect of symbiont phylogeny on protective phenotype. We conclude that multiple symbiont-mediated phenotypes can evolve independently from one another without trade-offs between them.

KEYWORDS:

Aphid, Male-killing, Parasitoid, Symbiont-mediated resistance, Symbiosis, *Spiroplasma*

INTRODUCTION

Many animals form close and persistent interactions with symbiotic microorganisms. These relationships are particularly common in insects, which harbour a variety of heritable symbionts that are predominantly vertically transmitted between generations at high frequency. There are two chief classes of mechanisms that allow these maternally-inherited symbionts to persist in host lineages. First, symbionts can manipulate reproduction so that females carrying maternally-transmitted symbionts are overrepresented in subsequent generations. Second, the symbiont can provide a service to the insect that increases host fitness. In insects which feed on nutrient-poor resources, microbial symbionts can provide vital nutrients such as amino acids or vitamins, and the symbionts are often essential (obligate) for host growth and reproduction [1]. Another potential benefit symbionts can provide is to increase their hosts' ability to ward off natural enemies (pathogens, parasitoids, predators) [2]. The risk of natural enemy attack often varies in time and space [3], there may be costs of carrying symbionts in the absence of attack [4], and there is strong specificity in enemy–symbiont interactions that means no single strain can provide universal protection [5]; together these may explain why defensive symbiotic associations tend not to become obligate (i.e. are facultative). Different symbiont persistence mechanisms thus have different ecological and evolutionary consequences for both partners in the symbiosis.

There is evidence that some symbionts switch from one type of persistence mechanism to another or display multiple phenotypes simultaneously. The bacterial symbiont *Wolbachia*, which is extremely widespread throughout insects and some other arthropods, is well-known as a parasite that manipulates the reproduction of its host through a variety of different mechanisms [6]. However, in bedbugs (Cimicidae), *Wolbachia* instead acts as a nutritional mutualist, producing B-vitamins for its blood-feeding host [7]. *Wolbachia* can also induce multiple phenotypes in a single host: in *Drosophila*, a *Wolbachia* that spreads through cytoplasmic incompatibility (that is, matings between symbiont-infected males and symbiont-free females are infertile) simultaneously reduces

its host's susceptibility to viral disease [8, 9]. In addition, single symbiont species or strains can confer multiple beneficial phenotypic effects on the same insect. For example, the bacterial symbiont *Serratia symbiotica* in aphids (Aphididae) can protect against parasitoids [10] and also mitigate the effects of heat shock [11]. Mechanisms of symbiont persistence are therefore not necessarily fixed nor mutually exclusive [12].

In spite of the wide array of symbiont-mediated effects that have now been described in insects, a question remains as to why single symbiont 'species' induce multiple host phenotypes. For example, different phenotypes could represent alternative strategies for persistence by different strains of the same symbiont species. Alternatively, beneficial symbiont-mediated traits could be compensating for costly parasitic effects, in which case we would expect to see both phenotypes conferred by a single strain and a positive correlation between the traits. Martinez and colleagues [13] showed that antiviral and cytoplasmic incompatibility traits of *Wolbachia* infecting *Drosophila* were uncorrelated across different strains; although the two traits co-occur, the strength of protection is not related to the degree of cytoplasmic incompatibility. The costs of symbiont carriage to the host were found to vary with the strength of antiviral protection only, thought to be because both costs and the extent of protection increase with symbiont titre [13]. However, apart from this example, the relationships between parasitic and mutualistic phenotypes are little known, while patterns of co-occurrence of beneficial phenotypes have not to our knowledge been investigated.

The symbiont *Spiroplasma* has been linked with a variety of different host phenotypes, and thus provides an opportunity to investigate how multiple symbiont-mediated phenotypes interact. Members of the genus *Spiroplasma* are widespread invertebrate endosymbionts [14]. Several different *Spiroplasma* clades, infecting diverse insect orders, are known to cause "male-killing", in which the male but not the female progeny of an infected mother are destroyed. It is generally assumed that male-killing benefits the maternally-transmitted symbiont by reallocating resources from sons to daughters [15]. In addition, a number of different defensive phenotypes have been

described, including resistance to hymenopteran parasitoids in several *Drosophila* species [16-18] and resistance to a sterilising nematode in *Drosophila neotestacea* [19-21].

The relationships between the different phenotypic effects of *Spiroplasma* remain unclear. Co-occurrence of male-killing and parasitoid protection in the *Drosophila* symbiont *Spiroplasma poulsonii* (also known as the male sex ratio organism, MSRO) shows that beneficial and reproductive manipulation phenotypes can coexist [18]. However, protective *Spiroplasma* in *D. neotestacea* and *D. hydei* do not induce male-killing [17, 22], suggesting that protection can provide an alternative persistence strategy for *Spiroplasma*. In pea aphids (*Acyrthosiphon pisum*), *Spiroplasma* is known to both cause male-killing [23] and protect against a common fungal pathogen [24]. It is also costly to its host aphid, reducing life-time reproduction [25]. However, neither of these phenotypes is found in all aphid *Spiroplasma* strains [24, 26] and we do not know whether the different phenotypes are induced by different symbiont strains, or if single strains can confer multiple phenotypes.

We studied the relationships between multiple symbiont-mediated phenotypes in pea aphids (*Acyrthosiphon pisum*) carrying different strains of *Spiroplasma*. We took 11 strains of *Spiroplasma* and assessed their protective phenotypes against (i) the fungus *Pandora neoaphidis*, (ii) the parasitoid *Aphidius ervi*, and (iii) the parasitoid *Praon volucre*. For a subset of five strains, we also assessed male-killing capability. We then looked for positive or negative correlations between the different phenotypes. Finally, we looked for an association between phenotypes and symbiont phylogeny to see whether particular symbiont lineages have consistent effects on their hosts. We were thus able to distinguish between three alternative hypotheses for the relationships between multiple symbiont-mediated phenotypes: whether (a) there are positive correlations between different phenotypes, (b) there are negative correlations between different phenotypes, or (c) symbiont-mediated phenotypes are evolving independently of one another.

