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Horizontal transfer of facultative endosymbionts is limited by host relatedness

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Heritable microbial symbionts can have important effects on many aspects of their hosts' biology. Acquisition of a novel symbiont strain can provide fitness benefits to the host, with significant ecological and evolutionary consequences. We measured barriers to horizontal transmission by artificially transferring facultative symbionts from the grain aphid, *Sitobion avenae*, and five other aphid species into two clonal genotypes of *S. avenae*. We found the symbiont *Hamiltonella defensa* establishes infections more easily following a transfer from the same host species and that such infections are more stable. Infection success was also higher when the introduced symbiont strain was more closely related to the strain that was originally present in the host (but which had previously been removed). There were no differences among successfully established symbiont strains in their effect on aphid fecundity. *Hamiltonella defensa* did not confer protection against parasitoids in our *S. avenae* clones, although it often does in other aphid hosts. However, strains of the symbiont *Regiella insecticola* originating from two host species protected grain aphids against the pathogenic fungus *Pandora neoaphidis*. This study helps describe the extent to which facultative symbionts can act as a pool of adaptations that can be sampled by their eukaryote hosts.

KEY WORDS: ***Acyrthosiphon pisum*, *Aphidius ervi*, *Hamiltonella defensa*, horizontal transfer, invasion, *Pandora neoaphidis*, *Regiella insecticola*, symbiosis.**

Organisms respond to ecological change through the action of natural selection operating on genetic variation. Many microorganisms have a further option, which is to acquire novel traits from mobile genetic elements that move between species such as plasmids, bacteriophages, and transposable elements (Ochman et al. 2000; Gogarten et al. 2002). There is growing evidence that higher eukaryotes can also rapidly acquire novel ecological capabilities and gain competitive advantage by hosting symbiotic microorganisms. Insects in particular are host to a diverse community of facultative endosymbionts that can profoundly influence host biology (Moran et al. 2008; Zug and Hammerstein 2012). These often beneficial microbes are primarily vertically (maternally) transmitted, but can also be transmitted horizontally at lower frequency between host lineages (Sandström et al. 2001; Haselkorn et al. 2009; Russell et al. 2009; Duron et al. 2010). Although not essential for the growth or reproduction of their hosts, facultative symbionts can strongly influence their hosts' fitness, for example, by conferring increased resistance to natural enemies (Oliver et al. 2003; Scarborough et al. 2005; Hedges et al. 2008; Teixeira et al. 2008; Jaenike et al. 2010; Łukasik et al. 2013c), tolerance to environmental extremes (Montllor et al. 2002), and the ability to use new food sources (Tsuchida et al. 2004). The acquisition and spread of a symbiont can thus have major consequences for the recipient host population (e.g., Jaenike et al. 2010; Himler et al. 2011; Cockburn et al. 2013), which can lead to cascading effects on the ecological community in which it is embedded (Ferrari and Vavre 2011; Jaenike and Brekke 2011). A critical question that we address in this article is the nature of the barriers to the transfer of microbial symbionts between hosts.

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Symbionts acquired by horizontal transfer can provide their hosts with immediate beneficial evolutionary innovations, which then spread through the population. But for this to happen, a number of steps need to occur successfully: (1) there needs to be an ecological opportunity for horizontal transfer, for example, the insect must occupy the same habitat or share a natural enemy or host plant that can vector or act as a conduit for the symbiont (e.g., Duron et al. 2010); (2) once transferred, the bacteria need to establish in the host, avoiding elimination by the new host's immune system (Hurst and Darby 2009); (3) they will then need to be transmitted to the new host's offspring, (4) avoid stochastic loss when rare (Jansen et al. 2008); and finally (5) to spread through a population they need to confer a net fitness benefit to the new host compared to uninfected individuals or have the capacity to manipulate host reproduction to increase symbiont transmission (Bright and Bulgheresi 2010).

Phylogenetic data have revealed numerous cases of successful horizontal transfer of facultative symbionts between species (e.g., Sandström et al. 2001; Russell et al. 2003; Haselkorn et al. 2009; Henry et al. 2013), and some mechanisms of transfer have been identified. Transmission routes include via natural enemies such as parasitoids or parasitic mites (Jaenike et al. 2007; Gehrer and Vorburger 2012) and through plant tissues (Caspi-Fluger et al. 2012). Aphid symbionts can also be paternally transmitted during the sexual generation, and this is a potential route of cross-species transfer via attempted interspecific matings (Moran and Dunbar 2006). Much less is known about which factors determine whether a symbiont establishes successfully after horizontal transfer, and whether it can express a phenotype that would facilitate its spread. Artificial transfers of endosymbionts which manipulate host reproduction—for example, strains of *Wolbachia* and *Spiroplasma*—are often more successful if they are made between closely related hosts (Rigaud et al. 2001; Russell and Moran 2006; Tinsley and Majerus 2007; Russell et al. 2009), although the relationships can be more complicated (Haselkorn and Jaenike 2015). Phylogenetic distance is likely to reflect physiological similarity and hence symbionts may be preadapted to the internal environment of closely related hosts. Hosts may also evolve to support beneficial symbiont genotypes with which they have had long-term associations. Closely related symbionts, even if currently residing in divergent hosts, are more likely to share traits such as the ability to function in an internal environment of a particular species or clade of hosts, as well as features that enable their recognition by the host and perhaps trigger host pathways that promote symbiosis.

