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Consequences of symbiont co-infections for insect host phenotypes

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Abstract

1. Most animals host communities of symbiotic bacteria. In insects, these symbionts may have particularly intimate interactions with their hosts: many are intracellular and can play important roles in host ecology and evolution, including protection against natural enemies.
2. We investigated how interactions between different species or strains of endosymbiotic bacteria within an aphid host influence the outcome of symbiosis for both symbiont and host.
3. We first asked whether different combinations of facultative symbiont species or strains can exist in stable co-infections. We then investigated whether the benefits that facultative bacteria confer on their hosts (protection against natural enemies) are enhanced, reduced or unaltered by the presence of a co-infecting symbiont. We asked this both for co-infecting symbionts that confer different phenotypes on their hosts (protection against fungal pathogens vs. parasitoid wasps) and symbionts with overlapping functions. Finally, we investigated the additional survival costs to aphids of carrying multiple infections of symbiont species or strains, and compared symbiont titres in double and single infections.
4. We found that stable co-infections were possible between all of the combinations of facultative symbiont species (*Regiella insecticola* + *Hamiltonella defensa*, *Regiella* + *Rickettsiella* sp., *Regiella* + *Spiroplasma* sp.) and strains (*Hamiltonella*) that we studied. Where symbionts provided protection against different natural enemies, no alteration in protection was observed in the presence of co-infections. Where symbionts provided protection against the same natural enemy, the level of protection corresponded to the higher of the two symbionts present. In some instances, aphid hosts suffered additional survival costs when hosting double infections. In the case of *Hamiltonella*, however, infection with multiple strains of the same symbiont led to lower symbiont titres than single infections, and actually improved aphid survival.
5. We conclude that the long-term maintenance of symbiont co-infections in aphids is likely to be determined primarily by costs of co-infections and in some instances by redundancy of symbiont benefits.

KEYWORDS
aphids, co-infection, host–parasite interactions, parasitoids, symbiont-mediated protection

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1 | INTRODUCTION

The majority of insects host symbiotic microbes (Duron & Hurst, 2013). These infections can be divided into obligate symbionts (without which the host cannot survive) and facultative symbionts (which are not required for successful growth or reproduction). While obligate symbionts perform vital roles such as supplementing otherwise limited diets (for example blood or plant sap) by synthesizing essential nutrients (Douglas, 2009), facultative symbionts are thought to be maintained either through provision of conditional benefits (Brownlie & Johnson, 2009) or by manipulation of host reproduction (Duron et al., 2008).

It has long been recognized that more than one symbiont species may persist within an individual host, sometimes in separate cells or organs, but sometimes alongside one another (Buchner, 1965; Goto, Anbutsu, & Fukatsu, 2006; Russell et al., 2013; Skaljac, Zanic, Ban, Kontsedalov, & Ghanim, 2010). Infections with multiple strains of the same symbiont species have also been identified (Hiroki, Tagami, Miura, & Kato, 2004; Kondo, Ijichi, Shimada, & Fukatsu, 2002; Mouton et al., 2004; Valette et al., 2013). In recent years, examples of intricate symbioses have been discovered where co-infecting endosymbionts play complementary roles in providing limiting nutrients for their host (Husnik et al., 2013; McCutcheon & Moran, 2007; Snyder, Deberry, Runyen-Janecky, & Rio, 2010). However, we still know little about how symbionts interact with one another within a host, and the consequences of symbiont co-infections for host phenotypes.

Facultative beneficial symbionts can be transferred between host lineages (horizontal transmission) allowing novel multiple symbiont combinations to arise. The new combinations may provide additive benefits to hosts, though it is also possible that there is negative or positive interference. Carriage of symbionts that provide conditional benefits to the host, for example after natural enemy attack, may also involve costs, for example in terms of reduced survival or fecundity (Oliver, Campos, Moran, & Hunter, 2008; Vorburger & Gouskov, 2011). Again, the costs of carrying multiple symbionts may combine additively, or there may be negative or positive interference. How benefits and costs interact is critical to understanding the processes shaping symbiont community structure.

Aphids, and in particular the pea aphid (*Acyrthosiphon pisum* Harris), provide an excellent system for studying facultative symbiont communities because in addition to a single obligate nutritional symbiont (*Buchnera aphidicola*) they possess a number of phenotypically well-characterized facultative bacterial symbionts (Oliver, Smith, & Russell, 2014), with co-infections occurring relatively frequently (Ferrari, West, Via, & Godfray, 2012; Henry et al., 2013; Russell et al., 2013). Observations of natural aphid populations suggest that certain combinations of symbiont species occur more or less often than would be expected by chance (Ferrari et al., 2012; Henry et al., 2013), providing circumstantial evidence that symbiont communities are not random. This suggests that aphid symbiont community composition depends either on interactions between symbionts, fitness effects of symbiont communities on their hosts, control of symbiont communities by the hosts, or some combination of the three.

A few studies have considered multiple facultative symbiont infections in aphids. (Oliver, Moran, & Hunter, 2006) created artificial co-infections of two symbionts previously shown to play a role in protecting aphids against parasitoid wasps: *Serratia symbiotica* and *Hamiltonella defensa*. Resistance to parasitism increased relative to single infections, but the aphids also suffered severe fecundity costs, which may explain why this co-infection is rare in the field. Co-infections between a strain of *Hamiltonella* and the bacterium currently known as X-type are more common than would be expected by chance (Henry et al., 2013), and this combination has been shown to provide better parasitoid protection following heat shock compared with either alone (Guay, Boudreault, Michaud, & Cloutier, 2009). Finally, Leclair et al. (2016) have found that the improved resistance to parasitoids provided by *Hamiltonella* is unaltered by co-infection with *Rickettsiella* (a commonly found combination), although the colour change (red-to-green) noted in aphids with *Rickettsiella* (which is thought to affect parasitoid and predator attack rates) is markedly reduced when *Hamiltonella* is also present (Leclair et al., 2016).

There is still much we do not know about interactions among co-infecting symbionts. In this study, we examined the consequences of endosymbiont co-infections for symbiont persistence and insect host phenotype. First, we asked whether different combinations of beneficial facultative symbiont species or strains could coexist in stable co-infections, and whether co-infections between symbionts with similar phenotypic effects were less likely to persist than co-infections between symbionts conferring different phenotypes. Secondly, we asked whether the benefits that facultative bacteria provide to their hosts are enhanced, reduced or unaltered by the presence of a co-infecting symbiont which provides a different benefit. Thirdly, we asked whether co-infections between symbionts providing the same beneficial function led to changes in host phenotypes. Finally, we asked whether there are additional survival costs to aphids carrying multiple infections of symbiont species or strains. By measuring symbiont titre we were also able to deduce whether co-infection affected individual or total symbiont populations. We are thus able to explore whether and how symbiont co-infections differ from single infections, and the consequences of symbiont community interactions for both symbionts and hosts.