METHODS

Experimental organisms and symbiont manipulations

Pea aphids were collected in southern England and in France (see Table 1 for details of collection host plants). Each experimental line derives from a single parthenogenetic female. Prior to experiments, aphids were maintained as asexually reproducing lines in the laboratory at 14 °C and 70 ± 10% relative humidity with a light: dark period of 16 : 8 hours. Aphids were kept in 9 cm Petri dishes containing a single leaf of *Vicia faba* inserted into 2% agar gel and transferred to fresh dishes every 7–10 days. Aphid lines were screened for all previously-reported species of facultative endosymbionts infecting pea aphids using diagnostic PCR [27, 28]. All collected aphids harboured a co-infection between *Spiroplasma* and one other facultative symbiont (see Table 1 for details); co-infecting symbionts were removed using oral administration of specific antibiotics (cefotaxime, gentamicin and ampicillin) via the food plant, as described previously [29]. This antibiotic combination does not impact either the aphid primary symbiont, *Buchnera aphidicola*, or *Spiroplasma*. A period of 6–8 generations with no detectable presence of non-*Spiroplasma* facultative symbionts using diagnostic PCR was allowed to elapse before the aphids were considered free of the target symbionts.

In order to compare the effects of different *Spiroplasma* strains in a common host genotype background, we introduced artificial infections of *Spiroplasma* to a symbiont-free pea aphid line ('Clone 145'). A small quantity of haemolymph (*c*. 0.25 µl) was removed from a naturally-infected adult aphid ('donor') using a microcapillary needle and injected into an uninfected first-instar aphid ('recipient'). Recipient aphids were kept until adulthood and their later offspring (>10 in birth order) were retained and tested for *Spiroplasma* once they themselves had begun to reproduce. Aphid lines testing positive for *Spiroplasma* using diagnostic PCR were maintained for a minimum of seven generations before they were used in experiments to allow the infection to stabilise. Immediately prior to each experiment, symbiont status was reconfirmed by diagnostic PCR. We found no

instances of subsequent symbiont loss for aphids which had tested positive for *Spiroplasma* at the second generation after injection. We used an alternative recipient aphid line for the male-killing assays ('Clone 200') to that used for the resistance assays, after preliminary experiments indicated that Clone 145 does not readily produce sexual aphids under laboratory conditions, but the infection procedure was otherwise identical.

Fungal pathogen resistance assay

Resistance to fungal pathogens was assessed by exposing aphids to a single strain of the specific aphid pathogen *Pandora neoaphidis*. The fungal strain (ARSEF 2588) was obtained from the USDA Agriculture Research Service collection of entomopathogenic fungi (ARSEF); this strain is the same as that used for previous studies [30-32] and details of isolate preparation to produce infectious stock aphid cadavers can be found in McLean *et al.* [30]. We compared resistance of symbiont-free aphids to that of aphids carrying 11 different *Spiroplasma* strains. Each treatment consisted of 44 eleven-day-old aphids with the same *Spiroplasma* strain, all of which had moulted to the final, adult, instar. The aphids were placed together in the base of a 4 cm diameter Perspex infection chamber with four sporulating stock cadavers above. Infectious cadavers were rotated between the different experimental chambers at regular intervals, so that each set of cadavers covered each chamber for an equal proportion of the entire experimental exposure period (2 hours). Since cadavers may vary slightly in their spore production, the rotation ensures that each aphid receives as equal a spore dose as possible. Control aphids (44 per *Spiroplasma* strain) were placed in chambers for an equivalent period, but without exposure to fungus.

Following fungal exposure, aphids were transferred to Petri dishes containing a single leaf of *Vicia faba* inserted into 2% agar gel and the dishes sealed using parafilm to elevate humidity to >95% which maximises fungal infection. Aphids were placed on dishes in groups of four; this means that for each aphid line there were at least 10 different dishes for each treatment for the post-exposure period. Dishes were kept at 20 °C. Aphids were transferred to fresh dishes, without parafilm, on days

3 and 6 after infection. Aphid survival and evidence of sporulation were scored every 24 hours for 8 days after infection. Observers were unaware of the treatment status of all dishes throughout the infection scoring process.

In addition to the experiment described above, a separate experiment with an identical protocol was carried out on a separate occasion using a subset of the *Spiroplasma* strains. This additional experiment allows us to determine whether our results were influenced by factors that might vary over time such as pathogen virulence.

Parasitoid resistance assays

We assessed aphid resistance to two species of braconid hymenopteran parasitoids, *Aphidius ervi* and *Praon volucre*. The experimental procedures were essentially identical for the two wasp species. We compared resistance of symbiont-free aphids to that of genetically-identical aphid lines carrying 11 different *Spiroplasma* strains. Experimental parasitoid stocks were obtained from Fargro (Arundel, UK) and reared in the laboratory on a highly susceptible pea aphid clone ('C256'), supplemented with a 5:1 water:honey solution, at 20 °C with a 16 h:8 h light:dark cycle. Prior to experiments, wasps of both sexes were kept together in cages (and thus were assumed to be mated), and were given access to aphids for oviposition experience. Female wasps were isolated from aphids and other wasps for between one and two hours before being used in experiments. For both parasitoid species, experiments were carried out in three temporal blocks at 20 °C.

Each replicate consisted of 15 third instar aphids placed on a Petri dish containing a single leaf of *Vicia faba*. A single female parasitoid was introduced to each dish for three hours, a period which previous observations had shown to be sufficient to allow parasitism of the majority of aphids present whilst minimising superparasitism (laying multiple eggs in a single host). Dishes were checked to confirm that all parasitoids were active and attacking aphids (individual oviposition events were not recorded). After parasitism, aphids were transferred to fresh leaves on new Petri dishes, and retained for 11 days, with further transfers to fresh leaves every 3–4 days. On day 11

following parasitism, the number of surviving and parasitized aphids were recorded. Both parasitoid species form distinctive 'mummies' on successful pupation: for *A. ervi*, aphids become swollen and golden, while for *P. volucre* the parasitoid larvae emerges beneath the swollen aphid and spins a cocoon. Parasitism was defined as the proportion of aphids that produced mummies (excluding any aphids which had died in the course of the experiment for unknown reasons). For each parasitoid, there were between five and 10 replicates per aphid line (mean = 6.8 for *A. ervi*; 7.6 for *P. volucre*).

In addition to the experiments using *Spiroplasma*, we investigated whether the aphid symbiont *Hamiltonella defensa* could protect against *Praon volucre*. The symbiont *H. defensa* is known to provide strong protection against diverse aphid parasitoids including *A. ervi* [33], but does not appear to protect against *Praon pequodorum* [34]. *Hamiltonella defensa* has not yet been tested against *P. volucre*, and we therefore conducted these experiments to provide a comparison with the results for *Spiroplasma*. The methodology was similar to that employed for *Spiroplasma*, with the exception that comparisons were conducted between naturally-infected aphids and their antibiotic-cured counterparts. Details of the methods can be found in the online supplementary material.