Following establishment, phenotypes conferred by a symbiont on its original host may or may not be expressed in a new host. For example, some aphid symbionts have been shown to protect both their original and new hosts against the same parasitoids or pathogens (Vorburger et al. 2010; Łukasik et al. 2013b). However, this is not always the case: the *Spiroplasma* symbiont which restores fertility to *Drosophila neotestacea* when it is infected by a castrating parasitic nematode has the same effect in only one of four other *Drosophila* species (Haselkorn et al. 2013). Strains of *Wolbachia* originating from diverse *Drosophila* species may or may not confer protection against viruses after introduction into a common host, *D. simulans* (Martinez et al. 2014)—although information about their fitness effects in native hosts is often lacking. There is a need for a systematic exploration of whether symbiont establishment and the benefits conferred by microbial mutualists are more likely to occur in transfers between closely related genotypes or species.

We report here a study in which we attempt to infect artificially the grain aphid (*Sitobion avenae*) with strains of the bacterial facultative endosymbiont *Hamiltonella defensa* (henceforth referred to as *Hamiltonella*) from other *S. avenae* clones as well other aphid species that differ in their phylogenetic relatedness to the grain aphid. *Hamiltonella* naturally infects a wide range of aphid species as well as some related insects (Sandström et al. 2001; Russell et al. 2003; Henry et al. 2015), and many strains protect their aphid hosts against parasitoid wasps (Oliver et al. 2005; Vorburger et al. 2009). Here, we ask whether phylogenetic distance between the donor and recipient host genotypes, or between the symbiont strain originally carried by the recipient aphid and the newly introduced symbiont strain, affects (1) the probability of establishment and (2) the expression of symbiont traits in the grain aphid. We also report on parallel experiments with a smaller number of strains of *Regiella insecticola* (*Regiella* below), an aphid facultative endosymbiont associated in particular with resistance to fungal pathogens (Tsuchida et al. 2004; Scarborough et al. 2005; Leonardo and Mondor 2006).

Material and Methods

APHID CULTURE AND GENERAL EXPERIMENTAL METHODS

Symbionts were injected into two "recipient" clones of *S. avenae* (grain aphid), Co23 and Co26, which we cultured on wheat (*Triticum aestivum*) or cocksfoot (*Dactylis glomerata*). "Donor" aphids came from six species: *S. avenae;* three grass-feeding species, *Sitobion fragariae*, *Sitobion* nr. *fragariae* and *Utamphorophora* sp. that were cultured on *D. glomerata*; and two legume-feeding species *Acyrthosiphon pisum* and *Aphis fabae* cultured on broad beans (*Vicia faba*; Table S1). Aphid colonies were established from insects collected in the field in southern England between 2003 and 2009 except for *A. fabae* which was originally collected in Switzerland in 2006 (Vorburger et al. 2009). All aphid lines were cultured under conditions that ensured indefinite asexual reproduction (14 ± 1°C, 70 ± 15% humidity and a L16:D8 light regime). Aphids were maintained in 90 mm

nonvented Petri dishes on plants that were kept fresh by placing their stems in 2% agar. Insects were transferred regularly to fresh plants in new dishes. All experiments were conducted at 20 ± 2°C, and aphids were acclimatized to this temperature for at least three generations. Throughout the study, the identity of any *S. avenae* lines was regularly confirmed using microsatellites (Łukasik et al. 2011).

SYMBIONT SCREENING AND MOLECULAR METHODS

DNA from adult aphids was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. To confirm the morphologically based identifications of aphid species, we amplified and sequenced a 658 bp fragment of mitochondrial cytochrome oxidase C subunit I (*COI*) gene, a region used for species barcoding, using primers LepF and LepR (Foottit et al. 2008). The sequences, unambiguous in all cases, were compared against the Barcode of Life database (Ratnasingham and Hebert 2007), and all matched existing records at 98.3–100% identity (see Supporting Information for details of the identification procedure). COI gene sequences have previously been used to reconstruct aphid phylogenies (Foottit et al. 2008), although this approach lacks resolution at deeper phylogenetic nodes compared to multigene approaches (Nováková et al. 2013).