2 | MATERIALS AND METHODS

2.1 | Aphid and symbiont manipulations

Under long daylight conditions, pea aphids reproduce asexually by apomictic parthenogenesis, allowing maintenance of clonal (genetically identical) lines in the laboratory. All clonal lines of pea aphids used in these experiments were derived from single individuals collected in the south of England, UK which were screened upon collection for the seven common secondary symbionts of pea aphids (Henry et al., 2013). Prior to experiments, aphids were maintained in 9 cm Petri dishes containing a single leaf of *Vicia faba* with the petiole inserted in 2% agar gel at 14°C with a 16:8 hr light:dark cycle. All experiments were carried out at 20°C.

Symbiont infections in aphids were manipulated using antibiotics to remove bacteria and haemolymph injection to create new associations. Antibiotic treatment followed McLean, van Asch, Ferrari, & Godfray (2011), modified in one case (Clone C207) as described in McLean & Godfray (2015). Novel secondary symbiont infections were introduced by removing *c*. 0.25 μl haemolymph from a naturally infected adult aphid ("donor") using a microcapillary needle and injecting the fluid into first instar uninfected aphids ("recipients"). When introducing two symbiont strains or species simultaneously, haemolymph was removed from the first donor and then immediately from the second donor using the same needle, and injected into the recipient with a single injury. The identity of the first donor was alternated between injections to prevent any bias arising from infection order. Injected aphids were maintained until they became adults, and their later offspring (>10th in birth order) were retained and tested for symbiont presence. Successful injections were identified using diagnostic PCR and aphid lines were kept for a minimum of seven generations before use in experiments (Koga, Tsuchida, & Fukatsu, 2003). Symbiont status of experimental aphid lines was confirmed immediately before and after all experiments using diagnostic PCR. Different symbiont species were distinguished using the same primers as in the initial screening (Henry et al., 2013). One experiment required us to differentiate reliably between two different strains of *Hamiltonella*. To do this, we designed strain-specific primers based on the murE gene (Strain 1; F: CTTTTGGGGCAATTGTCATTC & R: GATTCAGTACGGGGATTATCG. Strain 2; F: CTTTTGAGGCAATTGTCATCG & R: ATCAGGAGTATGA GCATAATCG). We conducted blinded preliminary trials to check that these primers did not produce false positives (i.e. no cross-amplification) and that amplification was reliable even when the target strain was at low concentration relative to the non-target strain.

2.2 | Fungal pathogen *Pandora neoaphidis*

Our protocol for fungal infections and stock maintenance is based on Scarborough, Ferrari, & Godfray (2005) and Parker, Garcia, & Gerardo (2014). We used a single genotype of the fungal pathogen *Pandora neoaphidis* (ARSEF 2588) which was obtained from the USDA Agriculture Research Service collection of entomopathogenic fungi (ARSEF). This strain had been collected from an infected pea aphid in Lansing, New York, and was preserved at −80°C. The isolate was thawed and grown on Sabouraud dextrose agar with egg yolk (SDAEY) as described in Papierok & Hajek (1997). We then transferred *c*. 1 cm^2 of fungal mycelium to 1% tap water agar for 12 hr, which induces sporulation, and exposed adult aphids (from Clone 145; a symbiont-free and *Pandora*-susceptible aphid line) to spores. After 4 days, visibly infected aphids were moved to Petri dishes at 4°C to dry. Sporulation of dried cadavers was induced by rehydration with 1% tap water agar for 12 hr, and fungal stock lines were maintained by infecting new aphids every 2–4 weeks for several months before use in experiments.

To obtain experimental fungal infections, aphids were exposed to sporulating stock cadavers. Eleven-day-old aphids (all of which had recently moulted to the final, adult instar) were placed at the bottom of infection chambers below sporulating cadavers. Cadavers were rotated among each treatment group such that each cadaver was present for the same time in each infection chamber to ensure equal spore doses across treatments. After infection, aphids were transferred to Petri dishes containing a *V. faba* leaf with the petiole inserted in 2% agar, the dishes then being sealed with parafilm wax to keep the humidity close to 100%. The aphids were transferred to new dishes, without parafilm, on the 3rd and 6th days of the infections. Survival and sporulation were scored every 24 hr for 8 days after infection, the treatment status of each dish being unknown to the recorder.

2.3 | Parasitoids

Inbred populations of the parasitoids *Aphelinus abdominalis* (Hymenoptera, Aphelinidae) and *Aphidius ervi* (Hymenoptera, Braconidae) were maintained on a susceptible pea aphid stock clone (Clone 145) at 20°C with a 16:8 hr light:dark cycle. Prior to use in experiments, wasps of both sexes were kept together in cages (and so were assumed to be mated) and were given access to aphids to ensure they had oviposition experience. Female wasps were kept singly without hosts for between 1 and 2 hr before being used in resistance assays.

The behaviour of the two species is very different and so requires different assay designs. For *A. ervi*, each replicate consisted of 15 third instar aphids placed on a Petri dish containing a single leaf of *V. faba* which were exposed to one female parasitoid for 3 hr. Previous observations had shown that this exposure time is sufficient to allow parasitism of a large proportion of the aphids present while limiting superparasitism (multiple eggs laid in a single host). During the first half-hour, parasitoids were observed to ensure that oviposition occurred, and if wasps were inactive they were replaced and the exposure time reset. The day after the experiment, aphids from a single replicate were split between three dishes with *V. faba* leaves and retained for 11 days post-parasitism, the aphids being transferred to fresh leaves every 3–4 days. After 11 days, the numbers of surviving and parasitized aphids were recorded. Successful parasitism was signalled by the presence of a parasitoid "mummy": the distinctive swollen dried husk formed by an *A. ervi* parasitoid on pupation. Parasitism frequency was calculated as the proportion of aphids producing mummies (omitting any that died for unknown reasons). For experiments involving two strains of *Hamiltonella*, "survival" was defined as the proportion of all the initial aphids that were alive on day 11.

For *A. abdominalis*, each replicate again consisted of 15 third instar aphids on a Petri dish with a single *V. faba* leaf. However, because *A. abdominalis* has a slower and more furtive oviposition strategy than *A. ervi*, an exposure period of 24 hr was used to ensure single parasitism of the majority of aphids present. Unlike *A. ervi*, females of *A. abdominalis* will feed on aphids which are then not used for oviposition and always die (Bai & Mackauer, 1990). Aphids exposed to *A. abdominalis* suffered 5%–20% mortality due to host feeding, a level that did not seem to vary amongst aphid lines. After parasitism, aphids from a single replicate were immediately divided amongst three dishes and maintained as above for 8 days, after which the number of aphids surviving and parasitized was recorded. Successful

A. abdominalis parasitism is indicated by a slender black mummy. Parasitism and survival were calculated as for *A. ervi*.

