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Evolutionary costs and benefits of infection with diverse strains of *Spiroplasma* in pea aphids

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Author Contributions

HK, PG, CV and HMH performed the experiments; PG, HMH and HK carried out the molecular analysis; HMH was responsible for the data analysis; HMH, HK and CV wrote the paper.

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Data Accessibility

The DNA sequences used in this study are available in Genbank (accession numbers: MG288511 to MG288588). The main dataset is deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.q0r84n4.

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Abstract

The heritable endosymbiont *Spiroplasma* infects many insects and has repeatedly evolved the ability to protect its hosts against different parasites. Defenses do not come for free to the host, and theory predicts that more costly symbionts need to provide stronger benefits to persist in host populations. We investigated the costs and benefits of *Spiroplasma* infections in pea aphids (*Acyrthosiphon pisum*), testing 12 bacterial strains from three different clades. Virtually all strains decreased aphid lifespan and reproduction, but only two had a (weak) protective effect against the parasitoid *Aphidius ervi*, an important natural enemy of pea aphids. *Spiroplasma* induced fitness costs were variable, with strains from the most slowly evolving clade reaching higher titers and curtailing aphid lifespan more strongly than other strains. Some *Spiroplasma* strains shared their host with a second endosymbiont, *Regiella insecticola*. Although the result of an unfortunate handling error, these co-infections proved instructive, because they showed that the cost of infection with *Spiroplasma* may be attenuated in the presence of *Regiella*. These results suggest that mechanisms other than protection against *A. ervi* maintain pea aphid infections with diverse strains of *Spiroplasma*, and that studying them in isolation will not provide a complete picture of their effects on host fitness.

Keywords

Acyrthosiphon pisum, cost of infection, defensive symbiosis, facultative secondary symbionts, lifespan, parasitoid

Introduction

Microbial endosymbionts of eukaryotes are ubiquitous, and have often become heritable through the evolution of mother-to-offspring transmission. Large-scale screens for symbionts like *Wolbachia* or *Cardinium* suggest that the majority of arthropod species are likely to carry heritable infections with endosymbionts (Zchori-Fein and Perlman 2004; Hilgenboecker et al. 2008). Microbial symbionts may provide their hosts with essential nutrients, especially in species with very imbalanced diets such as blood feeders like the tsetse fly (Chen et al. 1999) or phloem feeders like aphids (Douglas 1998). Some of these symbioses are ancient and have evolved to the point that the host is unable to survive without its bacterial partner, which is referred to as an obligate symbiont (Wernegreen 2002; Moran et al. 2008). Other endosymbionts are facultative associates for the host and not strictly required for host survival. These are referred to as secondary symbionts. A single arthropod species can host multiple species of secondary symbionts, but each symbiont typically infects only a part of the host population (e.g. Chiel *et al.* 2007; Ferrari *et al.* 2012). Additional variation may be present within symbionts. A secondary symbiont species infecting a particular host species often comprises multiple distinguishable strains (Raychoudhury et al. 2009; Ferrari et al. 2012; Russell et al. 2013). Explaining the evolutionary persistence and the high diversity of secondary symbionts in host populations requires an understanding of how different symbionts counterbalance the costs they impose on their host (Heath and Stinchcombe 2014).

One way for maternally transmitted symbionts to spread in a host population is to manipulate the host's reproduction in a way that favors symbiont transmission. Reproductive manipulation has evolved repeatedly in endosymbiotic bacteria like *Wolbachia*, *Arsenophonus*, *Cardinium*, *Rickettsia* or *Spiroplasma* (Duron et al. 2008). It can act via the induction of cytoplasmic

45 incompatibility, male-killing, parthenogenesis, or the feminization of genetically male offspring
46 (Werren et al. 2008).

47 In addition to reproductive manipulation, heritable symbionts can spread if they provide their
48 host with an evolutionary benefit. This strategy is not mutually exclusive with reproductive
49 manipulation. An important class of evolutionary benefits that has evolved repeatedly is protection
50 against natural enemies, i.e. defensive symbiosis (Oliver and Moran 2009; McLean 2019). Multiple
51 species of secondary symbionts increase the resistance of aphids against parasitoid wasps and
52 pathogenic fungi (Oliver et al. 2003; Scarborough et al. 2005; Vorburger et al. 2010; Łukasik et al.
53 2013), certain strains of *Spiroplasma* can protect flies against parasitoid wasps or parasitic
54 nematodes (Jaenike et al. 2010; Xie et al. 2010; Paredes et al. 2016), and *Wolbachia* can reduce
55 viral infection in flies and other insects (Hedges et al. 2008; Teixeira et al. 2008; Bian et al. 2010).
56 So why do these seemingly beneficial symbionts not go to fixation in host populations?

57 Most general explanations assume trade-offs between the benefits provided by the symbiont and
58 the costs associated with its possession, acting in combination with environmental heterogeneity.
59 For example, the secondary symbiont *Hamiltonella defensa* (Moran and Russell 2005) can protect
60 different aphid species against parasitism (Oliver et al. 2003; Schmid et al. 2012; Asplen et al.
61 2014), but *H. defensa* is selected against in the absence of parasitoids (Oliver et al. 2008), possibly
62 because of the reductions in host lifespan and lifetime reproduction or in nymphal growth it induces
63 (Vorburger and Gouskov 2011; Leybourne et al. 2018). Temporal and spatial variation in the risk
64 of parasitism may thus maintain coexistence between infected and uninfected hosts. Similarly,
65 species and strain diversity may partly be explained by unequal effects against different natural
66 enemies. For *H. defensa*, several studies have shown that protection of aphids against parasitoid
67 wasps can be highly specific (reviewed in Vorburger 2014). A given strain of *H. defensa* can
68 provide effective protection against some parasitoid species but not against others (Asplen et al.

2014; Cayetano and Vorburger 2014; McLean and Godfray 2015, 2017; Martinez et al. 2016), and this specificity can even extend to interactions within species. In black bean aphids (*Aphis fabae*), particular isolates of *H. defensa* protect strongly against some parasitoid genotypes but not or only weakly against other parasitoid genotypes, leading to strong genotype-by-genotype interactions between parasitoids and the hosts' defensive symbionts (Schmid et al. 2012; Cayetano and Vorburger 2013; Vorburger and Rouchet 2016). Similar genotype-specificity is observed in the interaction between the fungal pathogen *Pandora neoaphidis* and the secondary symbiont *Regiella insecticola*, which protects pea aphids (*Acyrthosiphon pisum*) against fungal infection (Parker et al. 2017). Variation in the local parasitoid and pathogen community may thus select for different secondary symbionts, and genotype-by-genotype specificity may further maintain strain variation via negative frequency-dependent selection (Kwiatkowski et al. 2012; Heath and Stinchcombe 2014).