Aphids were screened for the presence of the seven most common facultative symbionts of the pea aphid: *H. defensa*, *R. insecticola*, *Serratia symbiotica*, "X-type," *Rickettsia* sp., *Spiroplasma* sp., and *Rickettsiella viridis*, as well as two widespread insect endosymbionts that have been recorded occasionally from aphids, *Arsenophonus* sp. and *Wolbachia pipientis*, using diagnostic polymerase chain reactions (PCRs) with symbiont-specific primers (Table S2). The identity of any positives was confirmed by sequencing of the PCR product using Big Dye Terminator version 3.1. (Applied Biosystems, Foster City, CA). Sequence traces for each sample were individually assembled and edited using CodonCode Aligner version 2.06 (CodonCode Corp., Centerville, MA), a procedure we followed for all other sequences. Strain diversity in the focal symbiont species, *Hamiltonella*, was characterized further by sequencing six household genes, *accD*, *gyrB*, *hrpA*, *murE*, *recJ*, and *rpoS* (Degnan and Moran 2008; Henry et al. 2013), following Henry et al.'s (2013) protocols.

SYMBIONT STRAINS AND ARTIFICIAL INFECTION PROCEDURE

Hamiltonella strains from 12 aphid clones of six species were injected separately into each of two genotypes of *S. avenae*; six of these transfers were intraspecific, and the other six were interspecific. Both recipient genotypes naturally carried a *Hamiltonella* infection from which they had been cured in the laboratory by antibiotic treatment (Łukasik et al. 2011) prior to experimentation. The cured aphids were kept for at least 10 generations before being injected with other symbionts. Eleven of the donor clones hosted *Hamiltonella* in single infections with the 12th also being co-infected with *Regiella* (details in Table S1). Hemolymph was collected from fourth instar or adult donor aphids with an ultrafine needle pulled from a borosilicate glass capillary, connected with rubber tubing to a syringe. The needle was inserted beneath a posterior leg of a first-instar recipient aphid, just deep enough to puncture the cuticle, and the donor hemolymph injected; the precise amount introduced was not quantified. Typically, around 10 aphids from each recipient genotype were injected with hemolymph from each donor (generation A), although for some host genotype–symbiont strain combinations where establishment was difficult, the number of injected aphids was higher (up to 28). Fourteen days after the injections, the survivors were separated, and their subsequent offspring (generation B) retained. When these offspring (B) became adult, two or three of them were transferred to separate dishes. After they had produced offspring (generation C), they were screened for the presence of symbionts using diagnostic PCRs. Initially, a single generation B individual was tested, and if it was negative a further one or two were also tested. We considered an injected individual (from generation A) to be successfully infected if at least one of her (B) offspring tested positive for the symbiont. Thus, the rate of successful infection was calculated as the number of females that produced at least one infected daughter in the post-injection generation (B) divided by the number of survivors in the injected generation (A). On average, we tested the offspring of 7.7 (range 4–17) injected aphids.

For each combination of *Hamiltonella* strain and recipient genotype, we kept six separate sublines for four generations. At this point, they were reassessed using diagnostic PCR, and from then onwards four infected sublines were maintained. They were tested again at approximately the 10th generation. One subline per host–symbiont combination was used in the experiments described below. All lines were screened again for the correct symbiont (including sequencing the PCR products) two or three generations before each experiment.

We also injected three strains of a second symbiont, *R. insecticola* (two strains originating from *S. avenae*, one from *A. pisum*) into the two recipient aphid genotypes. Because of the low number of strains, we did not formally analyze correlations with genetic distance for these lines, but we briefly report the qualitative patterns observed.

EFFECTS OF FACULTATIVE ENDOSYMBIONTS ON APHID LIFE-HISTORY TRAITS

The effects on aphid fitness of infections with new symbiont strains were assessed at least eight generations after their successful introduction. We tested whether the successfully established symbionts affected aphid fecundity or their susceptibility to two

common natural enemies: the hymenopteran parasitoid *Aphidius ervi* and the entomopathogenic fungus *Pandora neoaphidis*.

The fecundity of winged aphids (which were considerably more abundant than wingless in the experimental generation) was measured following the protocol in Łukasik et al. (2013a). Briefly, we counted offspring produced by isolated females during three consecutive three-day periods (between the seventh and 16th day after birth), and used the total number produced as a measure of fecundity. The two recipient genotypes were assessed at different times, but all lines of each genotype were tested together. The fecundity of an average of 15.6 (range 7–16) females was measured for each line.

