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Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont

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Abstract

Many insects harbour facultative endosymbiotic bacteria, often more than one type at a time. These symbionts can have major effects on their hosts' biology, which may be modulated by the presence of other symbiont species and by the host's genetic background. We investigated these effects by transferring two sets of facultative endosymbionts (one *Hamiltonella* and *Rickettsia*, the other *Hamiltonella* and *Spiroplasma*) from naturally double-infected pea aphid hosts into five novel host genotypes of two aphid species. The symbionts were transferred either together or separately. We then measured aphid fecundity and susceptibility to an entomopathogenic fungus. The pathogen-protective phenotype conferred by the symbionts *Rickettsia* and *Spiroplasma* varied among host genotypes, but was not influenced by co-infection with *Hamiltonella*. Fecundity varied across single and double infections and between symbiont types, aphid genotypes and species. Some host genotypes benefit from harbouring more than one symbiont type.

Introduction

Many insects harbour endosymbiotic bacteria within their tissues and cells (Moran *et al.*, 2008), and it has been estimated that approximately 10% of species require nutritional contributions from obligate endosymbionts to survive and reproduce (Baumann, 2005). Facultative, nonessential endosymbionts are even more common (Duron *et al.*, 2008; Hilgenboecker *et al.*, 2008), and many insect species harbour multiple species and strains of these bacteria (e.g. Skaljac *et al.*, 2010; Toju & Fukatsu, 2011). Much of what we know about facultative symbiosis comes from work on the pea aphid, *Acyrthosiphon pisum*, which in addition to its obligate nutritional endosymbiont *Buchnera* (Douglas, 1998) can carry at least seven other species of bacteria. These facultative symbionts, which reside within secondary bacteriocytes and sheath cells, as well as in the haemolymph of aphids (Moran *et al.*, 2005; Sakurai *et al.*, 2005), are present at variable frequencies in different geographic locations and host plant-adapted races (Oliver *et al.*, 2010; Ferrari *et al.*, 2012; Russell *et al.*, 2013). Symbiont infection frequencies in a population are thought to be primarily determined by a balance between selection and vertical transmission failure, although they may also be affected by processes such as acquisition by horizontal transfer, drift and migration (Oliver *et al.*, 2013). Individual aphids may host between zero and four species of facultative endosymbionts (Ferrari *et al.*, 2012; Russell *et al.*, 2013).

Bacterial symbionts can provide major benefits for their hosts (Moran *et al.*, 2008), in particular conferring protection from natural enemies. All seven facultative endosymbionts of the pea aphid have been implicated in increasing their hosts' resistance to parasitoid wasps or pathogenic fungi (Oliver *et al.*, 2003; Scarborough *et al.*, 2005; Guay *et al.*, 2009; Łukasik *et al.*, 2013b). Other beneficial effects of pea aphid facultative endosymbionts

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include resistance to heat shock (Montllor *et al.*, 2002) and improved performance on certain host plants (Tsuchida *et al.*, 2004). However, symbiont carriage can also carry fitness costs through reduced fecundity and longevity (Vorburger & Gouskov, 2011), so that in the absence of natural enemies, symbiont-infected aphid lines can be outcompeted by symbiont-free conspecifics (Oliver *et al.*, 2008).

Surveys of symbiont distributions across pea aphid clones suggest that some species combinations occur more or less often than would be expected by chance (Ferrari *et al.*, 2012; Henry *et al.*, 2013; Russell *et al.*, 2013). A likely explanation for these observations is that different species when they occur in the same aphid interact to enhance or reduce their host's fitness. Different facultative endosymbionts may find themselves within the same hosts as a consequence of paternal transmission during the sexual generation (Moran & Dunbar, 2006), transmission by natural enemies (Jaenike *et al.*, 2007; Gehrer & Vorburger, 2012) or perhaps following oral acquisition from plant tissues or contaminated surfaces (Darby & Douglas, 2003; Oliver *et al.*, 2010). Double infections can also be engineered by microinjection in the laboratory (Oliver *et al.*, 2006). The presence of two symbionts may have positive, negative or no effect on life-history traits relative to single-infected aphids (Chen *et al.*, 2000; Oliver *et al.*, 2006). Benefits of hosting multiple symbiont types at the same time may include improved resistance to natural enemies (Oliver *et al.*, 2006; Guay *et al.*, 2009). However, we still know very little about the effects of multiple infections and how they may be affected by host genetic background.