Male-killing assay

In order to assess the male-killing activity of *Spiroplasma*, we used a subset of five aphid lines infected with different *Spiroplasma* strains, as well as an uninfected control line. These aphid lines were from a different genotype (200) to that used for the resistance assays (145), because the latter genotype does not produce sexual females or males under any experimental conditions we investigated. Time and resource constraints prevented us from testing reproductive manipulation across the entire panel of strains. We exposed the aphids to conditions that mimic autumn, and thus trigger the production of a sexual generation. To do this, we followed a protocol based on the second experiment described in Simon *et al.* [23]. Third instar aphids were placed on Petri dishes containing *Vicia faba*, in controlled temperature cabinets at 18 °C with a short light period (12 hours light) for seven days. The offspring of these initial aphids were the sexuparae (that is, individuals

capable of producing sexually-reproducing females, and males). The sexuparae were then transferred to an environment at 20 °C with a 16 hour light period. We used 12 sexuparae for each aphid line except Sp709 (for which n=6) and all offspring of the sexuparae were sampled. On becoming adult, the offspring were scored as being either asexual females, sexual females, or males (under this protocol, sexual females were not expected to be produced). Aphids which died before adulthood were placed in 100% ethanol and subsequently tested using microsatellite sequencing to determine whether they were male or female. In aphids, males have a single X chromosome (XO) and females two (XX). We could therefore use a sex-linked microsatellite locus AIA09M [35] on the X chromosome, for which the mother is heterozygous in the recipient aphid genotype, to detect whether offspring were male or female. In Clone 200, used in the experiment, females carry two alleles (four base pairs difference) while males carry only one.

Statistical analysis of phenotype data

Statistical analysis was carried out in R version 3.4.3 and 3.5.0 [36]. For fungal pathogen resistance, we analysed the proportion of aphids that produced a sporulating cadaver in the eight days following infection using generalized linear models with a quasibinomial error structure. Multiple comparisons were conducted with Dunnett contrasts against symbiont-free aphids using the MULTCOMP package [37]. The effect of symbionts on survival in the absence of fungal pathogens (the controls) was analysed using a GLM on survival at the end of the experiment (Day 8) as above. For parasitoid resistance, we analysed the proportion of aphids forming a parasitoid mummy, using GLM with a quasibinomial error structure. Multiple comparisons were carried out using Dunnett contrasts against the uninfected aphid line, implemented using MULTCOMP. We analysed the effect of *Spiroplasma* on male-killing by comparing the proportion of male offspring produced by the different aphid lines, again using generalized linear models with a quasibinomial error structure and Dunnett contrasts.

Phylogenetic analysis

All *Spiroplasma* strains occurring in pea aphids appear to belong to a single clade referred to as *Spiroplasma ixodetis*, a basal divergence within *Spiroplasma* [38]. We used an existing phylogeny of *Spiroplasma* strains [39] to look for an association between symbiont phylogeny and observed fungal and parasitoid resistance phenotypes; the male-killing phenotype was excluded from this analysis because of the low number of strains investigated. The phylogeny [39] was constructed with maximum likelihood techniques, using concatenated rpoB and dnaA sequences (available in GenBank, accession numbers MG288511–MG288588). For our analysis, we excluded from the original phylogeny all strains not used in our phenotype experiments. We tested for phylogenetic inertia by assessing whether *Spiroplasma* strains that are close in the phylogeny have similar effects on their host using Abouheif's C_{mean} index and Pagel's λ [40]. A power analysis showed that for our data, Abouheif's C_{mean} is the more powerful measure where phylogenetic inertia is weak while Pagel's λ is more powerful where phylogenetic inertia is strong. The analysis was performed in R using the 'Phylosignal' package version 1.2 [41].

RESULTS

Fungal pathogen resistance assay

The degree of protection provided by *Spiroplasma* against the fungal pathogen *Pandora neoaphidis* was highly variable (Figure 1A). Three strains had no significant impact on sporulation rates (Dunnett contrast with symbiont-free aphids; Sp237: $z = -2.255$, $p = 0.189$; Sp324: $z = 2.431$, $p = 0.125$; Sp710: $z = -0.927$, $p = 0.977$). Two strains reduced sporulation to below 10% (Sp161: $z = -6.180$, $p < 0.001$; Sp322: $z = -6.254$, $p < 0.001$) and the remaining strains all provided significant protection of varying strengths (Figure 1A). The additional experiment using a sub-set of *Spiroplasma* strains confirmed that the protective phenotypes observed are consistent over time (Figure S2).

The survival of control aphids (those not exposed to the fungus) was not significantly altered by the presence of *Spiroplasma* strains, with one exception, Sp161, that reduced survival of aphids relative

to symbiont-free individuals (z = -3.029, p = 0.025). *Spiroplasma* is thus generally not costly to aphid survival under the experimental conditions of brief starvation and humidity stress.

Parasitoid resistance assays

On average, 75% of symbiont-free aphids were successfully parasitized by *P. volucre* under our experimental conditions. We found that 10 out of 11 *Spiroplasma* strains investigated provided protection against the parasitoid *Praon volucre* (Figure 1B). For three symbiont strains, we did not observe any parasitoid mummies (Sp708, Sp709, Sp710) and the protection is therefore extremely effective under our experimental conditions. A single *Spiroplasma* strain (Sp700) made no difference to parasitism rates (Dunnett contrast with symbiont-free aphids; z= 1.018, p = 0.976; 79% mummies) (Figure 1B).

One strain of *Spiroplasma* (Sp146) provided significant protection against *Aphidius ervi* (Dunnett contrast with symbiont-free aphids; z = -3.407, p = 0.006), with the proportion of mummies falling from 86% in uninfected aphids to 15% in aphids with the symbiont. Another strain (Sp237) showed a weak trend towards protection (z = -2.517, p = 0.080; 28% mummies). No other strains (9/11) made a statistically significant difference to aphid resistance (Figure 1C).

We found no evidence that any of the five genetically-diverse strains of *H. defensa* we investigated provides protection against the parasitoid *P. volucre*. Details of the results of this experiment (Figure S1) can be found in the online supplementary material.

Male-killing assay

We recorded the number and sex of offspring produced by symbiont-free sexuparae, and by sexuparae of the same genotype carrying five different strains of *Spiroplasma*. Offspring of sexuparae can include parthenogenetic females, sexually-reproducing females, and males. As expected for our protocol, only parthenogenetic females and males were recorded in our treatments

(i.e. no sexually-reproducing females). Sexuparae of the experimental aphid line produced 44% male offspring when symbiont-free.