A promising system to investigate the evolutionary maintenance of symbiont strain diversity are bacteria of the genus *Spiroplasma*. These helical, cell wall-less bacteria belong to the class Mollicutes within the phylum Firmicutes (Gasparich et al. 2004). *Spiroplasma* bacteria are generally associated with arthropods, but they differ widely in their modes of transmission and their phenotypic effects on the hosts. Some are virulent, horizontally transmitted pathogens of insects and crustaceans that cause problems in apiculture and aquaculture (Clark et al. 1985; Wang et al. 2005), some are damaging plant pathogens that are vectored by phloem-feeding insects (Bové et al. 2003), and many are vertically transmitted endosymbionts (Williamson et al. 1998; Watts et al. 2009). It is estimated that between 5% and 10% of insects carry heritable infections with *Spiroplasma* (Duron et al. 2008). Similar to other heritable endosymbionts, some *Spiroplasma* have evolved the ability to defend their hosts against other infections (Ballinger and Perlman 2018). For example, the male killing strain MSRO of *S. poulsonii* protects *Drosophila melanogaster* against

parasitoid wasps (Xie et al. 2014; Paredes et al. 2016), illustrating that reproductive manipulation and protection are not mutually exclusive strategies of symbionts to spread in host populations. In the fungus-feeding *D. neotestacea*, infection with *Spiroplasma* induces tolerance to the parasitic nematode *Howardula aoronymphium* (Jaenike et al. 2010). In pea aphids, *Spiroplasma* has been shown to protect against fungal infections (Łukasik et al. 2013a) , and there is evidence for male-killing by at least one strain (Simon et al. 2011).

Spiroplasma infecting European pea aphids are subdivided into at least three clades that are similarly abundant in aphids feeding on different host plants, but share their hosts with different symbiont communities and have a different rate of molecular evolution, suggesting their maintenance in pea aphids might rely on different eco-evolutionary strategies (Mathé-Hubert et al. 2018). Here we provide insights in the ecology and evolution of these three clades. We investigate if protection against the pea aphid's main parasitoid *Aphidius ervi* might contribute to the evolutionary persistence of *Spiroplasma* in this species. Twelve *Spiroplasma* strains, evenly spread across the three clades, were tested for their ability to protect against three different lines of the parasitoid *A. ervi*, and we estimated *Spiroplasma* density in 10- and 20-day-old aphids, as well as *Spiroplasma*'s effects on aphid fitness in the absence of parasitoids. Although two out of the twelve *Spiroplasma* strains reduced aphid parasitism by at least one of three parasitoid lines, there was no global effect of *Spiroplasma* on the parasitism success. All *Spiroplasma* strains curtailed aphid lifespan and lifetime reproduction to various extents and the benefit provided by the two protective *Spiroplasma* strains is unlikely to counter-balance their cost. This suggests that *Spiroplasma* infection in pea aphids is maintained by another mechanism than the protection against *A. ervi*. An analysis of phylogenetic signal in the phenotypic data further revealed that the most slowly evolving of the three *Spiroplasma* clades attains the highest titer in aphids and reduces aphid lifespan more strongly than the other clades.

Material and Methods

Insect lines

To investigate phenotypic effects of *Spiroplasma* infections we used the European field survey and the phylogeny of *Spiroplasma* from pea aphids reported in Mathé-Hubert et al. (2018) to select 12 strains that are well spread across the phylogeny (Fig. 1). To control for the effect of aphid genotype, the selected *Spiroplasma* strains were transfected from their original host clones (the donors) into a common recipient clone called LSR1. This clone was originally collected in a field of alfalfa (*Medicago sativa*) near Ithaca, New York, in 1998 (Caillaud et al. 2002), and its genome has been sequenced for the pea aphid genome project (The International Aphid Genomics Consortium 2010). Four *Spiroplasma* strains were transfected into LSR1 at the University of Oxford, UK, and kindly provided to us by Ailsa McLean. The remaining transfections were carried out in our laboratory at Eawag, Switzerland. Before transfection of *Spiroplasma* with a microinjection pump (FemtoJet, Eppendorf) as described by Vorburger *et al.* (2010), the donor aphids were cured from all other secondary symbionts by feeding them on a mixture of antibiotics as described in McLean *et al.* (2011). For strain S383, this protocol failed to remove a co-infection with *Hamiltonella defensa* in the donor clone. We thus merged the curing and transfection step by injecting recipients with a small amount of a 20 mg/ml solution of the antibiotic cefotaxime, using a needle that was immersed into the donor's hemolymph prior to injection. This procedure succeeded in transmitting just *Spiroplasma* to the recipient clone. Depending on the strains, the transfections happened between 10 and approx. 150 generations before the experiments.

Although prior to transfections we had reconfirmed the genotypes and the secondary symbiont infections of the donors and the recipient clone with microsatellites and diagnostic PCRs, respectively, a handling error must have occurred between these checks and the actual transfections,

such that we used a *R. insecticola*-infected sub-line of clone LSR1 (LSR+Ri) as recipient rather than the sub-line without any secondary symbionts. As a consequence, seven of the 12 newly transfected sub-lines carried a co-infection with *R. insecticola* in addition to the different *Spiroplasma* strains. Only sub-line LSR1+S383 (presumably due to the simultaneous injection of an antibiotic – see above), the four sub-lines provided by the University of Oxford, and the secondary endosymbiont-free control did not carry *R. insecticola*. Figure 1 summarizes the infection status of each sub-line. That the *R. insecticola*-infected sublines indeed belonged to clone LSR1 was confirmed by microsatellite genotyping, and sequencing of five bacterial genes (*accD*, *gyrB*, *murE*, *recJ*, and *rpoS*; Henry et al. 2013) identified the co-infecting *R. insecticola* as a strain previously shown to provide no protection against *A. ervi* in pea aphids (Oliver et al. 2003; Hansen et al. 2012). Because we discovered this error only after all phenotyping experiments had been completed, we had to account statistically for the presence of *R. insecticola* during data analysis (see below).