The ability to manipulate the symbiont complement within clonal lines of aphids by antibiotic curing and microinjections makes them an ideal model system to explore symbiont–symbiont–host interactions. Here we study the interactions between two natural pairs of pea aphid symbionts, *Hamiltonella defensa* and *Spiroplasma* sp., and *Hamiltonella* and *Rickettsia* sp., both commonly found in particular populations of pea aphid adapted to different host plants [18% prevalence of *Hamiltonella–Spiroplasma* in aphids associated with *Medicago* and 41% of *Hamiltonella–Rickettsia* in *Lotus* aphids (Ferrari *et al.*, 2012)]. *Hamiltonella* (Gammaproteobacteria: Enterobacteriales) is a relatively well-studied bacterium occurring in a wide range of aphid species (Sandstrom *et al.*, 2001; Russell *et al.*, 2003). Its prevalence varies between 4 and 82% across pea aphid host plant populations (Ferrari *et al.*, 2012; Russell *et al.*, 2013), and it was detected in 46% of field-collected grain aphids, *Sitobion avenae* (Łukasik *et al.*, 2013a). *Hamiltonella* can increase resistance to parasitoids in pea aphids (Oliver *et al.*, 2003) and black bean aphids (*Aphis fabae*) (Vorburger *et al.*, 2009), although no such effects were detected in the grain aphid (Łukasik *et al.*, 2013a). In the absence of parasitoids, carrying *Hamiltonella* can be costly to hosts, at least under certain conditions (Oliver *et al.*, 2008; Simon *et al.*, 2011; Vorburger & Gouskov, 2011; Łukasik *et al.*, 2013a). *Hamiltonella* has not been reported to confer protection against fungal entomopathogens (Ferrari *et al.*, 2004; Łukasik *et al.*, 2013b). A wide range of *Rickettsia* (Alphaproteobacteria: Rickettsiales) and *Spiroplasma* (Mollicutes: Entomoplasmatales) have been recorded as insect endosymbionts (Gasparich, 2002; Weinert *et al.*, 2009), but so far only a few aphid species have been screened for infection with these bacteria. In the pea aphid populations, their prevalence varies between 0–40% and 0–36%, respectively (Tsuchida *et al.*, 2002; Ferrari *et al.*, 2012; Russell *et al.*, 2013), but neither was detected in a sample of 50 grain aphid clones collected in England (Łukasik *et al.*, 2013a). *Rickettsia* typically has a negative influence on pea aphid fecundity (Sakurai *et al.*, 2005; Simon *et al.*, 2007; but see Simon *et al.*, 2011), but improved tolerance to increased temperature has been observed, although only for a single aphid and symbiont genotype combination (Chen *et al.*, 2000). *Spiroplasma* was also shown to reduce fecundity and longevity in pea aphids (Fukatsu *et al.*, 2001; Simon *et al.*, 2011), and additionally, a single tested strain displayed a 'male-killing' phenotype in the sexual generation (Simon *et al.*, 2011). We recently showed that at least in a single pea aphid genotype, strains of both *Rickettsia* and *Spiroplasma* confer resistance to a fungal entomopathogen *Pandora neoaphidis* (Łukasik *et al.*, 2013b), an important natural enemy of aphids (e.g. Dean & Wilding, 1973; Pickering *et al.*, 1989).

We artificially transferred *Hamiltonella–Spiroplasma* and *Hamiltonella–Rickettsia* from their original pea aphid hosts into a total of five genotypes of the pea aphid and the grain aphid. Here we ask (i) whether pathogen resistance occurs in two further pea aphid genotypes and (ii) whether the same effects are found in a different species, the grain aphid. Single and double infections were created, which allowed us (iii) to test whether pathogen resistance conferred by *Rickettsia* and *Spiroplasma* was modified by the presence of *Hamiltonella* and whether this was influenced by host background (genotype and species). We then asked (iv) whether carriage of *Rickettsia* and *Spiroplasma* affected host fecundity and (v) tested the hypothesis that double infections are particularly costly.