The *A. ervi* stock used in the experiments was originally obtained from Syngenta Bioline and was maintained on a symbiont-free line of *S. avenae* for at least 20 months prior to use. Aphid susceptibility was measured following the protocol in Łukasik et al. (2013a). Groups of 30 aphid nymphs, 72–96 h old at the start of the experiment, were exposed in Petri dishes to single parasitoid females for 8 h. Fifteen days later, we counted (1) parasitoids which had successfully pupated within aphids (forming characteristic "mummies"), (2) aphids dying with no parasitoids pupating, and (3) aphids that survived. Susceptibility was calculated as the proportion of exposed aphids that formed mummies. Lines of aphid genotype Co23 were tested in two temporal blocks, with an average of 6.6 (range 5–8) replicates (groups of 30 exposed nymphs) per line, whereas lines of Co26 were tested in three blocks, with an average of 11.3 (range 5–16) replicates per line.

Aphid susceptibility to strain X4 of *P. neoaphidis* (kindly provided by J. Pell, Rothamsted Research) was measured following the protocol described in Łukasik et al. (2013b). Groups of 20 young wingless adult aphids were exposed to two fresh sporulating pea aphid cadavers for 90 min, and then transferred in groups of 10 to Petri dishes that were then wrapped in wet paper towels for the first 24 h to ensure the high humidity that is conducive to fungal infection. Aphids were transferred to fresh plants in new dishes every three days, and their survival and any fungal sporulation were assessed daily. As a measure of fungal susceptibility we used the proportion of the exposed aphids that formed sporulating cadavers within eight days from exposure. Lines of each recipient genotype were tested across three (Co23) or five (Co26) incomplete blocks, with an average of 5.8 (range 4–10) replicates (groups of 20 exposed aphids) per line.

ANALYSIS

The pairwise genetic distances between each newly introduced *Hamiltonella* strain and the original *Hamiltonella* strain of the recipient aphid clones were estimated using the concatenated sequences of the six household genes. We also estimated the genetic distance between the recipient aphid genotype and the donor aphid genotype using COI sequences. In both cases, this was done by calculating P-distances (proportions of nucleotide sites at which two sequences differ). Maximum likelihood phylogenies for hosts and symbionts were generated using the online PhyML server (Guindon et al. 2010). *Eulachnus rileyi*, a member of the subfamily Lachninae which is the sister group to the Aphidinae, to which all the donor aphid species belong (Foottit et al. 2008), was used as an outgroup for the aphid phylogeny. A *Hamiltonella* isolate from the whitefly *Bemisia tabaci*, a member of a distinct clade from that infecting aphids (Rollat-Farnier et al. 2015), served as an outgroup for the symbiont phylogeny. We determined the best-fitting models of evolution using MEGA6's model selection algorithm (Tamura et al. 2011). The phylogenies were bootstrapped 100 times.

The experimental data were analyzed using the statistical software R version 2.13.0 (R Development Core Team 2011). In the analyses of infection success, each host genotype-symbiont strain association was considered a replicate. To test which factors affected infection success, we used generalized linear modeling techniques to assess first the effect of recipient genotype, followed by intraspecific versus interspecific transfers. We then asked whether any of the remaining deviance could be explained by variables representing the genetic distance between the new and original host, and the genetic distance between the new *Hamiltonella* strain and the one originally present in the host. The probability of an infection being stable was analyzed using logistic regression, with intraspecific versus interspecific transfer and host and *Hamiltonella* genetic distances as explanatory variables.

To compare the fecundity or susceptibility to natural enemies of different aphid lines, we also used generalized linear modeling techniques, with symbiont presence, followed by symbiont strain, aphid genotype and their interaction sequentially entered into the model. Separate analyses were performed for aphid lines infected with *Hamiltonella* or *Regiella* (but including the same data from lines free of facultative symbiont). We found that Gaussian error variances were appropriate for the analysis of the fecundity data, and assumed quasibinomial error variances for the susceptibility data to account for observed overdispersion.

Because of the possible confounding effects of co-infections, only lines injected with hemolymph from donors that were infected with *Hamiltonella* alone were included in the formal comparison of infection success. Similarly, only lines where *Hamiltonella* was the sole established symbiont were included in formal comparisons of life-history traits.