2.4 | Experiment 1: Co-infection between symbiont species that have different phenotypic effects (*Hamiltonella* and *Regiella*)

We first investigated whether the effectiveness of a protective symbiont was influenced by the presence of a second symbiont that provides protection against a different natural enemy. The symbionts we used were *Hamiltonella* (Strain 1), which confers enhanced protection against the wasp *A. ervi*, and *Regiella insecticola*, which provides protection against the fungal pathogen *P. neoaphidis*. Both symbionts were introduced singly and simultaneously to symbiont-free aphids from Clone 145 and the resistance of uninfected, singly infected and double-infected lines to both *A. ervi* and *P. neoaphidis* compared.

2.5 | Experiment 2: Co-infection with symbiont species with the same phenotypic effects

We then explored whether co-infections of two different symbiont species, both of which provide protection against the same natural enemy, influence resistance. We used three different symbiont species known to provide protection against *P. neoaphidis*: *Regiella*, *Spiroplasma* sp. and *Rickettsiella* sp. We created single infections of each symbiont in a common host genetic background (Clone 145), and generated two of the double infection combinations (*Regiella* + *Spiroplasma* and *Regiella* + *Rickettsiella)* through simultaneous injection as described above. We determined whether the double infections could be stably maintained for seven generations, and then assessed the susceptibility of the lines to the fungal pathogen *P. neoaphidis*.

2.6 | Experiment 3: Co-infection with multiple strains of the same symbiont species (*Hamiltonella*)

Many strains of *Hamiltonella* confer enhanced resistance to parasitoid wasps, though this varies against different wasp species (McLean & Godfray, 2015). Preliminary work showed that an isolate (Strain 1) provides strong protection against *A. ervi* but lesser protection against *A. abdominalis*, while another isolate (Strain 2) displayed the reverse pattern. We investigated whether the strong protection provided by each strain was influenced by the presence of the other isolate in a double infection. The two strains were introduced into four aphid clonal backgrounds: Clone 145 (the line used in Experiments 1 & 2), Clone C207 (which had been cured of an infection of a strain of *Hamiltonella* providing protection against the *A. ervi*, and an X-type infection), Clone C74 (which had been cured of an infection of *Hamiltonella* providing protection against *A. abdominalis*) and Clone C313 (which had been cured of an infection of a *Regiella* strain which did not provide protection against parasitoids). We recorded infection status of aphids at every generation post-injection for eight generations to check whether the two strains were capable of being maintained as a stable co-infection.

After eight generations of stable double co-infection, we assayed lines of aphids without secondary symbionts, with single infections of one *Hamiltonella* strain, and with a double infection of both strains, against the parasitoids *A. abdominalis* and *A. ervi*. As all four aphid clones were capable of maintaining a double strain infection (see Results), we limited the number of clonal backgrounds used for parasitism assays. For the experiments with *A. abdominalis* we used three of the recipient backgrounds: Clone C207, Clone C74 and Clone 145. Clone C207 has near 100% innate (i.e. in the absence of the symbiont) resistance to *A. ervi*, and hence we used only Clones C74 and 145 for *A. ervi* assays. Prompted by the results of the parasitism assay, we carried out a subsequent experiment using only Clone 145 to examine mortality of the different infected lines in the absence of parasitism. This survival assay followed a protocol analogous to the *A. ervi* parasitism assays: 15 third instar aphids were placed in groups together for 24 hr and the group then divided amongst three dishes; after 11 days, the number of aphids surviving was recorded.

2.7 | Statistical analysis of symbiont effects on aphid phenotypes

All statistical analysis was carried out using R v 3.0.2 (R Core Team 2016). For all fungal pathogen infections, we analysed the proportion of aphids that produced a sporulating cadaver using generalized linear models with a quasibiomial error structure. Models were compared using *F*-statistics. Multiple comparisons were conducted using the MULTCOMP package in R (Hothorn, Bretz, & Westfall, 2008) which implements Tukey's HSD. A survival analysis of control (unexposed) aphids was conducted using a Weibull model using the SURVIVAL package in R (Therneau & Grambsch, 2000). For parasitoid infections, we analysed two independent measures of the symbiont effect. First, we analysed the proportion of mummified aphids amongst living (parasitized and unparasitized) insects ("parasitism"). Secondly, we examined the proportion of aphids that survived as opposed to being parasitized or dying of unknown causes. The "survival" measure allows us to assess one effect of the symbiont(s) on host fitness over the experimental period. We again used generalized linear models with a quasibinomial error structure and carried out multiple comparisons as for fungal pathogen infections.

2.8 | *Hamiltonella* co-infection titres

We used real-time quantitative PCR (qPCR) to measure *Hamiltonella* densities in single and co-infected aphid lines. DNA was extracted from groups of three aphids from each line using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA), and was quantified using a nanodrop. We first used general *Hamiltonella* primers, which amplified the DNAK gene in both Strain 1 and Strain 2 (F: GGTTCAGAAAAAAGTGGCAG & R: CGAGCGAAAGAGGAGTGA), to measure overall *Hamiltonella* titres using the standard curve method (Laughton, Fan, & Gerardo, 2014). We then designed strain-specific primers for both *Hamiltonella* strains amplifying the murE gene (Strain 1; F: GGACTTTTGGGGCAATTGTCATTC & R: TTCCATGGCGGCAAAAGTGG. Strain 2; F: GGACTTTTGAGG

CAATTGTCATCG & R: GCCATGATAATCGAGGTGATCCC), and compared the relative ratio of each symbiont in single vs. co-infected aphids using the comparative threshold (Ct) method. qPCR work was carried out on an Applied Biosystems Step One Plus machine using standard conditions. Primer efficiencies were calculated to be 100 ± 5% for all primer pairs used.

To generate qPCR standards for the *Hamiltonella* standard curve, a target PCR fragment was cloned using Invitrogen TOPO TA cloning kit with pCR2.1 vector into One Shot TOP10 competent cells. Plasmids were extracted using the GE Healthcare illustra plasmidPrep Mini Spin Kit under recommended conditions. The insert was sequenced with the M13F primer to confirm that the cloning was successful. We measured target amplification using a standard dilution series at a 1:5 ratio starting with 3.2×10^6 copies. We used three technical replicates at each concentration to calculate the dilution curve for each plate, where the Ct value was averaged across technical replicates. The absolute quantity of *Hamiltonella* DNAK in each sample of 50 ng total DNA was calculated using linear regression. Each plate included the standard curve, single infections of Strains 1 and 2, and co-infected samples for a single aphid genotype. Three technical replicates were included for each DNA sample, five biological replicates (each of which represented aphids from a different infection lineage), and three aphid genotypes were measured for each symbiont background. We treated *Hamiltonella* titres as over-dispersed count data, and analysed these data with generalized linear models with a quasipoisson error structure. Host genotype, infection type (single vs. double), and symbiont genotype (which was nested within infection type), were treated as fixed effects. Models were compared using *F*-statistics.