Materials and methods

Study organisms

The clonal genotypes of the pea aphid *Acyrthosiphon pisum* and the grain aphid *Sitobion avenae* used in the experiments were collected in south England between 2003 and 2009 (Table S1). Aphids were genotyped at four (*A. pisum*) or seven (*S. avenae*) microsatellite loci, as previously described (Ferrari *et al.*, 2008; Łukasik

et al., 2011). They were also screened for the major facultative endosymbionts of aphids (*Hamiltonella defensa*, *Regiella insecticola*, *Serratia symbiotica*, the species of Enterobacteriaceae known as 'X-type', *Rickettsia* sp., *Spiroplasma* sp. and *Rickettsiella* sp.) using a series of diagnostic PCRs targeting the 16S rRNA gene of each bacterium (Łukasik *et al.*, 2013a). Some positive records were confirmed by partial or full sequencing of the PCR product. Gram-staining of aphid haemolymph and terminal restriction fragment length polymorphism (T-RFLP) analysis did not indicate the presence of any other bacteria (P. Łukasik, unpublished). Aphids were maintained in 90-mm nonvented Petri dishes on broad bean (*Vicia faba*, var. The Sutton) or wheat leaves kept hydrated by placing their stems in 2% agar. Cultures were maintained at 14 ± 1 °C under a 16 : 8 h light–dark regime and at approximately 70% relative humidity. Experiments were conducted at 20 ± 2 °C, and aphids were acclimatized to these conditions for at least three generations before use.

Establishment of experimental lines

We used two pea aphid genotypes as symbiont donors: genotype 161 was naturally infected with a strain of *Hamiltonella* and a strain of *Spiroplasma*, whereas genotype 208 carried a strain of *Rickettsia* and a strain of *Hamiltonella,* which differed at the 16S rRNA gene locus from that carried by genotype 161. Oral administration of antibiotics resulted in lines of these two genotypes cured of *Hamiltonella*, but not the other two symbionts (McLean *et al.*, 2011). Haemolymph from single- and double-infected donor aphids was used to infect three genotypes of pea aphid (codes 145, J68 and J102) and two of grain aphid (Co23 and Co26). The recipient pea aphid genotypes were free of secondary symbionts when collected, whereas the grain aphids had been cured of *Hamiltonella* (Łukasik *et al.*, 2011). In some cases, when aphids were injected with haemolymph from double-infected donors, only *Hamiltonella* became established. Figure 1 summarizes the aphid–symbiont combinations that were available for experiments, and further details are provided in Table S1. We will refer to aphids with the same genotype but different symbiont complements as 'lines'. The genotype and infection status of all lines was confirmed prior to the experiments using microsatellites and diagnostic PCRs, as specified above.

Pathogen susceptibility assay

Aphid susceptibility to the common fungal entomopathogen *Pandora neoaphidis* was tested by exposing aphids to the isolate X4, which had been used in several previous studies of the protection against pathogens conferred by aphid endosymbionts (Łukasik *et al.*, 2013b and references therein). Replicate groups of 20 9–10-day-old apterous aphids were exposed for 90 min to spore showers from two infected aphid cadavers in cylinders approximately 25 mm high and of 15 mm diameter. Afterwards, the aphids were kept in Petri dishes with broad bean leaves or wheat seedlings that were replaced every 3 days (genotypes Co23, Co26 and 145), or on potted bean plants (genotypes J68, J102). For the first 24 h after the end of exposure, the relative humidity was maintained close to 100% to facilitate germination of fungal spores, and then over the next 5 days, the humidity was allowed to drop to approximately 70%. After 6 days, all insects were scored as alive, dead but not sporulating, or dead and sporulating. All dead aphids that were not sporulating at the time of the 6-day census were transferred onto wet filter paper for 24 h to test whether sporulation would occur later. For logistic reasons, lines of a given