Accounting statistically for co-infections with *R. insecticola*

For the three experiments described hereafter, we handled the presence of *R. insecticola* according to the following logic: We estimated the average effect of *R. insecticola* on each trait we analyzed and then used this estimate as an offset to correct for its presence in the coinfected sublines. Specifically, we first fit a '*Regiella*' model devised to estimate the average effect of *R. insecticola* in the presence of a *Spiroplasma* strain. In addition to the variables specific to each experiment (described in the corresponding sections), this model contains two dummy variables as fixed effects describing the presence (1) or absence (0) of *Spiroplasma* and *R. insecticola* (variables S_i and R_i respectively) and a random interaction between the aphid subline (*SUB*) and the fixed effect *S*. This random effect follows a normal distribution of mean zero and standard deviation σ. Mathematically, this gives $Y_i = Int + \alpha \times R_i + \beta \times S_i + S_i \times SUB + e_i;\ SUB \sim N(0, \sigma)$ eq. 1 , where Y_i is the

transformed explained variable, e_i are the residuals estimated by the models together with the coefficients of the fixed effects (α and β) and the standard deviation (σ). Because we used dummy variables, the intercept of the model (*Int*) is the mean of the control sub-line containing neither *Spiroplasma* nor *R. insecticola*. The coefficients α and β are the estimated mean effects of *R. insecticola* and *Spiroplasma*, and the random interaction between the sub-line and *S* accounts for the heterogeneity induced by the different *Spiroplasma* strains. This estimation of the effect of *R. insecticola* assumes that on average the *Spiroplasma* strains that are alone have the same effect as the *Spiroplama* strains that are with *R. insecticola*. The estimated effects of *R. insecticola* (coefficient α in eq. 1) is then used to construct an offset (Hutchinson and Holtman 2005) for the second '*Spiroplasma*' model estimating the effect of each *Spiroplasma* strain. This offset takes the value α when *R. insecticola* is present and 0 when it is absent. The '*Spiroplasma*' model contains the aphid subline as a fixed effect. Thus, for the sublines not containing *R. insecticola* there is no offset and each coefficient describes the effect of the subline's *Spiroplasma* strain, and for the sublines containing *R. insecticola*, the estimated effect of *R. insecticola* in the presence of *Spiroplasma* is absorbed by the offset, and each coefficient describes the effect of the corresponding *Spiroplasma* strain plus its eventual interaction with *R. insecticola*.

Experiment 1: Effect of *Spiroplasma* on *A. ervi* parasitism

We investigated the effect of the 12 *Spiroplasma* strains on the parasitism success of three different lines of the parasitoid wasp *A. ervi* (lines "B", "D" and "K"). We established the line "D" using wasps sampled in July 2015 at two sites in southern Germany during the field survey reported in Mathé-Hubert et al. 2018. This wasp line has been maintained in the laboratory for approx. 40 generations prior to the experiment. The two other *A. ervi* lines "K" and "B" were commercially supplied by the biocontrol companies Koppert (Berkel en Rodenrijs, The Netherlands) and Biobest

188 (Westerlo, Belgium), and were reared in the laboratory for one and two generations before the
189 experiment, respectively. We used three different lines of parasitoids to increase our chances of
190 detecting any protective effects of *Spiroplasma*, since previous studies on another bacterial
191 endosymbiont, *H. defensa*, have shown that the protection afforded by the symbiont can depend on
192 the parasitoid's genotype (e.g. Schmid et al. 2012; Cayetano and Vorburger 2013). All wasps were
193 bred on the same pea aphid clone (lab ID A06-01) that was free of protective endosymbionts and
194 different from the clone used in experiments (LSR1).

195 Parasitism success was measured using a factorial design in which the 13 aphid sublines (12
196 *Spiroplasma*-infected sublines plus uninfected control) were exposed to all three parasitoid lines in
197 six randomized complete blocks. To prevent maternal effects carried over from the aphid stock
198 cultures influencing our results, each of the 234 replicates (13 aphid sub-lines× 3 wasp lines × 6
199 replicates) was reared independently on seedlings of broad bean (*Vicia faba*) for one generation
200 before individuals of the second generation were tested. To start the test generation, five adults
201 from each replicate were used to obtain age-synchronized offspring born within 24 h. At the age
202 of 2-3 days twenty nymphs per replicate were placed on a new plant and exposed to a single female
203 wasp (approx. 2 days old) for 5 hours. Because a few aphid nymphs were harmed during the
204 exposure to wasps, the number of nymphs alive one day after the exposure was recorded. The
205 proportion of these surviving nymphs that were successfully parasitized and transformed into
206 mummies (parasitoid pupae within the dead aphid's exoskeleton) was recorded 11 days after
207 exposure to parasitoids. The proportion of mummies from which adult wasps had emerged
208 successfully (proportion emerged) was recorded 20 days after exposure. We conducted this
209 experiment at 22°C under a 16-h photoperiod.

210 For each of the two variables, proportion mummified and proportion emerged, we fitted the
211 '*Regiella*' and '*Spiroplasma*' models as described above. Both models additionally contained the

wasp line as a fixed effect as well as its interaction with the dummy variables '*R*' and '*S*' for the model '*Regiella*' and with the aphid sub-line for the model '*Spiroplasma*'. Both models also contained the random variable 'Block'.

If for the '*Spiroplasma*' model the wasp line × aphid subline interaction was significant, we re-fitted one model per wasp line to test for overall variation among aphid sublines and to assess the effect of each *Spiroplasma* strain using a Student's *t*-test. These tests compare each *Spiroplasma*-infected subline to the uninfected control subline by assessing the significance of the coefficients of the variable 'aphid subline'. We then used the package "multcomp" (Hothorn et al. 2008) to assess for each of the models fitted to one wasp line which *Spiroplasma* strains had a significant effect after accounting for multiple testing. When the wasp line × aphid subline interaction was not significant, we re-fitted the model without the interaction to test for the effect of each *Spiroplasma* strain.