Results

There was no association between the genetic distances of aphid genotypes and those of their natural *Hamiltonella* strains (Mantel's $r = 0.1287$, $P = 0.22$ for all *Hamiltonella* strains; $r = 0.1949$,

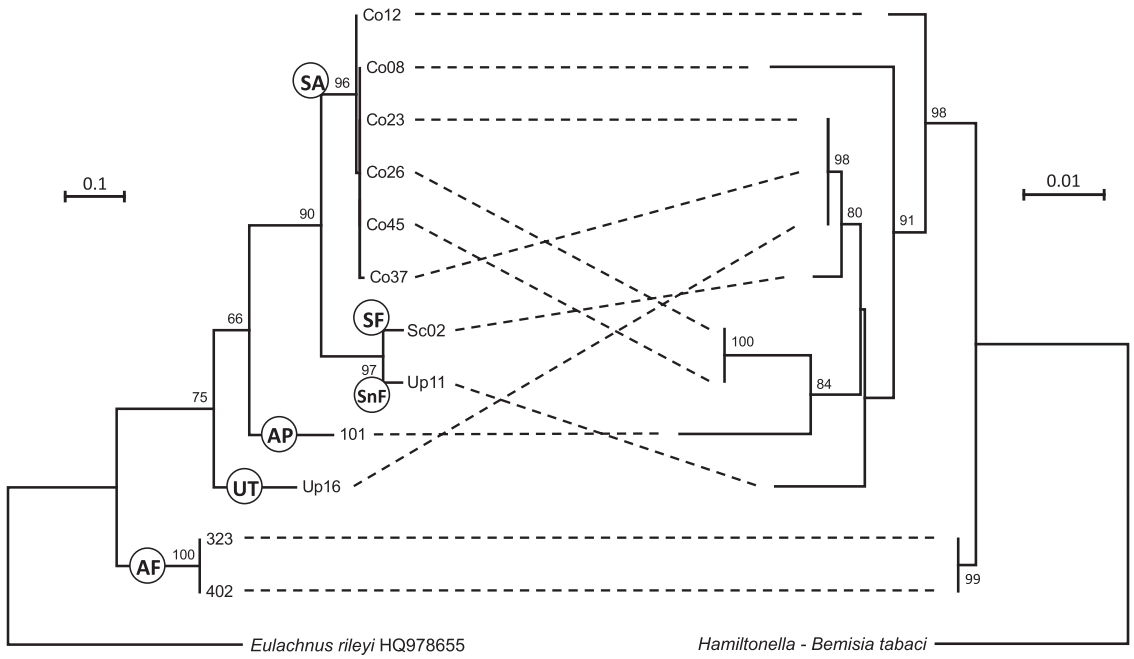

Figure 1. **The relationship between experimental aphid lines and the *Hamiltonella* strains that they naturally hosted. The maximum likelihood phylogeny of hosts (left) is based on a 641 bp alignment of mitochondrial cytochrome oxidase I (COI) gene, and the phylogeny of symbionts (right) is based on a concatenation of six household genes, *accD*, *gyrB*, *hrpA*, *murE*, *recJ*, and *rpoS*, of the total length of 3953 bp. Aphid specific names are indicated on branches of the host phylogeny and abbreviated as SA, *Sitobion avenae*; SF, *Sitobion fragariae*; SnF, *Sitobion* nr. *fragariae*; UT, *Utamphorophora* sp.; AP, *Acyrthosiphon pisum*; AF, *Aphis fabae*. Bootstrap support values over 60% are displayed.**

$P = 0.174$ for *Hamiltonella* strains in single infections only). There was also no congruence between host and symbiont phylogenies (Fig. 1).

SYMBIONT INFECTION RATE AND STABILITY OF NEW INFECTIONS

We injected hemolymph from 11 aphid genotypes with single infections of *Hamiltonella* into two recipient genotypes of *S. avenae*. The rate of successful infection was high overall, with 82% of aphids that survived for at least 14 days after injection producing at least one infected offspring (no difference between recipient genotypes: $F_{1,17} = 0.87$, $P = 0.36$; see Fig. S1 for details of individual lines). Successful establishment of *Hamiltonella* was more likely in intraspecific transfers ($F_{1,17} = 29.52$, $P < 0.001$), but the addition of the genetic distance between the donor and recipient aphid did not significantly increase the explanatory power of the model ($F_{1,17} = 0.02$, $P = 0.89$; Fig. 2A). However, the probability of successful infection was positively associated with the genetic similarity between the injected *Hamiltonella* strain and the strain originally present in the recipient host ($F_{1,17} = 12.51$, $P = 0.003$; Fig. 2B).

Most of these novel infections were stable over many generations, but there were exceptions. Both *Hamiltonella* strains from *A. fabae* were lost in all lines of both recipients within a few generations (for more details, see Supporting Information). Two other strains (from *S. fragariae* and *A. pisum*, respectively) were lost in some but not all sublines of the recipient clone Co23 (Fig. S1). Overall, stable infections were more likely to form after intraspecific transfers from *S. avenae* ($\chi_1^2 = 12.32$, $P = 0.0005$), with no additional effects of host genetic distance ($\chi_1^2 = 2.19$, $P = 0.14$) or *Hamiltonella* genetic distance ($\chi_1^2 = 0.04$, $P = 0.84$).