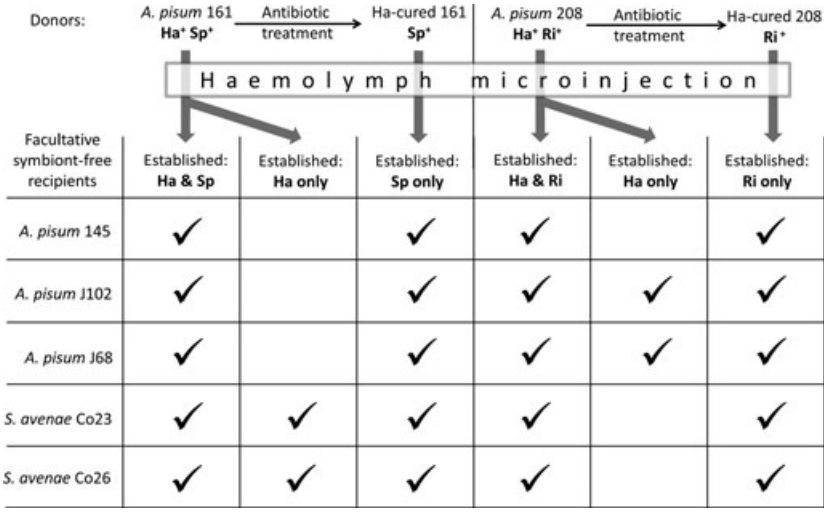

Fig. 1 Development of experimental aphid lines. Two multiply infected pea aphid (*Acyrthosiphon pisum*) donor clones (161 and 208) were cured of *Hamiltonella* leaving a single symbiont (*Spiroplasma* or *Rickettsia,* respectively). Haemolymph from naturally infected and *Hamiltonella*-free lines were injected into different pea aphid and grain aphid (*Sitobion avenae*) genotypes to produce the symbiont–host genotype combinations shown in the table.

genotype were always assessed at the same time, separately from lines of other genotypes; because of this design, the statistical effect of aphid genotype cannot be separated from the effect of temporal block. In total, we exposed 228 groups of 20 aphids, four to ten for each of the 29 experimental lines.

Fecundity assay

The effects of different symbiont complements on host fecundity were estimated by counting the number of offspring produced by isolated wingless aphids in the first 19 (*A. pisum*) or 16 days (*S. avenae*) of life. The experimental aphids, all born within 8 h of each other and the progeny of large, high-quality wingless females, were kept in groups of six in Petri dishes on leaves that were replaced every 3 days. Seven days after birth, before they started reproducing, young wingless adults or fourth-instar juveniles with no wing buds were randomly selected from across the groups and isolated in separate Petri dishes. Starting the study with larger numbers of aphids, only some of which were later selected for fecundity measurements, guaranteed that despite the variable proportion of wingless morph, sufficient numbers would be available for the experiments. Following isolation, reproducing aphids were transferred to fresh dishes with new plants every 3 days, and the offspring produced in the interval by every aphid were counted. The total number of offspring produced by females surviving for 19 (pea aphid) or 16 days (grain aphid) was used as a measure of fecundity. In all cases, lines of a given genotype were tested at the same time, separately from lines of other genotypes. The fecundity assay for genotype 145 was conducted in two blocks, with 18–20 replicates (isolated aphids) per line per block (although these were combined in the results reported below after initial analysis showed no main or interaction effects of block). Other genotypes were assessed in a single block each, with 12–22 replicates per line.

Statistical analysis

All data were analysed using generalized linear modelling in R v. 2.14.2 (R Development Core Team, 2011). Proportion data (sporulation and survival) were analysed with the assumption of a quasibinomial error distribution to account for overdispersion. Analyses of fecundity assumed Gaussian error distributions after tests for normality were conducted. In testing the different hypotheses, a full model involving all explanatory variables was fitted, and some significant interaction terms ($P < 0.05$) are mentioned in the Results. Post hoc comparisons of means were performed using the function *glht* in the package *multcomp* (sporulation and survival) or Tukey's HSD (fecundity).