Since the explained variables were proportions, we first fitted them using binomial GLMMs ("lme4" R package; Bates *et al*. 2014), which were strongly overdispersed. The attempt to mitigate overdispersion with the "observation level random effect" approach (Harrison 2015) resulted in severe underdispersion. Thus we fitted LMMs to the logit transformed proportions (Warton and Hui 2011). To assess the significance of the main effects, we used the "mixed" function of the "afex" R package (v0.18) to perform an F-test with the Kenward-Roger approximation for degrees of freedom (Halekoh and Højsgaard 2014). All statistical analyses were performed using the software R (version 3.5.2).

Experiment 2: Fitness cost of *Spiroplasma*

We assessed the fitness cost of *Spiroplasma* strains by measuring their effects on several life-history traits of their host using the surplus of nymphs produced in experiment 1: For three of the

six blocks, each containing three replicates of every aphid sub-line, we kept all leftover nymphs until they were six days old. Then, for each of the 117 replicates (13 aphid sub-lines × 9 replicates organised into 3 blocks), we selected two young aphids for the life table experiment. In 40% of the cases, one of the two aphids developed wings. They were excluded from the experiment. The 199 wingless aphids were raised individually on broad bean seedlings until their death. Every week, we moved the aphids to a new nine-day-old plant, and recorded the number of offspring they had produced on the former plant. We recorded the survival of the monitored aphids three times a week. The experiment was carried out at 18°C and under a 16-h photoperiod.

We used the life table data to estimate four fitness-related life history traits. The first two are lifetime reproduction (total number of offspring) and lifespan. We also computed the mean reproductive age of each aphid (age of mother at each birth, averaged across all offspring births). In comparison to the lifetime reproduction, the mean reproductive age accounts for the fact that two genotypes with the same lifetime reproduction could have different fitness if one of them produced its offspring earlier than the other. The fourth variable was the intrinsic growth rate, i.e. the constant r in the equation describing population growth in an unlimited environment: $N_t = N_0 e^{rt}$. The procedure to calculate it is described in (Birch 1948). This variable combines the information of the number of offspring and of the age of the mother when the offspring are produced.

To each of these four fitness-related variables we fitted the '*Regiella*' and '*Spiroplasma*' models. Both models also included the random variables block and replicate, the latter accounting for the non-independence of the two individuals taken from the same colony of experiment 1. The test procedure for these four variables is the same as described for experiment 1, except that a box-cox transformation was used to achieve normality of residuals and homoscedasticity instead of the logit function. For the survival data we used the "coxme" R package (v2.2-5) to fit a cox model

(Therneau 2015b). For this survival analysis, we checked the assumption of proportional hazard using the "cox.zph" function of the package "survival" (Therneau 2015a; v2.43-3) and the "survplot" function of the package "rms" (Harrell 2017; v5.1-2), with the argument "loglog" set to true. As in experiment 1, this model assessed the overall variation among aphid sublines and compared each *Spiroplasma*-infected subline to the uninfected control.

Experiment 3: Variation in *Spiroplasma* density

The density of *Spiroplasma* within its host may influence both the cost *Spiroplasma* inflicts on the aphid and the parasitism by *A. ervi*. Thus, we measured the density of *Spiroplasma* in 10- and 20-day-old aphids using quantitative PCR (qPCR). For each combination of age and strain, we measured five biological replicates, each consisting of a pool of three aphids that were reared on a nine-day-old plant, a different plant being used for each biological replicate. The biological replicates were reared within a single tray on randomized positions. DNA was extracted using either the QIAGEN "DNeasy 96 Blood & Tissue Kit" (extraction in plates; N=104 samples) or the QIAGEN "DNeasy Blood & Tissue Kit" (extraction in tubes; N=16 samples) after the aphids had been crushed by shaking them 30 times per second for 40 seconds with two glass marbles of 2 mm Ø on a bead mill (TissueLyser II, QIAGEN). These extractions typically yield approx. 5 µg of DNA in 200µl.

For each pool of three aphids, the number of *Spiroplasma* and aphid gene copies were estimated using a Roche LightCycler 480 2.0. Each 12.5 µl of qPCR reaction included 6.25 µl of GoTaq® qPCR Master Mix, 1.25 µl Dnase free Water, 2.5 µl of DNA template and 1.25 µl each of the 4.5 µM forward and reverse primers. Primers for the *Spiroplasma* dnaA gene were DnaA_F 5'-AAT GCT TGG ATC ATA ATT TAA AGA C-3' and DnaA_R 5'-GTT TTG AAG AAA GAA ATG TTT CAA G-3'. Primers for the *A. pisum* Ef1a gene were Ef1a_F 5'-TAG CAG TTA CAT CAA GAA AAT CGG-3' and Ef1a_R 5'-ATG TTG TCT CCA TTC CAT CCA G-3'. Cycling

conditions are described in Table S2. Gene copy numbers were estimated with reference to a standard curve generated with serial dilutions of a synthetic standard. We did not standardize the overall DNA concentrations among samples because we were mainly interested in the *Spiroplasma* titers (number of *Spiroplasma* gene copies relative to aphid gene copies), and because the randomization of biological replicates safeguarded us against any unwanted biases. However, to improve the precision of the measurements, samples with a very high concentration were re-run after a dilution devised to yield an expected Cp around 20. For each sample the number of gene copies per aphid individual was calculated from the average of triplicate qPCR reactions.

Since the format of the extraction kit (DNeasy 96 Blood & Tissue Kit [plate format] *vs.* DNeasy Blood & Tissue Kit [individual tubes]) had a strong effect on the estimated number of aphid gene copies and a minor effect on the estimated number of *Spiroplasma* gene copies (Fig. S1), we removed the estimated effect of the extraction kit using the function "removeBatchEffect" of the package "limma" (Smyth 2005, v3.38.3) prior to further analyses. These corrected numbers of *Spiroplasma* and aphid gene copies per individual are indicated as # *Spiroplasma* dnaA and # aphid EF1a, respectively. The number of *Spiroplasma* gene copies per aphid gene copy is defined as # *Spiroplasma* dnaA / # aphid EF1a.