Two strains of *Spiroplasma* caused complete male-killing (Sp161, Sp700) (Figure 2). One *Spiroplasma* strain significantly reduced the proportion of males, but males still occurred (Dunnett contrast with symbiont-free aphids; Sp237: 19% males, $z = 3.029$, $p = 0.012$). The two further strains had no significant effect on the proportion of males produced, although in both cases fewer males were produced (Dunnett contrast with symbiont-free aphids; Sp709: 21% males, $z = 1.619$, $p = 0.417$; Sp710: 33% males, $z = 1.266$, $p = 0.672$).

We used microsatellites to attempt to identify whether aphids that died before becoming adults were female or male; results were obtained for 84% of dead aphids (661 individuals). For symbiont-free aphids, the proportion of dead aphids that were male was 51%, and the number of dead aphids just 57 individuals (~7% of the total offspring). For strains conferring 'complete' male-killing (Sp161 and Sp700) the proportions of dead aphids that were males were 78% and 82%, respectively (dead aphids comprised 20% and 48% of the total offspring). Again, the other *Spiroplasma* strains showed intermediate phenotypes, but there was considerable variation: Sp709 had a much higher proportion of dead offspring that were males (64%) than either of the others (Sp237: 38% of dead individuals male; Sp710: 31%), although the proportion of all offspring which died before adulthood was very similar for all three (10–13%).

Correlation between phenotypes and effect of symbiont phylogeny

We found no evidence that resistance to the fungal pathogen was correlated with resistance to *P. volucre* (Spearman's correlation: $S = 1402.9$, $p = 0.353$, $rho = 0.208$). Although only one *Spiroplasma* strain showed significant protection against *A. ervi*, there was nevertheless variation in resistance to *A. ervi* between aphids infected with different *Spiroplasma* strains; we therefore tested whether resistance to the two different parasitoids was significantly correlated. We found that this was not the case (Spearman's correlation: $S = 1821.6$, $p = 0.900$, $rho = -0.029$).

Male-killing appears to be uncorrelated with fungal pathogen resistance and parasitoid resistance. We are cautious about our conclusions because the number of data points available for the correlation is very small and some values are zero. However, there is no evidence of any association between resistance against *Praon volucre* and strength of male-killing (Spearman's correlation: S = 124.76, p = 0.497, rho = 0.244), or between resistance against the fungal pathogen and male-killing (Spearman's correlation: S = 91.6, p = 0.197, rho = 0.445). A visual inspection of the data (Figure 1, Figure 2) shows that similar male-killing phenotypes are associated with different combinations of protective phenotypes.

We used an existing phylogeny of *Spiroplasma* strains [39] to test the hypothesis that different *Spiroplasma* protective phenotypes are associated with symbiont phylogeny (Figure 1).There was no evidence of phylogenetic effects on any of the phenotypes we tested (Table 2) and all estimates of phylogenetic inertia obtained are low, with values close to zero or even negative, indicating that there is not even a weak trend towards phylogenetic inertia. Although presence of poorly-supported nodes in our phylogenetic tree could theoretically prevent us from detecting real correlations between phylogeny and phenotype, this would require multiple errors that are each unlikely. Complete male-killing was found only in one clade of *Spiroplasma*, although more data would be needed to test whether this phenotype is linked to phylogeny. We were unable to test for evidence of phylogenetic effects on male-killing due to the small sample sizes involved.

DISCUSSION

The facultative endosymbiont *Spiroplasma* can confer multiple phenotypes on its pea aphid hosts. We investigated four potential phenotypes associated with this symbiont: fungal pathogen resistance, resistance against two different parasitoid species, and male-killing. Protection against fungal pathogens was found for eight of the 11 *Spiroplasma* strains (Figure 1A), with considerable variation in efficacy between strains. We also found that *Spiroplasma* can equip aphids with strong protection against the parasitoid *P. volucre*. All but one of the 11 strains investigated provided high

or very high levels of protection against *P. volucre*, compared with only one strain in our study providing significant protection against *A. ervi* (Figure 1B, 1C). Male-killing was absent, partial or complete, depending on the *Spiroplasma* strain (Figure 2). We found no evidence for positive or negative correlations between any of the different phenotypes, or an association between protective phenotypes and symbiont phylogeny. There is thus no evidence that different strains use alternative persistence mechanisms—reproductive manipulation versus fitness benefits, or one fitness benefit versus another—to maintain themselves in the host population. Our assays were conducted under relatively benign conditions, on a single food plant, and with minimal variation in our chosen antagonists; it is possible that under more challenging circumstances, trade-offs might be observed. However, it appears most likely that the different phenotypes are evolving independently.

We identified effective and specific protection by *Spiroplasma* against the aphid parasitoid *P. volucre*. Parasitoids of this genus appear unaffected by the well-known aphid symbiont *H. defensa* ([34] and this study Figure S1), whose protection is known to be highly specific to particular genera, species or even genotypes of parasitoids [5, 42-44]. It has been proposed that *Spiroplasma* provides protection against parasitoids in *Drosophila melanogaster* by competing for lipids , although there is also evidence to support *Spiroplasma* from *D. melanogaster* and *D. neotestacea* producing substances toxic to parasitoids [19]. In this last case, and also in *Spiroplasma*-induced protection against nematodes, ribosome inactivating proteins (RIPs) are believed to be involved. The specificity of action against *P. volucre* suggests that in aphids, production of toxins is a more likely mechanism than indirect resource competition (which would presumably act similarly against all parasitoids), though further work is needed to test this idea.

If protection against parasitoids requires multiple species-specific mechanisms, why do not these all occur in a single symbiont? Such a symbiont would presumably increase its host's fitness, possibly leading to selection on the host to strengthen the symbiosis. One possibility is the mechanisms have evolved independently and cannot transfer between species. Although parasitoid protection by

H. defensa [45] is provided by a toxin-encoding bacteriophage (APSE) [46-48], and these phage also occur in the symbiont *Arsenophonus* [49], APSE have not been identified in other any aphid symbionts. A second possibility is that the different mechanisms interfere with each other or are in some way incompatible if present in the same cell (different parasitoid-specific mechanisms might rely on different alleles of a single gene, for example). Finally, carriage and maintenance of the different mechanisms may entail costs to the symbiont, either in absolute terms (perhaps the risk of failing to be transmitted to aphid progeny) or when competing with other symbionts within the host. A combination of costs and fluctuating fitness benefits (depending on the risk of parasitoid attack) may help explain both why defensive mechanisms are not stacked up in the same individual and why multiple defensive symbionts can coexist in the same host population. Surveys of the frequency of the different *Spiroplasma*-induced phenotypes in natural aphid populations would be helpful in exploring these ideas.