Results

Do *Spiroplasma* and *Rickettsia* confer pathogen protection in multiple host genetic backgrounds of the pea aphid?

In the absence of facultative endosymbionts, all three pea aphid genotypes are highly susceptible to *P. neoaphidis*. We previously showed that the experimental strains of *Spiroplasma* and *Rickettsia* conferred protection on genotype 145 by decreasing sporulation rate and increasing survival (Łukasik *et al.*, 2013a). Here we demonstrate that single infection with *Rickettsia* has the same effect on all three pea aphid genotypes, almost completely eliminating the risk of infection (6-day sporulation: $F_{1,49} = 857.18$, $P < 0.001$; Fig. 2a–c) and dramatically increasing 6-day survival ($F_{1,49} = 347.42$, $P < 0.001$; Fig. S1a–c). We observed some differences between host genotypes in the effect of *Rickettsia* on survival (aphid genotype × infection status interaction: $F_{2,47} = 4.32$, $P = 0.019$).

Spiroplasma in single infections conferred a lower, but highly significant degree of protection to the three pea aphid genotypes (6-day sporulation: $F_{1,47} = 121.50$, $P < 0.001$; 6-day survival: $F_{1,49} = 86.38$, $P < 0.001$; Fig. 2a–c, Fig. S1a–c). Its protective effect was weaker in genotype J102 than in the other two (aphid genotype × infection status interaction in the effects on sporulation: $F_{2,45} = 5.00$, $P = 0.011$). Both symbionts thus increase resistance in multiple pea aphid genotypes, though, with a likely symbiont × host genotype interaction in the case of *Spiroplasma*.

Do *Spiroplasma* and *Rickettsia* confer pathogen protection in grain aphids?

The two genotypes of grain aphid are highly susceptible to the fungus in the absence of facultative symbionts, but establishment of either *Spiroplasma* or *Rickettsia* effectively prevented sporulation (*Spiroplasma*: $F_{1,34} = 73.03$, $P < 0.001$; *Rickettsia*: $F_{1,34} = 71.85$, $P < 0.001$; Fig. 2d–e). Both symbionts increased survival of the exposed grain aphids (*Spiroplasma*: $F_{1,34} = 14.83$, $P < 0.001$; *Rickettsia*: $F_{1,34} = 26.12$, $P < 0.001$; Fig. S1d–e), although the effects of *Spiroplasma* on survival differed between host genotypes (aphid genotype × infection status interaction: $F_{1,33} = 7.94$, $P = 0.008$).

Is pathogen resistance brought about by *Spiroplasma* and *Rickettsia* affected by the presence of the second symbiont, *Hamiltonella*?

Inspection of Fig. 2 suggests that the presence of *Hamiltonella* has no consistent effect on susceptibility to the fungal pathogen. In the case of *Rickettsia,* resistance is complete or nearly complete with and without *Hamiltonella*. Similarly, among *Spiroplasma*-infected grain aphids,