The pattern was qualitatively similar for the novel infections with the symbiont *Regiella*; infections with two strains of *Regiella* from *S. avenae* were all successful and stable, whereas the average infection rate with a *Regiella* strain from *A. pisum* was only 56% (in donor genotype Co23: 43%; in Co26: 70%), but if established these were also stable (Fig. S1).

EFFECTS OF INFECTION ON APHID TRAITS

We found no significant differences in the fecundity of aphids that were or were not infected by *Hamiltonella* ($F_{1,334} = 0.29$, $P = 0.592$). There were also no differences among lines hosting different *Hamiltonella* strains ($F_{9,325} = 1.54$, $P = 0.133$) or an interaction between *Hamiltonella* strain and recipient genotype ($F_{10,315} = 1.53$, $P = 0.127$; Fig S2). We also found no overall effect of *Regiella* on aphid fecundity ($F_{1,125} = 2.54$, $P = 0.114$). However, there were fecundity differences among lines infected with different strains of *Regiella* ($F_{2,123} = 3.88$, $P = 0.023$; Tukey's

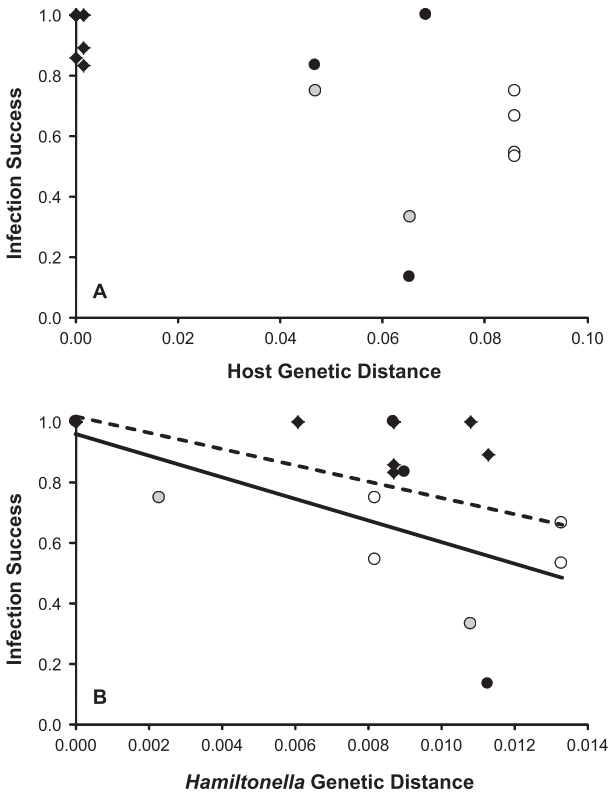

Figure 2. The relationship between the proportion of injected aphids passing the newly introduced *Hamiltonella* strain to their offspring and (A) the genetic distance between the new and original host, or (B) the genetic distance between the new *Hamiltonella* strain and the one the injected aphid was previously infected with. Black symbols indicate that the new associations were stable, gray—that we observed cases when they were lost within a few generations, and white—that we were unable to obtain stable associations. Diamonds represent intraspecific transfers of *Hamiltonella* from *S. avenae*; circles represent interspecific transfers from other Aphid specific. The dashed trend line includes all transfers, the solid line only interspecific transfers.

HSD test revealed a significant negative effect of a single pea aphid strain), but no differences in the effects of the different strains on the two host genotypes ($F_{3,120} = 2.23$, $P = 0.089$).

There was no overall effect of *Hamiltonella* presence on aphid susceptibility to parasitism by *A. ervi* ($F_{1,216} = 0.43$, $P = 0.511$), and no significant differences in the effects of hosting different *Hamiltonella* strains ($F_{9,215} = 0.98$, $P = 0.456$; Fig. S3A) or the response of the two host recipient genotypes ($F_{10,206} = 2.23$, $P = 0.089$). Similarly, the presence of *Regiella* had no effect on resistance to *A. ervi* in any of the lines (Fig. S3A).

In line with previous studies, we found no evidence that *Hamiltonella* can protect aphids against the fungal pathogen *P. neoaphidis* (main effect, $F_{1,132} < 0.01$, $P = 0.993$; differences among symbiont strains, $F_{9,131} = 1.54$, $P = 0.144$; interaction between symbiont strain and recipient genotype, $F_{10,122} = 1.54$, $P = 0.135$; Fig. S3B). In contrast, hosting *Regiella* did overall confer protection to the pathogen ($F_{1,44} = 55.8$, $P < 0.001$) although there were differences in the strength of protection provided by different *Regiella* strains ($F_{2,43} = 58.6$, $P < 0.001$) which were the same across recipient genotypes ($F_{3,41} = 1.27$, $P = 0.299$). Comparison of model coefficients revealed that two *Regiella* strains, one from *S. avenae* and another from *A. pisum*, reduced mortality from the fungal pathogen, whereas a third strain, from *S. avenae*, had no effect (Fig. S3B).