Spiroplasma is a costly symbiont in pea aphids: it shortens lifespan and reduces life-time reproduction [25]. Five of the strains considered in this study were examined for potential costs in a previous study [25]. The symbionts in that study were in a different aphid genetic host background from either of those used here, so we are cautious about comparing the results too closely, but the patterns observed in that study (both Sp227 and Sp161 were found to have a greater reproductive cost and to be associated with higher mean reproductive age than strains Sp322, Sp327 and Sp709) do not imply that previously reported fitness costs correlate (positively or negatively) with either fungal or parasitoid resistance as reported in the current study. In nature, the real costs may be different from those reported in laboratory studies, in part because presence of co-infecting symbionts may modify those costs [25]. Pea aphid *Spiroplasma* in Europe seems to be found predominantly in co-infections with other aphid symbionts (all strains in this study, see also [39]; however this may not be the case for *Spiroplasma* in the US [50]). Understanding the basis for this apparent propensity to form associations with other symbionts might assist in understanding how

aphids may accumulate multiple defences through acquisition of multiple symbionts, and also how symbiont costs are manifested under field conditions.

Given the apparent challenges of providing protection against multiple parasitoids, how and why is *Spiroplasma* capable of protecting against fungal pathogens in addition to protecting against parasitoids? If there is competition between symbionts for hosts, bacteria could be evolving to provide multiple potential benefits to outcompete other symbiont strains or species. Protection against fungal pathogens has arisen in many diverse aphid symbionts [24, 51, 52]. Perhaps it is more straightforward or less costly for bacterial symbionts to acquire the (as yet unknown) means of protecting against fungal pathogens than to evolve protection against a broad range of parasitoids.

Spiroplasma is unusual among aphid symbionts in causing male-killing: no other aphid symbiont has yet been found to bias the sex ratio towards females [23, 53]. Among male-killing bacteria, aphid *Spiroplasma* is also unusual because male death occurs relatively late in development with many male aphids surviving to the third or fourth nymphal instar. The results of our microsatellite sequencing show that in the two *Spiroplasma* strains causing total male-killing, a large proportion of the dead aphids (and therefore total offspring) were males; a similar effect was previously observed by Simon et al. (2011). Late male-killing has been reported for microsporidian parasites in mosquitoes [54, 55] and in a virus attacking a species of moth [56, 57], but we are not aware of other examples from bacterial reproductive manipulators. The delayed male-killing in mosquito microsporidians is suggested to benefit the parasite by increasing the potential for horizontal transmission . Little is known about aphid *Spiroplasma* transmission in nature, but it is possible that horizontal transmission is important. However, it is also possible that male-killing is a relict state for pea aphid *Spiroplasma*, and is no longer advantageous in its new host. We note that in *Drosophila*, male-killing by *Spiroplasma* can be deferred to the pupal stage when the gene responsible (*Sp*AID) has a deletion of the OTU domain [58]. It is therefore possible that both the partial and the late-killing phenotypes might represent degraded forms of an ancestral phenotype.

Spiroplasma strains can persist in aphid populations in spite of their cost to hosts [25], presumably due to the protective and male-killing phenotypes this symbiont can confer. We find that these diverse phenotypes are not correlated, suggesting that they do not represent alternative strategies for success. However, there is considerable variation between *Spiroplasma* strains in their protective capabilities and in the extent of male-killing. The variation could originate from co-evolutionary interactions between symbionts and natural enemies [59], or from variation in interactions with different host genotypes [31, 32]. We conclude that the multiple phenotypes of symbiotic *Spiroplasma* represent independent, flexible and potentially coevolving outcomes of ongoing interactions between host, symbionts, and in some cases natural enemies.

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REFERENCES

[1] Douglas, A.E. 2009 The microbial dimension in insect nutritional ecology. *Functional Ecology* **23**, 38-47. (doi:10.1111/j.1365-2435.2008.01442.x).

[2] Brownlie, J.C. & Johnson, K.N. 2009 Symbiont-mediated protection in insect hosts. *Trends in Microbiology* **17**, 348-354. (doi:10.1016/j.tim.2009.05.005).

[3] Hrček, J., McLean, A.H.C. & Godfray, H.C.J. 2016 Symbionts modify interactions between insects and natural enemies in the field. *J. Anim. Ecol.* **85**, 1605-1612. (doi:10.1111/1365-2656.12586).

[4] Vorburger, C. & Gouskov, A. 2011 Only helpful when required: a longevity cost of harbouring defensive symbionts. *Journal of Evolutionary Biology* **24**, 1611-1617. (doi:10.1111/j.1420-9101.2011.02292.x).

[5] McLean, A.H.C. & Godfray, H.C.J. 2015 Evidence for specificity in symbiont-conferred protection against parasitoids. *Proc. Roy. Soc. Lond. B* **282**, 20150977. (doi:10.1098/rspb.2015.0977).

[6] Werren, J.H., Baldo, L. & Clark, M.E. 2008 *Wolbachia*: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741-751.

[7] Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.Y. & Fukatsu, T. 2010 *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 769-774. (doi:10.1073/pnas.0911476107).

[8] Martinez, J., Longdon, B., Bauer, S., Chan, Y.-S., Miller, W.J., Bourtzis, K., Teixeira, L. & Jiggins, F.M. 2014 Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains. *PLoS Pathogens* **10**, e1004369. (doi:10.1371/journal.ppat.1004369).

[9] Hedges, L.M., Brownlie, J.C., O'Neill, S.L. & Johnson, K.N. 2008 *Wolbachia* and virus protection in insects. *Science* **322**, 702. (doi:10.1126/science.1162418).

[10] Oliver, K.M., Russell, J.A., Moran, N.A. & Hunter, M.S. 2003 Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1803-1807.

[11] Montllor, C.B., Maxmen, A. & Purcell, A.H. 2002 Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. *Ecological Entomology* **27**, 189-195.

[12] Zug, R. & Hammerstein, P. 2015 Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biological Reviews* **90**, 89-111. (doi:10.1111/brv.12098).

[13] Martinez, J., Ok, S., Smith, S., Snoeck, K., Day, J.P. & Jiggins, F.M. 2015 Should symbionts be nice or selfish? Antiviral effects of *Wolbachia* are costly but reproductive parasitism is not. *PLoS Pathogens* **11**, e1005021. (doi:10.1371/journal.ppat.1005021).