To measure strain-specific symbiont titres (using the same DNA samples as above), we measured the comparative threshold (C_t) values of each strain in single vs co-infection. We used the *A. pisum* ef1α gene as an endogenous control (Wilson et al., 2006). ΔC_t values were calculated by subtracting endogenous control C_t values from the target C_t values. The relative ratio of symbiont titres in co- vs. single infections was calculated using the $\Delta\Delta C_t$ value, with relative ratio = $2^{\Delta\Delta C_t}$. As above, three technical replicates were conducted for each DNA sample, and five biological replicates across three aphid genotypes were measured for each symbiont background. We analysed the ΔC_t for each symbiont in each host genotype to compare titre in single vs co-infections across biological replicates. We log transformed ΔC_t values for each symbiont strain, and analysed single vs. co-infection ΔC_t values using two-factor ANOVAs with infection type (single vs. co-infection) and host genotype as main effects.

3 | RESULTS

3.1 | Experiment 1: Co-infection between *Hamiltonella* and *Regiella*

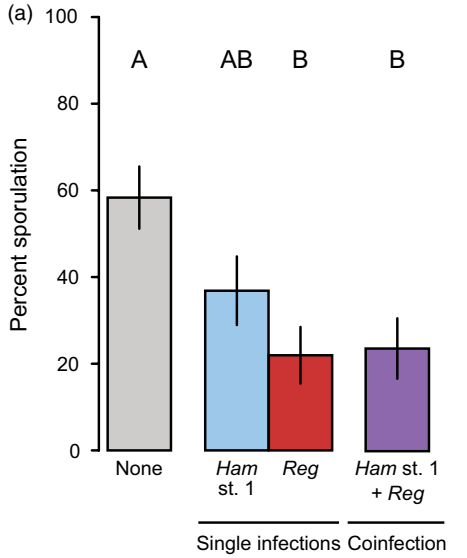
First, we tested whether a co-infection between two symbionts conferring different defensive phenotypes affected the protection each provided to aphids. As expected, symbiont background influenced the percent of aphids infected with the fungal pathogen *Pandora* and so produced a sporulating cadaver ($F = 18.2$, $df = 3$, $p < .001$). *Regiella* conferred significant protection against *Pandora* (Figure 1a) compared with symbiont-free aphids. There was no evidence that the protection conferred by *Regiella* was either improved or reduced in the presence of co-infecting *Hamiltonella* (Figure 1a). Aphids with a single infection of *Hamiltonella* (Strain 1) showed fungal resistance that was intermediate between uninfected and *Regiella*-carrying aphids (and not statistically significantly different from either in post hoc tests, Figure 1a). The survival of control aphids (i.e. those not exposed to fungal pathogens) was not significantly influenced by their symbiont strain ($\chi^2 = 2.43$, $df = 3$, $p = .49$; Figure 1b).

Similarly, we found no evidence that the protective phenotype associated with *Hamiltonella* was altered by co-infection with *Regiella*. Symbiont background significantly influenced parasitism rates by *A. ervi* ($F_{3,26} = 12.4$, $p < .001$). *Hamiltonella* Strain 1 conferred protection against wasps, but this benefit was unaffected by the presence of *Regiella* (Figure 2a). As expected, carrying *Regiella* alone did not significantly alter parasitism rates relative to symbiont-free aphids (Figure 2a). Although symbionts had a significant effect on wasp success rates, the proportion of aphids alive at the end of the experiment was not affected by symbiont status ($F_{3,27} = 0.837$, $p = .486$; Figure 2b). This is due to additional mortality that cannot be directly attributed to the parasitoid, either because symbionts prevented the successful development of parasitoids but did not prevent fatal damage to aphids, or because symbionts independently imposed a survival cost.

3.2 | Experiment 2: Co-infection with symbiont species protective against fungal pathogens

We successfully established co-infections of *Regiella* with *Spiroplasma* or *Ricketsiella*, with all of the double-injected aphids ($n = 10$ attempts) accepting and maintaining the co-infection for seven generations before use in the experiments. As expected, symbionts influenced aphid resistance to the fungal pathogen *Pandora* ($F = 87.7$, $df = 5$, $p < .0001$). All three symbiont species in single infections protected aphids against *Pandora*, with *Rickettsiella* providing significantly stronger protection than the other two (Figure 3a). In co-infections, we found that aphids experienced a level of protection as good as the more protective symbiont (Figure 3a). *Rickettsiella* confers almost total resistance that was not affected by the presence of *Regiella* while the resistance of single or double infections of *Spiroplasma* and *Regiella* were all approximately equal.

We also tested whether aphids carrying co-infections had lower survival relative to aphids with no symbionts or with single infections. As in a previous study (Parker, Spragg, Altincicek, & Gerardo, 2013), we found that control aphids (not exposed to *Pandora*) experienced a survival cost to harbouring *Regiella* (Figure 3b). This is in contrast with our findings in Experiment 1 where we did not find an effect of *Regiella* on control survival (see Discussion). We observed no effects on survival of carrying either *Spiroplasma* or *Rickettsiella* in single infections (Figure 3b). There were no additional costs to the aphid of carrying *Rickettsiella* in addition to *Regiella*. However, a double infection of



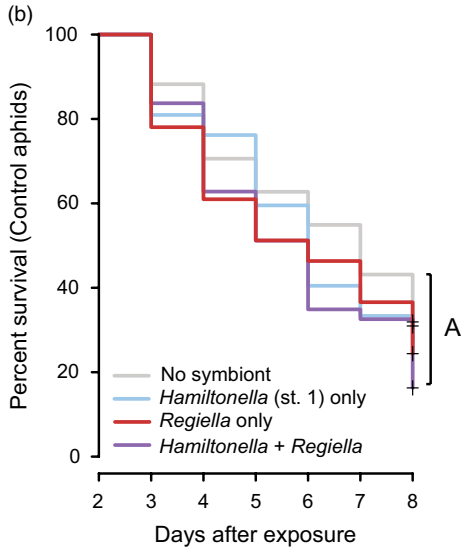
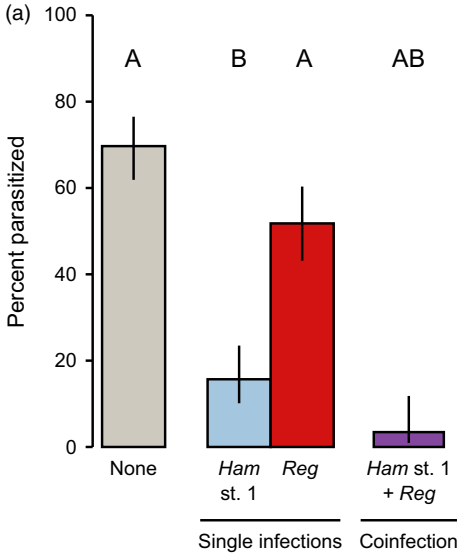