We fitted the '*Regiella*' and '*Spiroplasma*' models to each of the three variables # *Spiroplasma* dnaA, # aphid EF1a and # *Spiroplasma* dnaA / # aphid EF1a. Since the uninfected sub-line was not included in this part of the study, the '*Regiella*' model did not contain the dummy variable '*S*' (i.e., all the investigated sub-lines carried *Spiroplasma*). The '*Regiella*' and '*Spiroplasma*' models additionally contained the aphid age (10 or 20 days) as a fixed effect as well as its interaction with the dummy variables '*R*' for the model '*Regiella*' and with the aphid subline for the model '*Spiroplasma*'. The test procedure is the same a described for experiment 1, except that since there is no random effect in the '*Spiroplasma*' model, the main effects were tested using *F*-tests, and we

additionally fitted a model separately for each aphid age to assess differences between sub-lines using Tukey's tests.

Phylogenetic analyses

We performed two analyses using the phylogeny of *Spiroplasma* strains inferred by Mathé-Hubert *et al.* (2018). This phylogeny (Fig. 1) showed that *Spiroplasma* of pea aphids are divided into at least three clades. The first analysis tested if the *Spiroplasma* induced phenotypes correlate with the phylogeny (phylogenetic inertia), which is expected if these phenotypes evolve slowly in comparison to the sequences used to discriminate *Spiroplasma* strains. Such phylogenetic inertia would mean that in pea aphids, different clades of *Spiroplasma* have different effects on their host. Then we tested if clade 3, which appears to have short branches in the phylogeny, has a lower rate of molecular evolution than the two other clades.

To test for phylogenetic inertia and to investigate the links among the *Spiroplasma* induced phenotypes, we characterized the variation in the effects of *Spiroplasma* strains on the phenotype of their host by the coefficients of the '*Spiroplasma*' models from the three experiments. These coefficients were used rather than the raw data because they represent the estimated effect of *Spiroplasma* after accounting for *Regiella*. A PCA was used to summarise this phenotypic variation. In this PCA, individuals (rows) are the *Spiroplasma* strains which are characterised by the coefficients of the '*Spiroplasma*' models on the different traits (i.e. one column per trait). These traits (columns) were weighted to ensure that the three experiments had the same weight whatever the number of traits we measured during the experiment. Since the intrinsic growth rate is a composite variable of other variables, it was included in the PCA as a supplementary variable: it was projected onto the PCA after the PCA was inferred. We tested for phylogenetic inertia on the first two PCA axes which jointly explained 57.03% of the phenotypic variation. Two measures of

phylogenetic inertia are generally recommended, the lambda index and Abouheif's C_{mean} index (Münkemüller *et al.* 2012). For our phylogeny, the latter has more power (Fig. S2). Hence we used C_{mean} to measure phylogenetic inertia and tested its significance by performing 10 000 randomizations using the package "phylosignal" (Keck *et al.* 2016).

For the *Spiroplasma* strains that share their host with *R. insecticola*, the coefficients used in the analysis describe the effect of the strain plus its potential interaction with *R. insecticola*. However, because strains with and without *R. insecticola* are similarly distributed in the phylogeny, potential interactions would only add noise to the analysis. This would decrease statistical power and thus should not create any false positives.

In the *Spiroplasma* phylogeny, clade 3 appears to have a lower rate of molecular evolution than clades 1 and 2. We used the local-clock permutation test developed by Lanfear (2010) to assess whether this difference was significant. This test is independent of the above mentioned experiments and only concerns the molecular phylogeny. It uses the ratio between the likelihood of two models that are fitted to the phylogeny and its underlying sequences (GenBank IDs MG288511 to MG288588). The first model assumes a strict clock, meaning that all strains are evolving equally fast, while in the second model (local clocks), strains of clade 3 are allowed to evolve at a different rate than other strains. The *p*-value is obtained by comparing the observed ratio between the likelihoods of the two models to the null distribution of this ratio, which is estimated by refitting the strict and the local clocks models to 10 000 permutations of the sequences. This test has been shown to be more conservative than the usual likelihood ratio-test (Lanfear 2010). The local clocks model applied to the real data was also used to estimate the effect size of the difference of rates of molecular evolution.

Results

Experiment 1: Effect of *Spiroplasma* on *A. ervi* parasitism

The '*Regiella*' model detected highly significant variation among wasp lines in the proportion of aphids that were mummified (i.e. parasitized successfully), but no overall effects of the presence of either *Regiella* or *Spiroplasma* (Table 1). The '*Spiroplasma*' model also recovered the strong differences among wasp lines, with line B being the most and line D the least virulent line (Fig. 2), as well as significant variation among aphid sub-lines, also in interaction with the wasp line (Table 1). Separate analyses for each wasp line showed that this was mostly due to variation in susceptibility to the most virulent wasp line B (Table 1), for which the presence of *Spiroplasma* strains S227 and S385(+Ri) reduced parasitism significantly (Table S3). In the case of wasp line K, aphids infected with strain S161 were more likely to be successfully parasitized than the uninfected control sub-line (Table S3). Wasp lines also differed in proportion emerged, line K having the highest and line D the lowest emergence rate. However, this difference was detected by model '*Spiroplasma*' but not by model '*Regiella*', likely because of the higher complexity of the latter model.

Experiment 2: Fitness cost of *Spiroplasma*

The overall effect of the symbionts *Spiroplasma* and *R. insecticola* on the fitness of their host is summarised by the intrinsic growth rate. We repeat the caveat that the interpretation of these effects hinges on strong assumptions, namely that the average effect of *Spiroplasma* strains that are alone is comparable to that of strains that are sharing their host with *R. insecticola*, and that there are no interactive effects of *Spiroplasma* and *R. insecticola* on aphid phenotypes. Under these – admittedly untested – assumptions, it appears that *Spiroplasma* reduced the intrinsic growth rate

377 significantly while *R. insecticola* increased it or at least counteracted the negative effect of
378 *Spiroplasma* (Table 2 and Fig. 3A). Correcting for the estimated effect of *R. insecticola*, the
379 '*Spiroplasma*' model shows that all *Spiroplasma* strains except S322, S383 and S237 decreased
380 the intrinsic growth rate significantly. This was still significant for more than half of the strains
381 after correcting for multiple testing (Table S3).

382 Infection by *R. insecticola* did not affect aphid lifespan, but all *Spiroplasma*-infected sub-lines
383 had shorter lifespans than the *Spiroplasma*-free sub-line, on average by about eight days (Table 2,
384 Fig. 3B). Only the effect of strains S27 and S385(+Ri) on host survival was no longer significant
385 after accounting for multiple testing (Table S3). *Spiroplasma* also reduced lifetime reproduction
386 while *R. insecticola* – with the caveat mentioned above – appeared to increase it or at least to
387 counteract the negative effect of *Spiroplasma* (Table 2 and Fig. 3C). Neither infection with *R.*
388 *insecticola* nor infection with *Spiroplasma* had a significant overall effect on the mean reproductive
389 age of the aphid host (Tables 2, S3).