[14] Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L.Q., Engelstadter, J. & Hurst, G.D. 2008 The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biol.* **6**, 12. (doi:2710.1186/1741-7007-6-27).

[15] Majerus, M.E.N. & Hurst, G.D.D. 1997 Ladybirds as a model system for the study of male-killing symbionts. *Entomophaga* **42**, 13-20. (doi:10.1007/bf02769875).

[16] Mateos, M., Winter, L., Winter, C., Higareda-Alvear, V.M., Martinez-Romero, E. & Xie, J. 2016 Independent origins of resistance or susceptibility of parasitic wasps to a defensive symbiont. *Ecol. Evol.* **6**, 2679-2687. (doi:10.1002/ece3.2085).

[17] Xie, J., Vilchez, I. & Mateos, M. 2010 *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. *PLoS One* **5**, e12149.

[18] Xie, J., Butler, S., Sanchez, G. & Mateos, M. 2014 Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. *Heredity* **112**, 399-408. (doi:10.1038/hdy.2013.118).

[19] Ballinger, M.J. & Perlman, S.J. 2017 Generality of toxins in defensive symbiosis: Ribosome-inactivating proteins and defense against parasitic wasps in *Drosophila*. *PLoS Pathogens* **13**, e1006431. (doi:10.1371/journal.ppat.1006431).

[20] Jaenike, J., Unckless, R., Cockburn, S.N., Boelio, L.M. & Perlman, S.J. 2010 Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science* **329**, 212-215.

[21] Haselkorn, T.S. & Jaenike, J. 2015 Macroevolutionary persistence of heritable endosymbionts: acquisition, retention and expression of adaptive phenotypes in *Spiroplasma*. *Molecular Ecology* **24**, 3752-3765. (doi:10.1111/mec.13261).
[22] Jaenike, J., Stahlhut, J.K., Boelio, L.M. & Unckless, R.L. 2010 Association between *Wolbachia* and *Spiroplasma* within *Drosophila neotestacea*: an emerging symbiotic mutualism? *Molecular Ecology* **19**, 414-425. (doi:10.1111/j.1365-294X.2009.04448.x).
[23] Simon, J.C., Boutin, S., Tsuchida, T., Koga, R., Le Gallic, J.F., Frantz, A., Outreman, Y. & Fukatsu, T. 2011 Facultative symbiont infections affect aphid reproduction. *PLoS One* **6**, 10. (doi:e2183110.1371/journal.pone.0021831).
[24] Łukasik, P., van Asch, M., Guo, H., Ferrari, J. & Godfray, H.C.J. 2013 Unrelated facultative endosymbionts protect aphids against a fungal pathogen. *Ecology Letters* **16**, 214-218. (doi:10.1111/ele.12031).
[25] Mathé-Hubert, H., Kaech, H., Ganesanandamoorthy, P. & Vorburger, C. 2019 Evolutionary costs and benefits of infection with diverse strains of *Spiroplasma* in pea aphids. *Evolution* **online early**. (doi:10.1111/evo.13740).
[26] McLean, A.H.C., Ferrari, J. & Godfray, H.C.J. 2018 Do facultative symbionts affect fitness of pea aphids in the sexual generation? *Entomologia Experimentalis et Applicata* **166**, 32-40. (doi:10.1111/eea.12641).
[27] Henry, L.M., Maiden, M.C.J., Ferrari, J. & Godfray, H.C.J. 2015 Insect life history and the evolution of bacterial mutualism. *Ecology Letters* **18**, 516-525. (doi:10.1111/ele.12425).
[28] Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T. & Fukatsu, T. 2002 Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrthosiphon pisum*. *Molecular Ecology* **11**, 2123-2135.
[29] McLean, A.H.C., van Asch, M., Ferrari, J. & Godfray, H.C.J. 2011 Effects of bacterial secondary symbionts on host plant use in pea aphids. *Proc. Roy. Soc. Lond. B* **278**, 760-766. (doi:10.1098/rspb.2010.1654).
[30] McLean, A.H.C., Parker, B.J., Hrček, J., Kavanagh, J.C., Wellham, P.A.D. & Godfray, H.C.J. 2018 Consequences of symbiont co-infections for insect host phenotypes. *J. Anim. Ecol.* **87**, 478-488. (doi:10.1111/1365-2656.12705).
[31] Parker, B.J., Hrček, J., McLean, A.H.C. & Godfray, H.C.J. 2017 Genotype specificity among hosts, pathogens, and beneficial microbes influences the strength of symbiont-mediated protection. *Evolution* **71**, 1222-1231. (doi:10.1111/evo.13216).
[32] Parker, B.J., McLean, A.H.C., Hrček, J., Gerardo, N.M. & Godfray, H.C.J. 2017 Establishment and maintenance of aphid endosymbionts after horizontal transfer is dependent on host genotype. *Biology Letters* **13**, 20170016. (doi:10.1098/rsbl.2017.0016).
[33] Oliver, K.M. & Higashi, C.H.V. 2019 Variations on a protective theme: *Hamiltonella defensa* infections in aphids variably impact parasitoid success. *Current Opinion in Insect Science* **32**, 1-7. (doi:10.1016/j.cois.2018.08.009).
[34] Martinez, A.J., Kim, K.L., Harmon, J.P. & Oliver, K.M. 2016 Specificity of multi-modal aphid defenses against two rival parasitoids. *PLoS One* **11**, e0154670. (doi:10.1371/journal.pone.0154670).
[35] Caillaud, M.C., Mondor-Genson, G., Levine-Wilkinson, S., Mieuzet, L., Frantz, A., Simon, J.C. & Coeur d'Acier, A. 2004 Microsatellite DNA markers for the pea aphid *Acyrthosiphon pisum*. *Mol Ecol Notes* **4**, 446-448. (doi:10.1111/j.1471-8286.2004.00676.x).
[36] R Development Core Team. 2013 R: a language and environment for statistical computing. Vienna, Austria.
[37] Hothorn, T., Bretz, F. & Westfall, P. 2008 Simultaneous Inference in General Parametric Models. *Biometrical J.* **50**, 346-363.
[38] Regassa, L.B. & Gasparich, G.E. 2006 Spiroplasmas: evolutionary relationships and biodiversity. *Frontiers in Bioscience* **11**, 2983-3002. (doi:10.2741/2027).