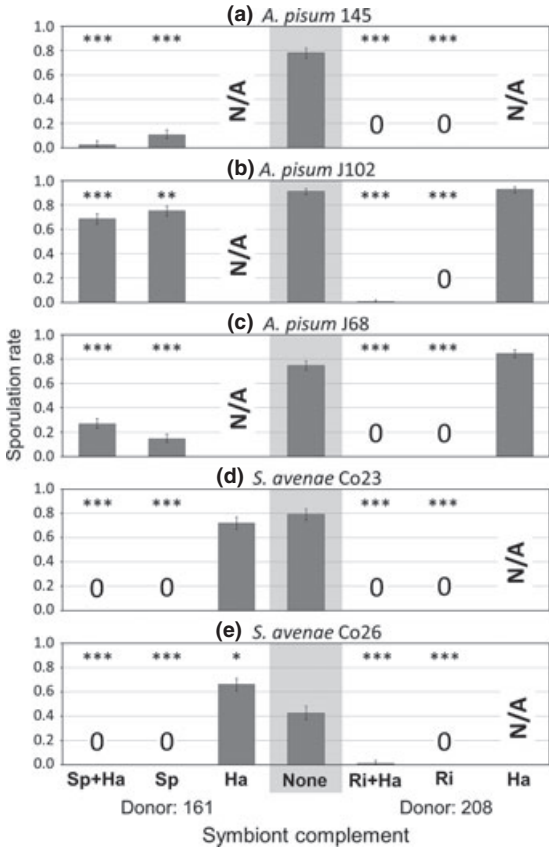

Fig. 2 The sporulation rate (mean ± SE) of *Pandora neoaphidis* 6 days after exposure in aphids representing five clonal genotypes of two species (a–c: *Acyrthosiphon pisum*; d–e: *Sitobion avenae*), either free from infection with facultative endosymbionts or infected with *Spiroplasma* (Sp), *Rickettsia* (Ri) and/or *Hamiltonella* (Ha) originating from pea aphid clones 161 and 208. Genotype–symbiont combinations marked 'N/A' were not available. Asterisks indicate lines with significantly different sporulation than the noninfected line of a given genotype: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

sporulation rate is zero regardless of *Hamiltonella* co-infection, but the survival of double-infected aphids is lower ($F_{1,21} = 6.48$, $P = 0.019$). We observed differences between pea aphid genotypes in relative sporulation rate (aphid genotype × infection status interaction: $F_{4,62} = 2.53$, $P = 0.049$) and survival ($F_{4,62} = 4.69$, $P = 0.003$) of *Spiroplasma*-only and *Spiroplasma–Hamiltonella*-infected lines, but the main effect of *Hamiltonella* co-infection was not significant ($P > 0.25$).

In four cases, we were able to compare the susceptibility of the same aphid genotype when it had no secondary symbionts or carried *Hamiltonella* alone. The presence of *Hamiltonella* has a slight positive effect on sporulation rate ($F_{1,72} = 5.72$, $P = 0.019$) and a slight negative effect on survival ($F_{1,72} = 4.71$, $P = 0.033$). There was some variability in response among host genotypes (Fig. 2, Fig. S1), but the interaction terms were not significant ($P > 0.30$).

Does carriage of *Spiroplasma* or *Rickettsia* reduce aphid fecundity?

Single infection with *Rickettsia* strongly reduces the fecundity of all three pea aphid genotypes ($F_{1,151} = 75.95$, $P < 0.001$), although the magnitude of the effect varies between genotypes (host–infection status interaction: $F_{2,149} = 8.03$, $P < 0.001$; Fig. 3a–c). In grain aphids, *Rickettsia* has no significant effect ($F_{1,55} = 2.65$, $P = 0.109$; Fig. 3d–e). We found no significant effect of single infections with *Spiroplasma* on the fecundity of either species ($P > 0.05$).

Are double infections more costly than single infections?

There are no consistent additional costs of double infection, although for some symbiont combinations and aphid genotypes, higher costs were observed (Fig. 3). In *Rickettsia*-infected pea aphids, co-infection with *Hamiltonella* ameliorates reductions in fecundity ($F_{1,154} = 37.87$, $P < 0.001$), although the effect is much more pronounced in genotype 145 than in the two others (aphid genotype × infection status interaction: $F_{2,152} = 16.39$, $P < 0.001$). No such effects were observed in *Rickettsia*-infected grain aphids. In *Spiroplasma*-infected pea aphids, *Hamiltonella* either had no effect on fecundity or in one case had a positive influence (infection status: $F_{1,160} = 4.46$, $P = 0.036$, host–infection status interaction: $F_{2,158} = 3.84$, $P = 0.023$). In contrast, in *Spiroplasma*-infected grain aphids, *Hamiltonella* decreases fecundity ($F_{1,55} = 12.68$, $P < 0.001$), particularly in genotype Co26 (host–infection status interaction: $F_{1,54} = 4.12$, $P = 0.047$). Fecundity was only analysed for aphids surviving until the end of the study. However, we noted that *Spiroplasma–Hamiltonella* co-infection markedly increased the mortality of genotype Co26 (Fisher's exact test: $P < 0.001$, though note unplanned comparison), with 60% of the aphids dying by the end of the experiment. Mortality was much lower (average 2.8%, max. 13.3%) in all other lines of the five experimental genotypes irrespective of infection status.