Discussion

We show that the probabilities of a symbiont establishing in a new host, and of the infection persisting, are higher in intraspecific transfers and when the new symbiont strain is more similar to the strain previously carried by the recipient host. Under our experimental conditions, carriage of *Hamiltonella* had no costs in terms of reduced fecundity, regardless of their origin. We found no *Hamiltonella* strain that conferred a significant degree of protection against either the parasitoid or the pathogen in *S. avenae*. However, two of three *Regiella* strains, including one originating from a different species, conferred increased resistance to the fungal pathogen.

These results support the findings of previous studies using fewer host–symbiont combinations (Rigaud et al. 2001; Russell and Moran 2006; Tinsley and Majerus 2007; Haselkorn et al. 2013; Haselkorn and Jaenike 2015) which also reported barriers to interspecific transmission. The ability of symbionts to establish in novel host environments, and to be vertically transmitted to future generations, appears to be an important factor limiting their spread (Bright and Bulgheresi 2010). The mechanisms underlying the effects of phylogenetic distance may be similar in parasitic and mutualistic systems and involve the immune reaction mounted by the novel host (Hurst and Darby 2009). The aphid immune system is thought to be reduced compared to that of other insects (Gerardo et al. 2010), but they do possess phagocytes (Laughton et al. 2011) which provide a general response to infection. It is possible that symbionts evolve ways to avoid the immune response in specific host backgrounds, but also that hosts fine-tune their immune system so that it does not respond to a specific and beneficial long-established symbiont strain. This could explain why intraspecific symbiont transfers were more successful, as well as why strains more similar to the one originally present in a particular recipient genotype had higher establishment success. Even if the immune system is not involved, successful symbiosis after novel transfer, and particularly vertical transmission, may require some degree of genetic adaptation by the symbiont and/or the host which will be easier if either partner has had previous evolutionary experience with the other. In the case of interspecific transfer, this condition could be fulfilled if particular symbiont strains are regularly transmitted horizontally within a group of closely related species,

but it might also occur among more distantly related species if they presented similar physiological environments for the bacteria, for example, because of shared ecology. Links between infection success in a novel host and host genetic distance have been reported in some host–parasite systems. RNA viruses tend to be more successful (achieve higher viral titer) in drosophilid species that are more closely related to the original host; however, more phylogenetically distant host taxa differ in their susceptibility to infection with that virus (Longdon et al. 2011). Similarly, parasitic nematodes are more likely to infect *Drosophila* species more closely related to their native host, but there is much variation in infection success (Perlman and Jaenike 2003).

After introduction into a novel host environment, symbionts should be under strong selection to increase transfer efficiency across host generations. This was observed in two *Drosophila* species after introduction of non-native *Spiroplasma* strains (Haselkorn et al. 2013; Nakayama et al. 2015). However, we found no increase in transmission efficiency in the generations following the successful introduction of *Hamiltonella* from *A. fabae* into *S. avenae*.

Symbiont spread in a new host would be hindered if novel associations had higher fitness costs for the aphids. Our data show no effect of the presence of *Hamiltonella* on aphid fecundity, regardless of strain origin, though other studies of novel associations have reported a range of fecundity effects, from very detrimental to positive (Chen et al. 2000; Russell and Moran 2005; Vorburger et al. 2010; Łukasik et al. 2013b). For example, Russell and Moran (2005) showed that an *Arsenophonus* strain established easily in a novel pea aphid host but then imposed a large fitness cost. Different effects on host fitness have also been reported from experiments involving symbiont transfer between genotypes of a single host species (Vorburger and Gouskov 2011; Łukasik et al. 2013a,b). However, fitness effects are notoriously difficult to measure and estimates often depend quite critically on the details of the experimental protocol. In particular, the costs of infection are often more pronounced when the aphid is in some way stressed (Oliver et al. 2008). The fitness costs of infection may also decrease as the symbiont and the host become co-adapted, as was recently demonstrated in the *Drosophila–Spiroplasma* system (Nakayama et al. 2015).