[39] Mathé-Hubert, H., Kaech, H., Hertaeg, C., Jaenike, J. & Vorburger, C. 2019 Nonrandom associations of maternally transmitted symbionts in insects: the roles of drift versus biased cotransmission and selection. *Molecular Ecology* **28**, 5330-5346. (doi:10.1111/mec.15206).
[40] Münkemüller, T., Lavergne, S., Bzeznik, B., Dray, S., Jombart, T., Schiffers, K. & Thuiller, W. 2012 How to measure and test phylogenetic signal. *Methods in Ecology and Evolution* **3**, 743-756. (doi:10.1111/j.2041-210X.2012.00196.x).
[41] Keck, F., Rimet, F., Bouchez, A. & Franc, A. 2016 phylosignal: an R package to measure, test, and explore the phylogenetic signal. *Ecol. Evol.* **6**, 2774-2780. (doi:10.1002/ece3.2051).
[42] Asplen, M.K., Bano, N., Brady, C.M., Desneux, N., Hopper, K.R., Malouines, C., Oliver, K.M., White, J.A. & Heimpel, G.E. 2014 Specialisation of bacterial endosymbionts that protect aphids from parasitoids. *Ecological Entomology* **39**, 736-739. (doi:10.1111/een.12153).
[43] Cayetano, L. & Vorburger, C. 2015 Symbiont-conferred protection against Hymenopteran parasitoids in aphids: how general is it? *Ecological Entomology* **40**, 85-93. (doi:10.1111/een.12161).
[44] Rouchet, R. & Vorburger, C. 2012 Strong specificity in the interaction between parasitoids and symbiont-protected hosts. *Journal of Evolutionary Biology* **25**, 2369-2375. (doi:10.1111/j.1420-9101.2012.02608.x).
[45] Oliver, K.M., Moran, N.A. & Hunter, M.S. 2005 Variation in resistance to parasitism in aphids is due to symbionts not host genotype. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 12795-12800.
[46] Lynn-Bell, N.L., Strand, M.R. & Oliver, K.M. 2019 Bacteriophage acquisition restores protective mutualism. *Microbiology*. (doi:10.1099/mic.0.000816).
[47] Brandt, J.W., Chevignon, G., Oliver, K.M. & Strand, M.R. 2017 Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. *Proceedings of the Royal Society B: Biological Sciences* **284**, 20171925. (doi:10.1098/rspb.2017.1925).
[48] Oliver, K.M., Degnan, P.H., Hunter, M.S. & Moran, N.A. 2009 Bacteriophages encode factors required for protection in a symbiotic mutualism. *Science* **325**, 992-994.
[49] Duron, O. 2014 *Arsenophonus* insect symbionts are commonly infected with APSE, a bacteriophage involved in protective symbiosis. *FEMS Microbiol. Ecol.* **90**, 184-194. (doi:10.1111/1574-6941.12381).
[50] Rock, D.I., Smith, A.H., Joffe, J., Albertus, A., Wong, N., O'Connor, M., Oliver, K.M. & Russell, J.A. 2018 Context-dependent vertical transmission shapes strong endosymbiont community structure in the pea aphid, *Acyrthosiphon pisum*. *Molecular Ecology* **27**, 2039-2056. (doi:10.1111/mec.14449).
[51] Heyworth, E.R. & Ferrari, J. 2015 A facultative endosymbiont in aphids can provide diverse ecological benefits. *Journal of Evolutionary Biology* **28**, 1753-1760. (doi:10.1111/jeb.12705).
[52] Scarborough, C.L., Ferrari, J. & Godfray, H.C.J. 2005 Aphid protected from pathogen by endosymbiont. *Science* **310**, 1781.
[53] Moran, N.A. & Dunbar, H.E. 2006 Sexual acquisition of beneficial symbionts in aphids. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 12803-12806.
[54] Andreadis, T.G. 1985 Life-cycle, epizootiology, and horizontal transmission of *Ambylospora* (Microspora, Ambylosporidae) in a univoltine mosquito, *Aedes stimulans*. *J. Invertebr. Pathol.* **46**, 31-46.
[55] Andreadis, T.G. & Hall, D.W. 1979 Significance of transovarial infections of *Amblyospora* sp (Microspora, Thelohaniidae) in relation to parasite maintenance in the mosquito *Culex salinarius*. *J. Invertebr. Pathol.* **34**, 152-157.
[56] Morimoto, S., Nakai, M., Ono, A. & Kunimi, Y. 2001 Late male-killing phenomenon found in a Japanese population of the oriental tea tortrix, *Homona magnanima* (Lepidoptera: Tortricidae). *Heredity* **87**, 435-440.
[57] Nakanishi, K., Hoshino, M., Nakai, M. & Kunimi, Y. 2008 Novel RNA sequences associated with late male killing in *Homona magnanima*. *Proc. Roy. Soc. Lond. B* **275**, 1249-1254. (doi:10.1098/rspb.2008.0013).

564 [58] Harumoto, T. & Lemaitre, B. 2018 Male-killing toxin in a bacterial symbiont of Drosophila.
565 *Nature* **557**, 252-255. (doi:10.1038/s41586-018-0086-2).
566 [59] Vorburger, C., Sandrock, C., Gouskov, A., Castaneda, L.E. & Ferrari, J. 2009 Genotypic variation
567 and the role of defensive endosymbionts in an all-parthenogenetic host-parasitoid interaction.
568 *Evolution* **63**, 1439-1450. (doi:10.1111/j.1558-5646.2009.00660.x).

569

570 **Table 1**. Details of aphids and symbiont strains used in experiments.

Aphid clone name	Symbiont manipulation role	Original symbiont infection	Strain name	Collection plant	Host biotype*	Collection location/year
146	Donor	*Sp* + *Fukatsuia***	Sp146	*Medicago sativa*	*Trifolium*	UK/2010
161†	Donor	*Sp* + *Hamiltonella*	Sp161	*Medicago sativa*	*Medicago sativa* 2	UK/2003
217	Donor	*Sp* + *Fukatsuia***	Sp217	*Medicago sativa*	*Trifolium/Medicago*	UK/2010
227	Donor	*Sp* + *Regiella*	Sp227	*Medicago sativa*	*Medicago sativa 1*	UK/2012
237	Donor	*Sp* + *Fukatsuia***	Sp237	*Medicago sativa*	*Trifolium*	UK/2010
322	Donor	*Sp* + *Fukatsuia***	Sp322	*Trifolium pratense*	*Trifolium*	UK/2003
324	Donor	*Sp* + *Fukatsuia***	Sp324	*Trifolium pratense*	*Trifolium*	UK/2003
700††	Donor	*Sp* + *Hamiltonella*	Sp700	*Medicago sativa*	*Medicago sativa*	France/1999
708	Donor	*Sp* + *Regiella*	Sp708	*Trifolium pratense*	*Trifolium*	France/2011
709	Donor	*Sp* + *Serratia*	Sp709	*Vicia cracca*	*Vicia cracca*	France/2011
710	Donor	*Sp* + *Serratia*	Sp710	*Vicia cracca*	*Vicia cracca*	France/2011
200	Recipient	*Hamiltonella*		*Medicago sativa*	*Medicago sativa* 2	UK/2012
145	Recipient	none		*Lathyrus pratensis*	Hybrid	UK/2003