390

391 ## Experiment 3: Variation in *Spiroplasma* density

392 Infection by *R. insecticola* did not have any detectable effect on # *Spiroplasma* dnaA, # aphid
393 EF1a or their ratio in either 10- or 20-day-old aphids (Table 3). The # aphid EF1a did not change
394 significantly from age 10 to 20, but # *Spiroplasma* dnaA increased strongly (Table 3, Fig. 4B, C),
395 on average by a factor of 4.86, which corresponds to an average doubling time of 4.38 days for
396 *Spiroplasma*. Accordingly, the ratio of *Spiroplasma* to aphid gene copies increased as well and
397 reached very high values (approx. 40-130) in 20-day-old aphids. There was substantial variation in
398 the densities and growth achieved by different *Spiroplasma* strains, reflected in the highly
399 significant sub-line and age × sub-line effects on # *Spiroplasma* dnaA (Table 3). This variation
400 appeared to have a limited effect on aphid gene copy number, as the differences among sub-lines

401 for # aphid EF1a were not statistically significant (P = 0.08, Table 3). *Spiroplasma* strain S227 was
402 notable, however, because this sub-line showed very low # aphid EF1a in 10-day-old aphids,
403 resulting in a high ratio of # *Spiroplasma* dnaA / # aphid EF1a (Fig. 4A). This is the sub-line that
404 exhibited the lowest susceptibility to parasitoids but also high costs of infection by *Spiroplasma*
405 (Figs. 2 & 3).
406
407 ## Phylogenetic signal in *Spiroplasma* phenotypes and rate of molecular evolution
408 The first two axes of the PCA that were tested for a phylogenetic signal summarized 57.03% of
409 the phenotypic variation in the 12 *Spiroplasma*-infected pea aphid sublines. The first axis mainly
410 summarized the negative effect that *Spiroplasma* strains with a high density had on the lifespan of
411 their host (Fig. 5A). This negative effect on lifespan had little effect on the aphids' intrinsic growth
412 rate because this first axis has only a low correlation with the lifetime reproduction and a negative
413 correlation with the mean reproductive age (i.e. short-lived aphids produced offspring earlier in
414 life). The second axis encompasses variation related to aphid health and suitability for parasitoids.
415 This axis was positively correlated to # aphid EF1a, the aphid growth rate, the lifetime reproduction,
416 and negatively correlated to the mean reproductive age. Sub-lines with a higher score on this axis
417 (i.e., more fecund sub-lines) also showed higher rates of mummification by parasitoids and
418 parasitoid emergence (Fig. 5B). The variation in the reproductive fitness of the sublines was not a
419 function of *Spiroplasma* titers, as the variation in # *Spiroplasma* dnaA was only weakly correlated
420 with this axis.
421 These two axes were used to investigate the correlation between the *Spiroplasma*-induced
422 phenotypic variation and the *Spiroplasma* phylogeny using the Abouheif's Cmean statistic. Only
423 the first axis was significantly correlated (PC1: Cmean = 0.34, p = 0.02; PC2: Cmean = -0.01, p =
424 0.29), with most strains of clade 3 having a high score on the first axis (Fig. 5A).

The local clock model estimated that the sequences of clade 3 are evolving 5.6 times more slowly than those of clades 1 and 2. The local-clock permutation test revealed that this difference was marginally significant ($P = 0.043$).

Discussion

In the absence of reproductive manipulation or frequent horizontal spread, heritable endosymbionts must provide a net fitness benefit to persist in host populations (Oliver et al. 2014). We investigated protection against the parasitoid wasp *A. ervi* as a potential benefit provided by 12 different strains of *Spiroplasma* in pea aphids, and we estimated their costs to the host in terms of life-history traits.

Evidence for protection was very limited and restricted to one of the three lines of *A. ervi* we used. Only *Spiroplasma* strains S227 and S385 reduced parasitism by the most virulent wasp line B significantly. In the case of S227, however, this was associated with very low reproductive fitness of the aphids in the absence of parasitoids, suggesting that S227-infected aphids were generally of poor health. On the other hand, when the aphids were exposed to wasp line K, one strain of *Spiroplasma* (S161) even seemed to represent a significant liability and made aphids more susceptible to parasitism. The effects of some *Spiroplasma* strains tended to be unequal across the three parasitoid lines, which resulted in a near-significant genotype-by-genotype interaction (Table 1). In principle, such interactions could contribute to the maintenance of strain diversity in parasites as well as symbionts (Kwiatkowski and Vorburger 2012; Ford et al. 2017; Vorburger and Perlman 2018), although their importance is questionable here, because the majority of *Spiroplasma* strains had no detectable effects on parasitism. We do not know why the three wasp lines varied so strongly in their parasitism efficacy. The conspicuously low success of line D could be related to the long time it has been reared in our laboratory at relatively small population size, which might have

448 resulted in negative effects of inbreeding. The difference between the two commercially available
449 lines may be related to their long-term rearing conditions in the production and/or their genetic
450 background. Genetic variation in parasitism success is commonplace in parasitoids (Kraaijeveld
451 and Godfray 1999; Colinet et al. 2010; Sandrock et al. 2010) and likely related to variation in the
452 cocktail of virulence factors parasitoids employ. For example, parasitoid wasp venom is a major
453 source of virulence factors and generally shows a high level of intraspecific variation (Colinet et
454 al. 2013; Mathé-Hubert et al. 2015), also in *A. ervi* (Colinet et al. 2014). Interactions between
455 parasitoid virulence factors and *Spiroplasma* in the aphid hosts could potentially explain the
456 somewhat uneven effects of the different *Spiroplasma* strains in the three parasitoid treatments.
457 Even though we find little evidence for protection against *A. ervi* in the present study, it should
458 be added that *Spiroplasma* may still reduce the risk of parasitism indirectly via a plant-mediated
459 effect, because *A. ervi* is more attracted to volatiles from plants infested by *Spiroplasma*-free aphids
460 than from plants with *Spiroplasma*-infected aphids, as recently shown by Frago et al. (2017). Such
461 an effect would have been missed by our non-choice assays.
462 Due to an unfortunate handling error in the preparation of our experimental lines, about half of
463 the *Spiroplasma* strains shared their hosts with a coinfection of *R. insecticola*. However, the
464 presence of *R. insecticola* did not have any detectable effects on susceptibility to *A. ervi*. This
465 outcome is consistent with earlier studies that tested the same strain of *R. insecticola* deliberately
466 and reported no significant effects on parasitism by *A. ervi* (Oliver et al. 2003; Hansen et al. 2012).
467 In contrast to the potential benefits we investigated, the costs of infection with *Spiroplasma*
468 were rather clear. All strains curtailed aphid lifespan significantly, on average by more than 8 days.
469 A reduction of host lifespan is also characteristic of *Spiroplasma* infection in *Drosophila*
470 *melanogaster* (Herren et al. 2014). Because old aphids are less fecund than young adults (e.g. Zeng
471 et al. 1993; Vorburger and Ramsauer 2008), and because offspring produced early in life are more