We could only estimate the effects of single infections with *Hamiltonella* in four cases, but they all had similar fecundity to uninfected aphids ($P > 0.15$; Fig. 3).

Discussion

Facultative endosymbionts of aphids, including *Rickettsia*, *Spiroplasma* and *Hamiltonella*, are known to protect their hosts against important natural enemies (Oliver *et al.*, 2003; Łukasik *et al.*, 2013b). But although the parasitoid-resistant phenotype conferred by *Hamiltonella*

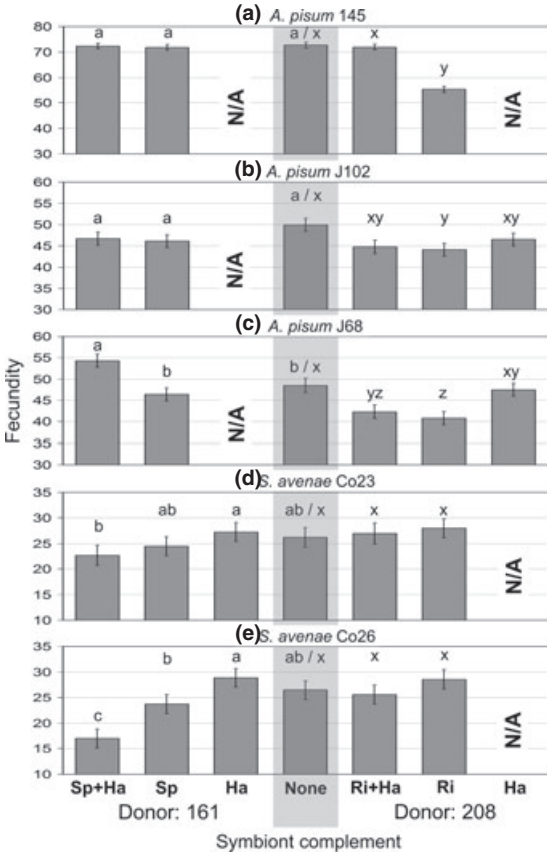

Fig. 3 The number of offspring (mean ± SE) produced within 19 days (*Acyrthosiphon pisum*) or 16 days (*Sitobion avenae*) from birth by aphids representing five clonal genotypes of two species (a–c: *A. pisum*; d–e: *S. avenae*), either symbiont-free or infected with *Spiroplasma* (Sp), *Rickettsia* (Ri) and/or *Hamiltonella* (Ha) originating from one of the two donor clones. Genotype–symbiont combinations marked 'N/A' were not available. For each genotype, lines infected with symbionts from a particular donor were compared with each other and with a noninfected line, but not with lines infected with symbionts from another donor. Letters identify lines not significantly different from others within the same comparison according to Tukey's HSD test with 95% confidence interval.

is known to be expressed across multiple host genotypes (Oliver *et al.*, 2005), the ability of *Rickettsia* and *Spiroplasma* to confer resistance to fungal pathogens had previously been demonstrated only in a single pea aphid clone. Here we show that this resistant phenotype can be expressed in other pea aphid genetic backgrounds as well as in the grain aphid. Both the pea and grain aphids were highly resistant to fungus when carrying *Rickettsia*. The presence of *Spiroplasma* conferred partial resistance on the pea aphid, the host from which it was isolated, but complete resistance on two clones of the grain aphid, a species from which it has yet to be recorded. Further studies of more symbiont strains as well as host genotypes and species would be valuable, but this study adds to the growing body of evidence that horizontal transmission of symbiotic microorganisms between genotypes and species can result in the instantaneous acquisition of ecologically important traits (Oliver *et al.*, 2010). This can have important population- and community-level implications (Ferrari & Vavre, 2011).