571 * Biotypes assigned according to the microsatellite scheme described in Peccoud et al. (2009); note that not all
572 biotypes correspond to the host collection plant
573 ** This symbiont was previously referred to as 'X-type'
574 † Strain used previously to demonstrate fungal pathogen resistance in pea aphid *Spiroplasma* (Lukasik et al.
575 2013)
576 †† Strain used previously to demonstrate male-killing in pea aphid *Spiroplasma* (Simon et al. 2011)
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581 **Table 2.** Results of statistical correlations between different *Spiroplasma* phenotypes and symbiont
582 phylogeny.

Explanatory variable 1	Explanatory variable 2	C_{mean} index	P-value	Pagel's λ	P-value
Phylogenetic relatedness	Resistance to fungal pathogen *Pandora neoaphidis*	-0.337	0.889	$<1.10^{-3}$	1.000
Phylogenetic relatedness	Resistance to parasitoid *Praon volucre*	0.037	0.165	$<1.10^{-3}$	1.000
Phylogenetic relatedness	Resistance to parasitoid *Aphidius ervi*	-0.034	0.327	0.112	0.782

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Figure legends

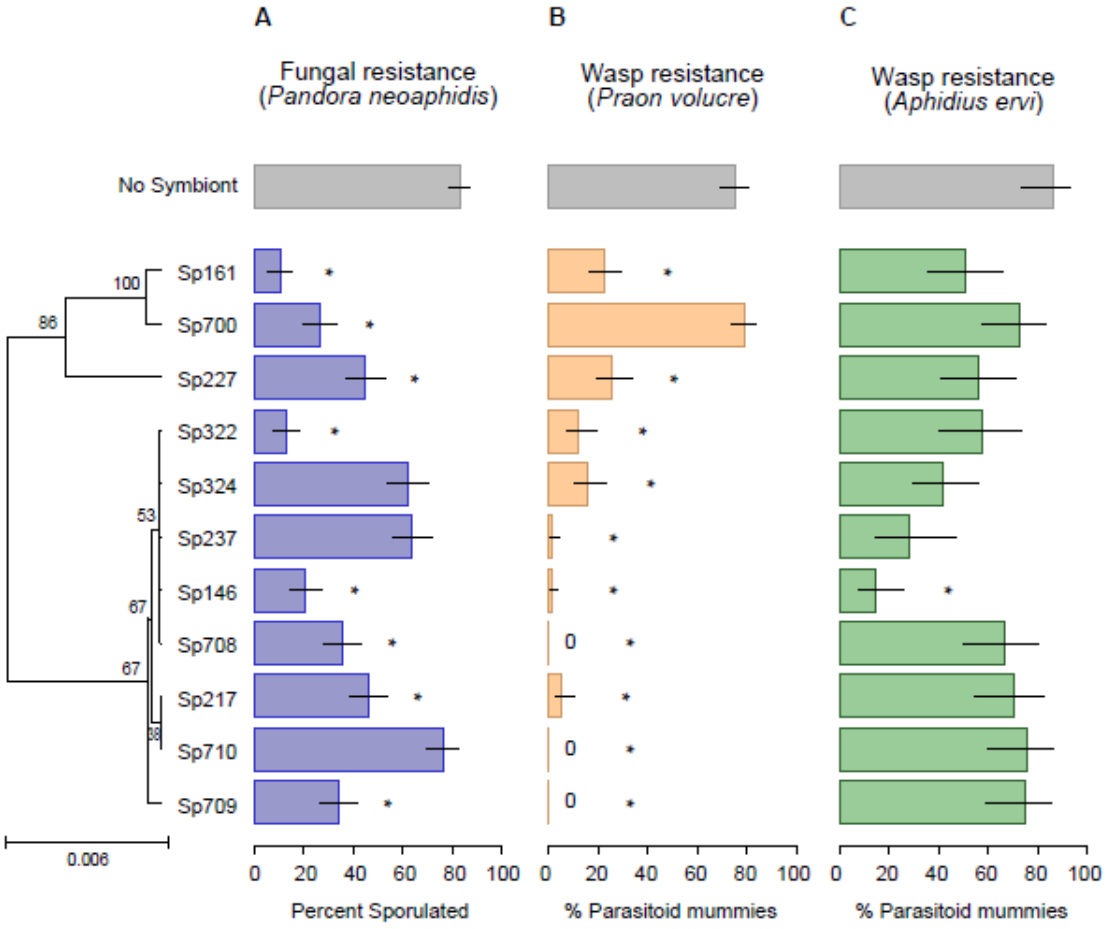
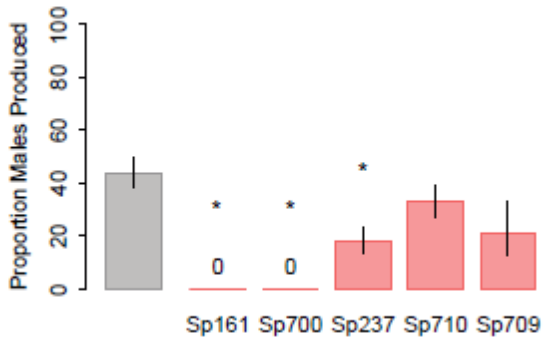


Figure 1. Resistance phenotypes of aphids carrying different 11 strains of *Spiroplasma*. Grey bar indicates control aphids without the symbiont. Phylogenetic relationships of the strains are shown on the left-hand side of the figure; these were obtained from the tree in [39] by dropping all strains that were not included in this study. The tree was inferred by maximum likelihood from the concatenated sequences of the rpoB and dnaA genes; scale bar indicates substitution rate. For strain details, see Table 1. **A** susceptibility to the fungal pathogen *Pandora neoaphidis*, **B** susceptibility to the parasitoid wasp *Praon volucre*, **C** susceptibility to the parasitoid wasp *Aphidius ervi*. Error bars denote standard error of the mean.



597

598 **Figure 2.** Proportion of male offspring produced by aphids without any symbiont (grey bar) and

599 carrying different strains of *Spiroplasma* (red bars). For strain details, see Table 1. Error bars denote

600 standard error of the mean.