FIGURE 1 *Pandora* fungal infection of co-infections between symbionts with different functions: *Regiella* ("*Reg*") and *Hamiltonella* strain 1 ("*Ham* st. 1"). (a) Proportion of aphids killed by *Pandora*. The *y*-axis shows the percentage of aphids that form a sporulating cadaver. Symbiont background is shown along the *x*-axis. Bars show standard error; letters indicate significance groups based on post-hoc tests. (b) Survival analysis of control (uninfected) aphids. The *y*-axis indicates percent of aphids from each symbiont background alive at each day of the experiment (shown along the *x*-axis). Significance groups are shown to the right of the figure



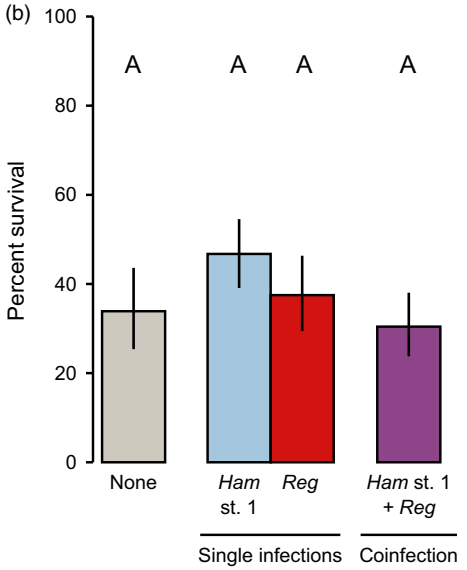


FIGURE 2 Parasitoid wasp (*Aphidius ervi*) infection of co-infections between symbionts with different functions: *Regiella* ("*Reg*") and *Hamiltonella* strain 1 ("*Ham* st. 1"). Symbiont background is along the *x*-axis. Bars show standard error; letters indicate significance groups. (a) Proportion of aphids successfully parasitized by *A. ervi*. The *y*-axis shows the percentage of aphids that formed a parasitoid "mummy" (dead aphids were excluded from analysis). (b) Proportion of all aphids surviving after exposure to *A. ervi*

Regiella with *Spiroplasma* did lead to lower survival than *Regiella* alone (Figure 3b).

3.3 | Experiment 3: Co-infection with different protective strains of *Hamiltonella*

We first established that a stable infection of two *Hamiltonella* strains could be maintained in four different aphid clonal backgrounds. Aphids from the first generation after double injection ("Generation 1") were tested for the presence of both strains using diagnostic PCR; 76% (37/49) of aphids tested showed a successful transfer of both strains. Of the 37 successful double infections, two aphid lines died by Generation 3, and of the remaining 35 lines, 71% (25/35) had retained both infections to Generation 3. In all cases of loss it was Strain 2 that failed to establish (the numbers of losses were too small to examine differences between aphid clonal backgrounds). All lines which tested positive for both symbiont strains at Generation 3 retained their double infection until Generation 8 (when experiments were carried out on a randomly chosen set of lines). Later tests on the experimental lines found that infections were still retained at Generation 12.

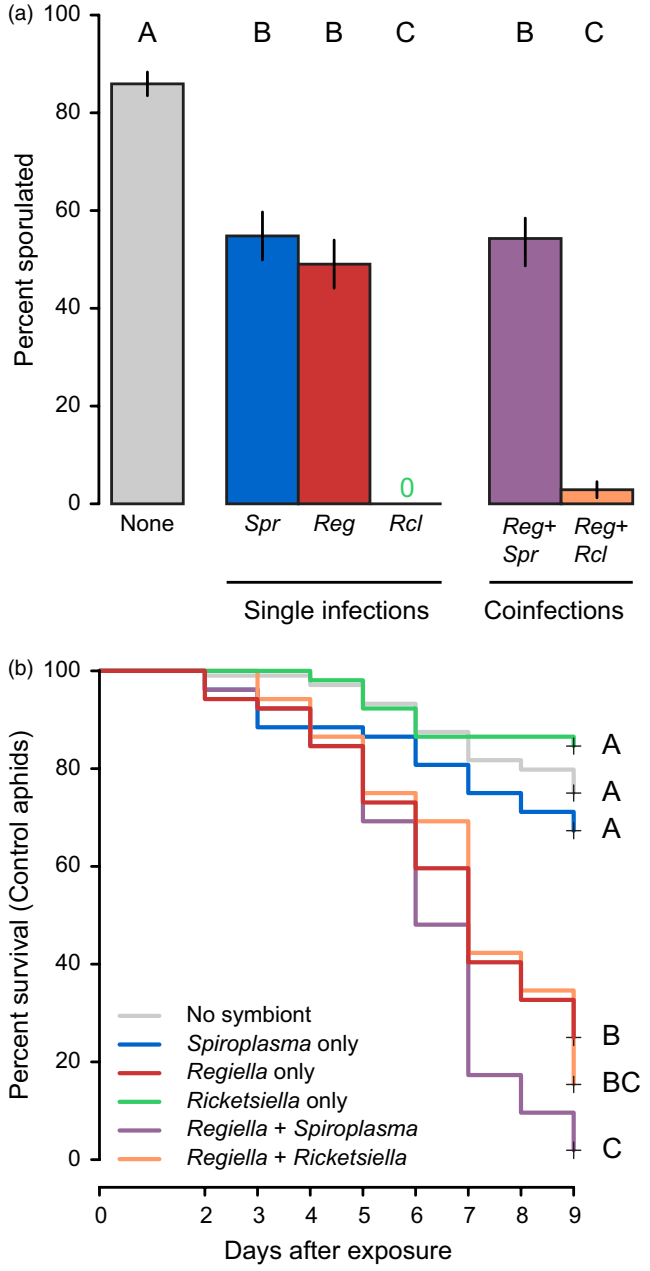


FIGURE 3 Co-infections between symbionts with related functions. (a) Results of a *Pandora* infection on co-infected lines. The *y*-axis shows the percentage of aphids that formed a sporulating cadaver. Symbiont species is indicated along the bottom of the figure: *Spiroplasma* ("*Spr*"), *Regiella* ("*Reg*"), *Ricketsiella* ("*Rcl*"). Error bars show standard error; significance groups are indicated along the top of the figure. (b) Shows survival of control aphids (not infected with *Pandora*) at each day of the experiment (show along the *x*-axis). Colours correspond to the symbiont backgrounds in Figure 3A. Significance groups are show to the right of the figure

As expected, we found that the presence of *Hamiltonella* had a significant effect on parasitism by *A. abdominalis* ($F_{3,97}$ = 206, p < .001). Both *Hamiltonella* strains significantly reduced parasitism rates in single infections compared to uninfected aphids (Figure 4a). Strain 2 provided stronger, almost complete, protection which was not compromised by the presence of Strain 1 (Figure 4a). The recipient aphid background had no effect on the phenotypes observed ($F_{2,100}$ = 0.385, p = .682).