important for a clone's growth rate than offspring produced late (Lenski and Service 1982), the strong negative effect on lifespan did not translate into equally strong effects on lifetime reproduction and the intrinsic rate of increase (Fig. 3). Nevertheless, two of the five sublines infected only by *Spiroplasma* showed significantly lower intrinsic rates of increase than the uninfected control. The sublines in which *Spiroplasma* co-occurred with *R. insecticola* showed similar trait values to the uninfected subline. This would suggest that the presence of *R. insecticola* counter-balanced the costs imposed by *Spiroplasma*. The *'Regiella'* models indeed showed a positive overall effect of *R. insecticola* on lifetime reproduction as well as the intrinsic rate of increase. This interpretation of the results is, however, conditional on the validity of the assumption that *Spiroplasma* strains associated with *R. insecticola* have the same average effect as those that are not. A positive effect of *R. insecticola* on host fitness has also been reported by Tsuchida et al. (2004) for pea aphids feeding on clover, but this does not seem to be a general property of this symbiont (Leonardo 2004; Ferrari et al. 2007), and other studies reported negative fitness effects of this symbiont, for example in the grain aphid, *Sitobion avenae* (Da Wang et al. 2016; Luo et al. 2017). Nonetheless, it has been observed before that one heritable endosymbiont can compensate the costs imposed by another. Doremus and Oliver (2017) found that the large costs associated with the possession of X-type endosymbionts in pea aphids were ameliorated by coinfection with *H. defensa*. When the influence of *R. insecticola* was corrected for statistically in the present data, the majority of *Spiroplasma* strains were inferred to reduce lifetime reproduction and intrinsic rate of increase significantly (Table S3). Thus we conclude that under laboratory conditions and in the absence of any other selective forces, infection with *Spiroplasma* generally has a negative effect on pea aphid reproductive fitness.

To some extent, the *Spiroplasma*-induced fitness costs were related to the symbionts' densities in the host. The *Spiroplasma* titers in pea aphids increased strongly from the age of 10 days to the

age of 20 days, suggesting that the host has limited control over the proliferation of *Spiroplasma*. This is also observed in *D. melanogaster*, and it may be related to the fact that cell wall-less bacteria like *Spiroplasma* can escape the attention of the insect immune system (Herren and Lemaitre 2011; Herren et al. 2014). Not all strains were equally prolific, though. *Spiroplasma* titers varied substantially among aphid sublines, and there was no indication that they were influenced by coinfecting *R. insecticola*. The links among the estimated effects of the different *Spiroplasma* strains on the various traits we measured was investigated with a PCA on the coefficients estimated by the models analyzing these traits. In this PCA, the first PC was chiefly associated with high *Spiroplasma* densities and short aphid lifespan, providing correlative evidence that higher *Spiroplasma* titers are more harmful to the host. Interestingly, there was a weak but significant phylogenetic signal in the variation along this axis (Fig. 5). This was mostly because strains from clade 3 showed higher scores for PC1 on average, i.e. these strains achieved higher densities and tended to be associated with shorter host lifespans. High *Spiroplasma* densities have also been shown to curtail host lifespan in flies (Herren and Lemaitre 2011). Clade 3 also exhibited a lower rate of molecular evolution than the other two clades, and it is tempting to speculate about a causal link with the seemingly more parasitic lifestyle of these *Spiroplasma* strains. Endosymbiotic bacteria generally exhibit increased rates of sequence evolution than their free-living relatives, which is attributed to the lower effective population size that comes with maternal transmission and the associated bottlenecks between host generations (Moran 1996; Woolfit and Bromham 2003; Boscaro et al. 2013). Long-term vertical transmission is also expected to reduce the costs that symbionts impose on their hosts. Endosymbionts are thus a good model of how organisms can move along the parasitism-mutualism continuum (Ewald 1987; King 2019). It might seem that *Spiroplasma* strains from clade 3 occupy a space further towards the parasitic end of this continuum than the other clades. Whether this reflects a shorter association with the host, which would be

consistent with the slower rate of molecular evolution, or whether other selective forces have created this situation, is currently unknown. In this context it could be relevant that the different clades of *Spiroplasma* tend to be associated with different communities of co-infecting symbionts in natural populations of pea aphids. For example, clade 3 *Spiroplasma* are positively associated in the field with the X-type symbiont and negatively with *H. defensa*, while those from clade 2 tend to be positively associated with *Rickettsia*, and this seems to be unrelated to the host plants from which pea aphids were collected (Mathé-Hubert et al. 2018). Regular coinfections with other bacteria certainly have the potential to affect the evolution of endosymbionts and possibly their virulence (Vorburger and Perlman 2018). Interactions with other species of endosymbiotic bacteria thus clearly deserve attention to better understand *Spiroplasma*'s influence on host ecology and evolution.