We found no evidence that the pathogen-protective phenotype conferred by *Spiroplasma* and *Rickettsia* was influenced by co-infection with another symbiont, *Hamiltonella*, a species known to help aphids defend themselves against parasitoid wasps (Oliver *et al.*, 2003). *Hamiltonella* genotypes vary in the parasitoid protection they confer, and the status of the two experimental *Hamiltonella* strains is not known, but it is likely that the natural multiple infections in the donor clones complement each other, resulting in resistance to a widened range of natural enemies. Symbionts have been shown to act synergistically, together protecting the host against a specific natural enemy better than either symbiont alone (Oliver *et al.*, 2006), or allowing resistance to operate effectively in a wider range of environmental conditions (Guay *et al.*, 2009). Such synergy between maternally co-transmitted facultative mutualists may be reinforced by natural selection (Vautrin *et al.*, 2008; Vautrin & Vavre, 2009), and this could explain higher than expected prevalence of certain symbiont combinations in natural populations of insects (Jaenike *et al.*, 2010; Ferrari *et al.*, 2012; Russell *et al.*, 2013).

Although the symbiont strains used in our experiments originated from pea aphids, there was no consistent pattern of greater costs (or lesser benefits) when they were introduced into the two genotypes of the grain aphid. However, the effects on fecundity of single and double infections varied between symbiont types, aphid species and genotypes (Fig. 3). Notably, single infections with *Rickettsia* negatively affected the fecundity of all pea aphid genotypes, but these deleterious effects were reduced in one of the lines co-infected with *Hamiltonella*. In one of the grain aphid genotypes, fitness was dramatically decreased by *Spiroplasma–Hamiltonella* co-infection, although there was no significant effect in the other genotype. Note that the experimental symbiont pairs we worked with were collected in the field and are typical of those found in natural populations (Ferrari *et al.*, 2012). Possibly these symbiont strains have (or have evolved) a degree of relative compatibility higher than that would be found in a randomly combined pair. Oliver *et al.* (2006) found that in artificially created (and rare in nature) double infections between the two symbiont Enterobacteriaceae species, *Hamiltonella* and *Serratia,* the densities of *Serratia* increased dramatically, perhaps in a response to interspecific competition, and this harmed the obligate symbiont *Buchnera* and hence the aphid host. *Buchnera* populations can also be negatively affected by a *Rickettsia* infection (Sakurai *et al.*, 2005), and antagonistic

relationships between heritable symbionts have been reported in other systems (Goto *et al.*, 2006). However, our knowledge of how different endosymbionts in multiple-infected hosts interact and how these interactions translate into host fitness is limited. Tools such as quantitative PCR, fluorescent *in situ* hybridization and transcriptome sequencing can provide an insight into how endosymbiont densities, within-host distributions or gene expression patterns are affected by the presence of another symbiont species (e.g. Sakurai *et al.*, 2005; Oliver *et al.*, 2006; Gottlieb *et al.*, 2008). However, to understand fully the importance of these effects in natural populations will require assessing how different symbiont combinations influence all components of host fitness (Chen *et al.*, 2000; White *et al.*, 2011) and how this changes across the range of environmental conditions that the insects encounter in nature (Oliver *et al.*, 2013).

Recent advances in molecular techniques have led to the discovery of many symbiotic microorganisms, and it is becoming clear that a large number of eukaryotes form stable obligate and facultative associations with multiple species of bacteria. An exciting challenge is to understand how these microorganisms interact with each other and with their host to determine the compound phenotype of these intimate associations.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The survival rate (mean ± SE) of aphids representing five clonal genotypes of two species (a–c: *Acyrthosiphon pisum*; d–e: *Sitobion avenae*), either free from infection with facultative endosymbionts or infected with *Spiroplasma* (Sp), *Rickettsia* (Ri) and/or *Hamiltonella* (Ha) originating from pea aphid clones 161 and 208, for 6 days from exposure to spores of *Pandora neoaphidis*.

Table S1 Characteristics of the aphid genotypes used in this study.

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