Strain 1 alone did not improve overall aphid survival relative to uninfected aphids when exposed to *A. abdominalis* in spite of the reduction in parasitism rates (z = 1.04, p = .726; Figure 5a). This is because non-parasitism related mortality was comparatively high for aphids carrying Strain 1. However, aphids with Strain 2, both singly and in co-infection, had markedly greater survival compared to uninfected aphids (Strain 2 vs. none: z = −7.28, p < .001; Co-infection vs. none: z = −7.08, p < .001) and those with Strain 1 alone (Strain 2 vs. Strain 1: z = 7.43, p < .001; Co-infection vs. Strain 1: z = 7.24, p < .001; Figure 5a).

When we examined the effect of *Hamiltonella* on parasitism by *A. ervi* we found that the presence of Strain 1, both singly and in co-infection, markedly decreased parasitism relative to uninfected aphids ($F_{3,42}$ = 27.2, p < .001; Figure 4b). Strain 2 did not overall improve resistance to this parasitoid ($F_{1,22}$ = 3.72, p = .07) nor was there a main effect of aphid recipient background (genotype) ($F_{1,45}$ = 0.160, p = .69). However, there was a significant interaction between symbiont presence and aphid genotype ($F_{3,39}$ = 3.43, p = .026) suggesting that Strain 2 might confer benefits in some aphid genotypic backgrounds (Figure 4b). The overall survival of aphids exposed to *A. ervi* was not affected by the symbionts they carried, despite the positive effects on parasitism rates shown by Strain 1 ($F_{3,42}$ = 1.83, p = .159; Figure 5b).

Prompted by the unexpected overall survival result, we conducted a further experiment examining survival in the absence of parasitoids for aphid Clone 145. We found that carriage of Strain 1 resulted in lower aphid survival ($F_{3,56}$ = 4.92, p = .004; Strain 1 vs. none: z = −2.88, p = .020). However, there were no significant survival costs for the aphid in carrying Strain 2 or, surprisingly, both *Hamiltonella* strains (z = −0.563, p = .941; Figure 5c).

3.4 | Quantitative PCR on *Hamiltonella* co-infection titres

Unexpectedly, co-infected aphids had significantly lower overall *Hamiltonella* titres than singly infected aphids ($F_{1,40}$ = 16.6, p < .001) (Figure 6a). Host genotypes also differed in overall *Hamiltonella* titres ($F_{2,41}$ = 38.5, p < .001). Symbiont genotype, and the interaction between host genotype and symbiont infection type, and between host and symbiont genotypes, did not significantly influence overall titre.

The ratio of symbiont titres in co-infections vs. single infections for individual *Hamiltonella* strains (measured using strain-specific qPCR primers) was less than 1 for both symbiont strains (Strain 1: F = 25.7, df = 1, p < .0001; Strain 2: F = 47.1, df = 1, p < .0001; Figure 6b). Symbiont titres of each strain differed across host genotypes (Strain 1: F = 4.51, df = 2, p = .02; Strain 2: F = 11.2, df = 2, p < .001), but the interaction between infection type and host genotype was not significant for either strain. The Ct values of the endogenous control gene ef1α were similar in single vs. co-infected samples (20.15 vs. 19.90, respectively). The qPCR method we used did not allow us to determine whether the drop in titres in co-infected symbionts was more severe in one strain than the other.

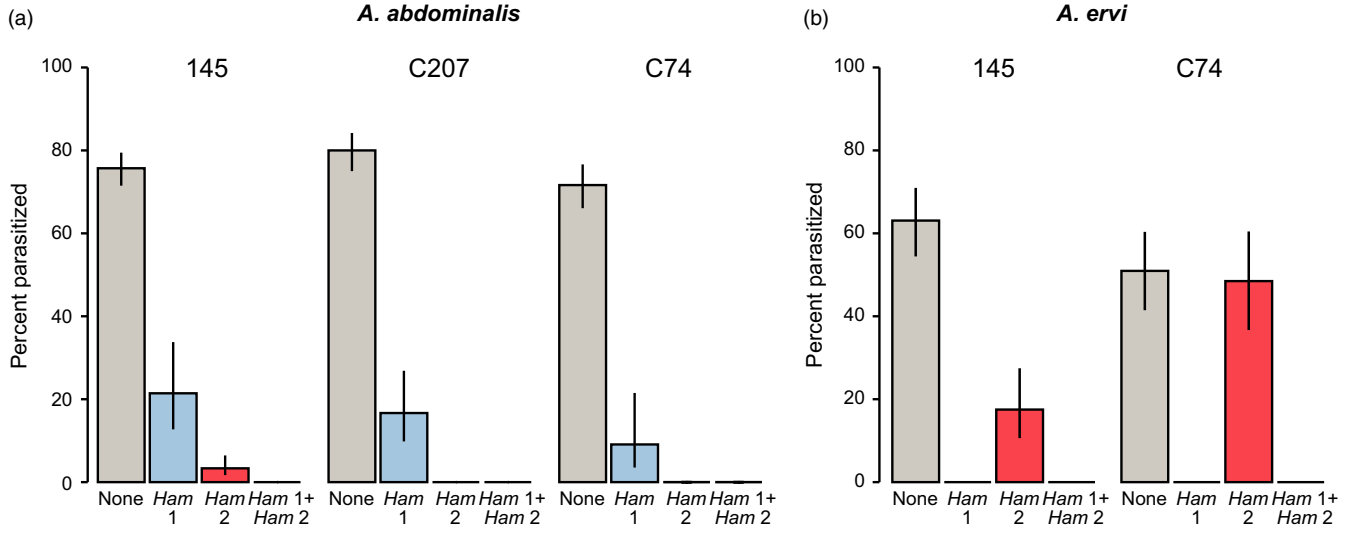


FIGURE 4 Parasitoid wasp infections of aphids carrying co-infections between strains of *Hamiltonella*. Symbiont infection status is shown along the *x*-axes; recipient aphid genotypes are shown along the top of the figures. The *y*-axes show the percentage of aphids that formed a parasitoid "mummy". Bars show standard error. (a) Proportion of aphids successfully parasitized by *Aphelinus abdominalis*. (b) Proportion of aphids successfully parasitized by *Aphidius ervi*