Protection against natural enemies is a common benefit of hosting facultative endosymbionts, and in aphids the resistant phenotype is typically expressed across a range of host genotypes (Oliver et al. 2005; Vorburger et al. 2010; Łukasik et al. 2013b). However, a number of recent studies have shown that the protection afforded by *Hamiltonella* strains can be quite specific to parasitoid species or even genotypes (Vorburger et al. 2009; Rouchet and Vorburger 2012; Asplen et al. 2014; Cayetano and Vorburger 2015; McLean and Godfray 2015). We found that none of the experimental *Hamiltonella* strains that successfully established in *S. avenae* conferred protection against the line of *A. ervi* we employed. In the better-studied pea aphid, nonprotective *Hamiltonella* strains exist that lack the bacteriophage known as (Oliver et al. 2009) but the virus was present in all the *Hamiltonella* strains used in the experiments (P. Łukasik, unpubl. ms.). It is not the case that our line of *A. ervi* is "super-virulent" as several strains of *Hamiltonella* confer protection against it in the pea aphid (Łukasik et al. 2013a McLean and Godfray 2015). Furthermore, we previously demonstrated that the grain aphid genotypes we used can be protected by facultative endosymbionts against the experimental parasitoid line: outside of this project we introduced *Hamiltonella* and X-type strains into *S. avenae* Co26 and obtained successful protection (Łukasik et al. 2013a). What we seem to have is further heterogeneity in the protection conferred by *Hamiltonella* beyond the simple presence or absence of APSE (Acyrthosiphon pisum Secondary Endosymbiont). It would be interesting to explore whether the symbiont strains used in the experiment confer protection against other parasitoids or in other host genotypes.

Although we studied fewer strains, we also found differences in the fungal resistance conferred by different *Regiella* strains. As far as we know, we report here the first example of an endosymbiont conferring protection against a fungal entomopathogen in an aphid species other than *A. pisum*.

There is growing evidence that interspecific horizontal transmission of facultative endosymbionts occur relatively frequently in insects (Degnan and Moran 2008; Haselkorn et al. 2009; Henry et al. 2013), even on ecological timescales (Duron et al. 2010). Indeed, the fact that the *Hamiltonella* strains found in *Utamphorophora* sp. and *S. avenae* clones Co23 and Co37 were identical across all sequenced genes strongly suggests a recent horizontal transmission event between these grass-feeding species. Horizontal transmission should be much more common within host species, with more opportunities for transfer and fewer barriers to symbiont establishment, but it is notoriously difficult to measure under natural conditions. Smith et al. (2015) have recently reported dramatic seasonal changes in prevalence of facultative endosymbionts in pea aphid populations, and even within common clonal genotypes. These changes are thought to be primarily caused by the differential success of lines carrying different symbionts, but symbiont exchange between clones might be playing a role. In contrast, in some cases the phylogenies of symbiont clades are congruent with those of hosts (Degnan and Moran 2008) indicating transitions to more stable and possibly obligate associations that may be evolving toward solely vertical transmission (McCutcheon and Moran 2012).

The importance of facultative symbionts in affecting many aspects of insect biology and evolution has only become apparent in the last two decades. Many insect phenotypes that had always been assumed to be standard nuclear-encoded traits are now

known to be determined by endosymbiont bacteria. Phenotypes that evolve in one particular insect-bacteria context can through horizontal transfer become available to other insect species, even possibly to ecological competitors of the original host (Duron et al. 2010). If horizontal transfer is common and promiscuous then endosymbionts will act as a horizontal gene pool akin to those made up of plasmids and other mobile elements in prokaryote communities (Ferrari and Vavre 2011; Jaenike 2012). How far this pool of adaptations extends will depend on both the ease with which establishment occurs as bacteria transfer between species of different phylogenetic relatedness, and the extent to which advantageous phenotypes can be expressed in different host backgrounds. It will be fascinating to explore to what extent the physiological barriers to horizontal transmission, which so far have only been tested under artificial laboratory conditions, influence the dynamics of the vast array of symbiotic bacteria in natural populations of insects.

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DATA ARCHIVING

DNA sequences have been deposited in GenBank (accession numbers: KM375934–KM376051). Experimental data underlying this article are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.rd71s.

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Supporting Information

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Supporting Material

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

Table S2. Sequences of primers used for diagnostic PCR reactions during this study.

Figure S1. Proportions of females of two recipient genotypes which produced offspring infected with the introduced symbiont(s) after microinjection of hemolymph from one of the infected donor genotypes containing *Hamiltonella* only, *Regiella* only, or co-infected with two different symbiont species.

Figure S2. Fecundity of two grain aphid genotypes, either facultative symbiont-free or infected with one of 13 strains of two symbiont species.

Figure S3. Susceptibility to two species of natural enemies, parasitoid *Aphidius ervi* and fungal pathogen *Pandora neoaphidis*, of two grain aphid genotypes, either facultative symbiont-free or infected with one of 13 strains of two symbiont species.