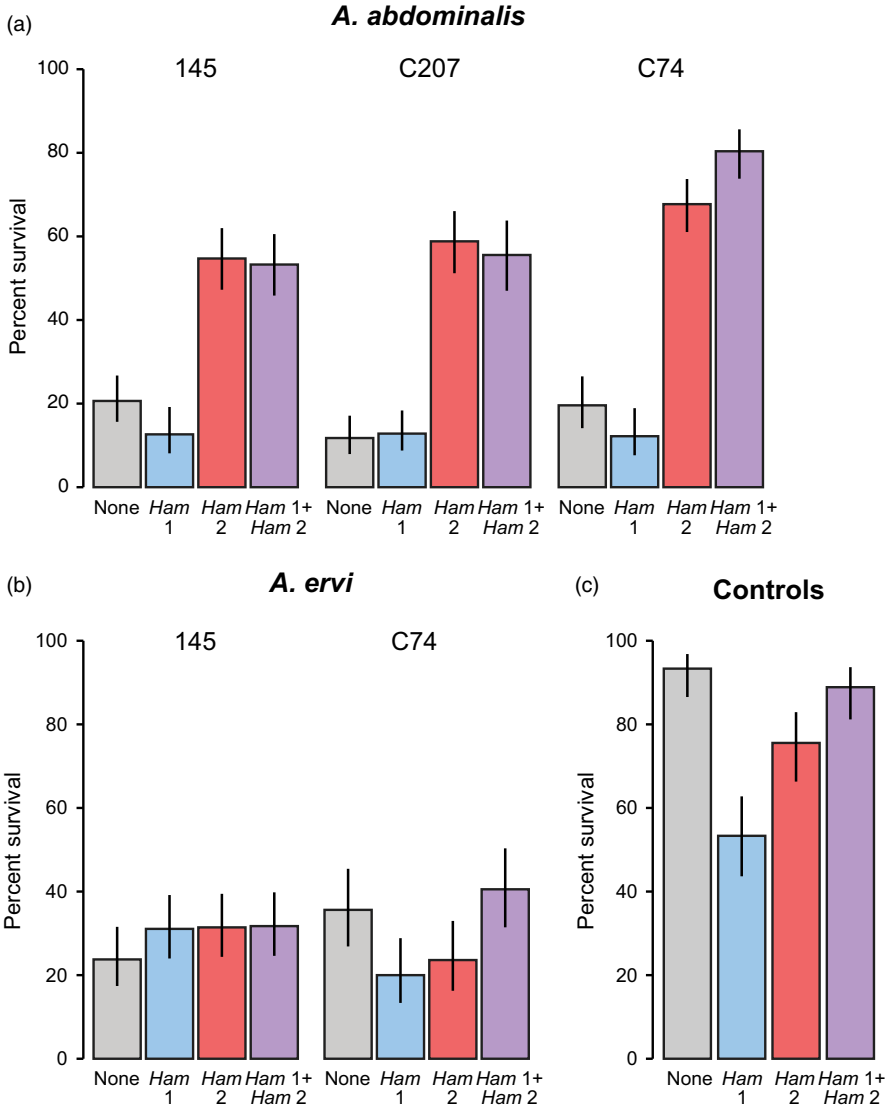


FIGURE 5 Survival of aphids carrying co-infections between strains of *Hamiltonella*. The *y*-axes show proportion of aphids surviving. Symbiont background is shown along the *x*-axis. Bars show standard error. (a) Survival of aphids parasitized by *Aphelinus abdominalis* (day 8). (b) Survival of aphids parasitized by *Aphidius ervi* (day 11). (c) Survival of non-parasitized aphids, measured in a separate experiment, after 11 days

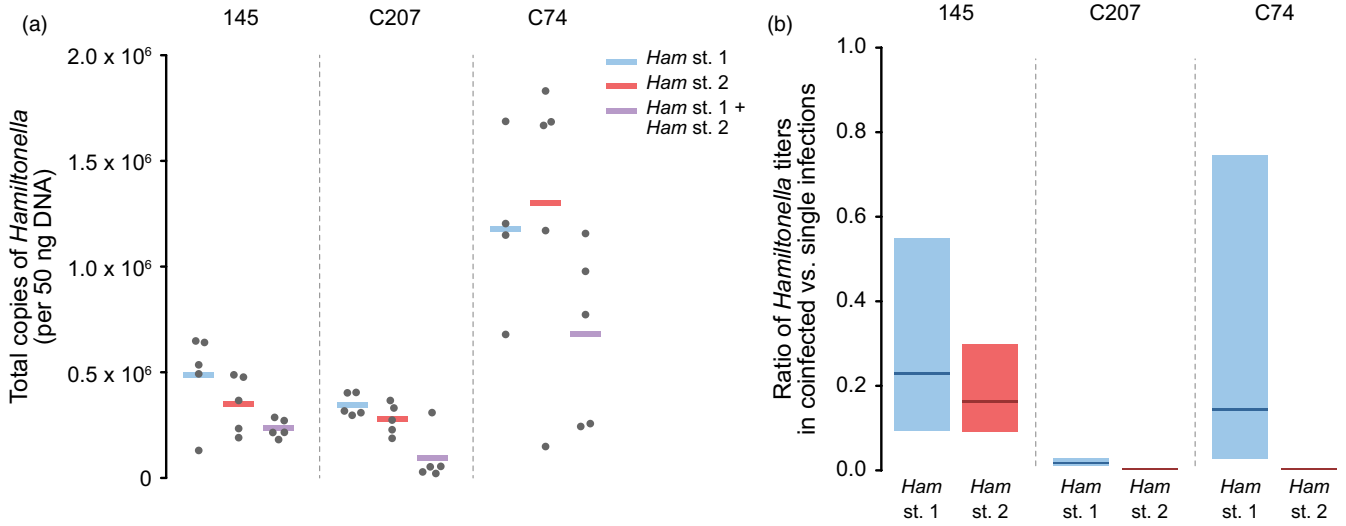


FIGURE 6 Symbiont titre of *Hamiltonella* co-infections measured using quantitative PCR. (a) Total number of copies of *Hamiltonella* DNAK in 50 ng DNA, measured using the standard curve method. Each biological replicate is shown in grey; means are shown by the coloured bars. Symbiont background (single infections of Strain 1 or Strain 2, or a co-infection of both strains) is shown by the three different colours as indicated. Aphid genotypes are indicated at the top of the figure. (b) Ratio of *Hamiltonella* in co-infections vs. in single infections, measured using strain-specific primers and the relative Ct method. In all cases, strains of *Hamiltonella* were found at significantly lower concentrations in co-infected vs. single hosts. Symbiont strain is shown along the bottom of the figure. Bars show mean ± 1 *SD*. Aphid genotype is indicated at the top of the figure

4 | DISCUSSION

Our aim in this study was to test whether interactions between symbionts carried by the same host could affect both symbiont persistence and host phenotype. To do this, we created artificial co-infections of different species and strains of aphid facultative symbionts. All the combinations of symbionts we explored were capable of forming stable, transmissible co-infections. This suggests that antagonistic competitive intra-host interactions between symbionts are not sufficient to explain symbiont distributions in nature.

We asked whether protective phenotypes are altered in the presence of a co-infecting symbiont species active against a different natural enemy (Figures 1a and 2a). *Hamiltonella* and *Regiella* are found in high frequencies in some of the same aphid host–plant adapted populations, suggesting that opportunities for co-infections are likely to arise. However, co-infections of these two symbionts are found in nature less often than would be expected by chance (Ferrari et al., 2012; Henry et al., 2013). We found that the protection conferred by single infections of *Regiella* and *Hamiltonella* against fungus and wasps, respectively, was equally strong in co-infections, and thus that mutual interference does not explain patterns of *Hamiltonella* and *Regiella* distribution in natural populations.

Where a co-infecting symbiont species provided the same protective function (resistance to fungus), host aphids experienced protection corresponding to the level of the most protective single infection (Figure 3a). This is in contrast with previous findings for parasitoids where Oliver et al. (2006) showed that aphids with a co-infection of *Hamiltonella* and *Serratia symbiotica* were better able to resist wasps than singly infected individuals. The resistance to parasitoids conferred by *Hamiltonella* is associated with a bacteriophage carried by the symbiont that encodes eukaryote toxins (Oliver, Degnan, Hunter, & Moran, 2009). How *S. symbiotica* influences aphid resistance is not known but does not seem to involve bacteriophages, and thus likely involves a different mechanism than *Hamiltonella*. Little is yet known about how symbionts affect resistance to fungi, and it will be interesting to explore if additive interactions are more common in symbionts that affect natural enemy success using different as opposed to the same physiological mechanisms.

We found no significant survival costs associated with some symbiont species co-infections (*Regiella* with *Hamiltonella* [Figure 1b] and *Regiella* with *Ricketsiella* [Figure 3b]), but observed that a co-infection between *Regiella* and *Spiroplasma* led to reduced survival (Figure 3b). Similarly, Oliver et al. (2006) found that aphids harbouring both *Hamiltonella* and *S. symbiotica* experienced lower fecundities, longer generation times, and lower adult weights than aphids infected singly. They attribute these costs to increased densities of *S. symbiotica* in co-infections. Together, these results suggest that the costs to aphids of co-infections may be species-specific, or dependent on other factors such as host genotype. We note that there may be other fitness effects of co-infections (e.g. on competitive ability or lifetime fecundity) that we do not measure here. We note that in Experiment 2 (Figure 3b), and in other work (Parker et al., 2013), single infections of *Regiella* led to lower survival, but we did not find this effect in Experiment 1 (Figure 1b). Survival of uninfected (control) aphids without *Regiella* was low in Experiment 1 for unknown reasons, and we suspect that this experimental artefact has masked the effects of *Regiella* on aphid survival.

Aphids were able to maintain stable co-infections of two different strains of the symbiont *Hamiltonella* over at least 12 generations, and

the individual protective phenotypes of both strains were maintained in co-infected hosts. In about 30% of cases one symbiont strain was lost after initial establishment, something we never saw in infections with multiple symbiont species. Contrary with our expectations, we found that *Hamiltonella* titres were lower in co-infections than in single infections. This result was surprising because coinfection between genotypes of horizontally transmitted parasites has been shown to produce similar (Hughes et al., 2004) or higher (Choisy & de Roode, 2010; Davies, Fairbrother, & Webster, 2002) parasite densities. Further empirical and theoretical work on the relationship between density, transmission and virulence specifically of predominantly vertically transmitted infections is needed to explain these results.

Using strain-specific primers, we then showed that both *Hamiltonella* strains had lower titres when in co-infection, though this did not impair the benefits they provided to the aphid. We do not yet know enough about the population dynamics of single and multiple bacterial strains within the host body to explain these results. However, our findings suggest that the strength of the protective phenotype conferred by *Hamiltonella* is not directly proportional to titre, which is in contrast with results from other systems (Martinez et al., 2014). Interestingly, we found no evidence that infection with multiple strains increased survival costs in uninfected controls, and in fact we found that aphids carrying both *Hamiltonella* strains had higher survival than those infected only with *Hamiltonella* Strain 1. One possibility is that higher survival is due to the reduced symbiont density we found in *Hamiltonella* co-infections.

The ability of aphids to maintain multiple strains of a single facultative symbiont species raises a number of questions. Are such infections found in nature but not detected by the diagnostic screening techniques which most aphid researchers routinely employ? Multiple strains of *Wolbachia* occur in many species of insects and other arthropods (Hiroki et al., 2004; Kondo et al., 2002; Mouton et al., 2004) though they spread and maintain themselves through a very different mechanism. Alternatively, do the opportunities for multiple infections arise rarely in the field? We injected both strains simultaneously in an attempt to maximize the opportunities for double establishment. In nature, double infections are more likely to arise sequentially and it may be much harder for a strain to invade a host already containing an established infection. Aphids with co-infecting strains may also be subject to costs in the field which we have failed to identify in the laboratory, such as increased susceptibility to other natural enemies, or reduced competitive ability (Oliver et al., 2008).

Our results suggest that the protection conferred to aphids against natural enemies by facultative symbionts is not influenced by symbiont co-infections. Natural aphid populations are typically under simultaneous pressure from multiple natural enemies (Hrček, McLean, & Godfray, 2016; McLean, Parker, Hrček, Henry, & Godfray, 2016) and symbiont co-infections can therefore allow effective protection in such environments. We have found some but not consistent evidence that co-infections can affect the costs of carrying symbionts, and in one case co-infection actually increased host survival. Our data also suggest that symbionts interact in unexpected ways within the host, for example leading to lower net bacterial densities. The seven relatively common facultative endosymbionts of pea aphid can be considered as constituting a type of "microbiome": an interacting assemblage of micro-organisms that influence their hosts' fitness in different ways (McFall-Ngai et al. 2013). Further research on how component species interact may help us to understand the processes structuring this very simple microbiome, providing insights into aphid biology and possibly the analogous processes occurring in much more complex microbiomes.

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AUTHORS' CONTRIBUTIONS

A.H.C.M., B.J.P., J.H. and H.C.J.G. conceived the ideas and designed methodology; A.H.C.M., B.J.P., J.H., J.C.K. and P.A.D.W. collected the data; A.H.C.M. and B.J.P. analysed the data and wrote the first draft of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.20d67 (McLean et al., 2017).

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