In conclusion, our experiment showed that infections with various strains of the heritable endosymbiont *Spiroplasma* are rather costly to their pea aphid hosts, and that protection against *A. ervi* is unlikely to compensate for these costs. We tested for protection against *A. ervi* because it is the pea aphid's most common parasitoid, but multiple parasitoids include pea aphids in their host range and we cannot exclude that *Spiroplasma* may be protective against other species. Already demonstrated is a protective effect of certain strains of *Spiroplasma*, including strain S161 used here, against the entomopathogenic fungus *Pandora neoaphidis* (Łukasik et al. 2013), but also this is not a general property of all *Spiroplasma* found in pea aphids. Once a symbiont has evolved maternal transmission, it is under strong selection to keep its host alive until reproduction. This can be achieved via protection against natural enemies or by providing other ecological benefits, e.g. by increasing tolerance to abiotic stressors (Oliver et al. 2010). The specific mechanisms may well vary among different strains of the same symbiont species, and with the high diversity of strains

544 structured into at least three clades, *Spiroplasma* of pea aphids is an attractive model to investigate
545 this variation further.
546

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Tables

Table 1: Analyses of parasitism by the parasitoid wasp *Aphidius ervi*.

Models 1-5 explain the proportion of the sets of 20 nymphs exposed to one wasp that were mummified. Models 6 and 7 explain the proportion of mummies from which a wasp emerged. Models 1 and 6 estimate the effect of *R. insecticola* and were used to build the offsets correcting for the presence of *R. insecticola* in the other models. Models 3-5 investigate the interaction between wasp line × aphid subline that is significant in model 2.

Explained variable	Model	Wasp lines	Effect	df	F	p.value
Proportion mummified	**1** '*Regiella*'	All	Wasp	2, 210	5.98	0.003 **
			Spiro. (0/1)	1, 78.73	0.09	0.763
			Regi. (0/1)	1, 10	1.83	0.206
			Wasp × *Spiro*.	2, 210	0.44	0.646
			Wasp × *Regi*.	2, 210	0.18	0.836
	2 '*Spiroplasma*'	All	Wasp	2, 190	58.82	< 0.001 ***
			Sub-line	12, 190	2.17	0.009 **
			Wasp × Sub-line	24, 190	1.57	0.050**.**
	3 '*Spiroplasma*'	B	Sub-line	12, 60	4.91	< 0.001 ***
	4 '*Spiroplasma*'	D	Sub-line	12, 60	0.73	0.72
	5 '*Spiroplasma*'	K	Sub-line	12, 60	1.25	0.273
Proportion emerged	**6** '*Regiella*'	All	Wasp	2, 185.32	0.64	0.528
			Spiro. (0/1)	1, 160.60	0.28	0.594
			Regi. (0/1)	1, 10.53	3.08	0.108
			Wasp × *Spiro*.	2, 186.07	0.27	0.758
			Wasp × *Regi*.	2, 186.91	0.26	0.770
	7 '*Spiroplasma*'	All	Wasp	2, 164.64	5.63	0.004 **
			Sub-line	12, 164.70	0.55	0.880
			Wasp × Sub-line	24, 164.65	0.87	0.638

Table 2: Analyses of the fitness costs of *Spiroplasma*

Models 1, 3, 5 and 7 estimate the effect of *R. insecticola* on four variables related to fitness. They were used to build the offsets correcting for the presence of *R. insecticola* in the other models estimating the effect of each *Spiroplasma* strain (models 2, 4,6 and 8).

Explained variable	Model		Effect	df	F (or χ^2)[#]	p-value
Lifetime reproduction	**1**	'*Regiella*'	*Spiro*. (0/1)	1, 41.86	9.27	0.004 **
			Regi. (0/1)	1, 10.06	6.61	0.028 *
	2	'Spiroplasma'	Sub-line	12, 90.21	5.46	< 0.001 ***
Lifespan	**3**	'*Regiella*'	*Spiro*. (0/1)	1, 173.99	11.46	< 0.001 ***
			Regi. (0/1)	1, 173.99	0.73	0.392
	4	'Spiroplasma'	Sub-line	12, 172.99	95.61	< 0.001 ***
Mean reproductive age	**5**	'*Regiella*'	*Spiro*. (0/1)	1, 81.66	0.18	0.673
			Regi. (0/1)	1, 10.07	1.65	0.227
	6	'Spiroplasma'	Sub-line	12, 141.42	4.58	< 0.001 ***
Intrinsic growth rate	**7**	'*Regiella*'	*Spiro*. (0/1)	1, 51.57	8.84	0.004 **
			Regi. (0/1)	1, 10.01	10.61	0.009 **
	8	'Spiroplasma'	Sub-line	12, 85.97	4.19	< 0.001 ***

[#]: For lifespan, we used a Cox model, for which fixed effect were tested with LRT. In this case, we report the χ^2 statistics.

Table 3: Analyses of the *qPCR* estimates of the number of gene copies in 10 and 20 days old aphids

Models 1, 5 and 9 estimate the effect of *R. insecticola* on # *Spiroplasma* dnaA / # aphid EF1a, # *Spiroplasma* dnaA and # aphid EF1a. They were used to build the offsets correcting for the presence of *R. insecticola* in the other models estimating the effect of each *Spiroplasma* strain (models 2, 6 and 10). When there was a significant interaction between aphid age and subline, separate models were fitted for each age group to investigate the interaction (models 3, 4, 7 and 8).

Explained variable	Model		Aphid age	Effect	df	F	p-value
#*Spiro.* dnaA / #aphid EF1a	**1**	'*Regiella*'	Both	*Regi.* (0/1)	1, 10.01	121.34	0.765
				Age	1, 106.05	0.25	< 0.001 ***
				Age × *Regi.*	1, 106.20	2.12	0.148
	2	'*Spiroplasma*'	Both	Age	1, 96	314.95	< 0.001 ***
				Sub-line	11, 96	8.07	< 0.001 ***
				Age × Sub-line	11, 96	1.96	0.041 *
	3	'*Spiroplasma*'	10 days	Sub-line	11, 49	4.50	< 0.001 ***
	4	'*Spiroplasma*'	20 days	Sub-line	11, 47	5.38	< 0.001 ***
#*Spiro.* dnaA	**5**	'*Regiella*'	Both	*Regi.* (0/1)	1, 10.01	0.00	0.993
				Age	1, 106.04	262.80	< 0.001 ***
				Age × *Regi.*	1, 106.14	0.00	0.985
	6	'*Spiroplasma*'	Both	Age	1, 96	728.20	< 0.001 ***
				Sub-line	11, 96	12.31	< 0.001 ***
				Age × Sub-line	11, 96	2.63	0.006 **
	7	'*Spiroplasma*'	10 days	Sub-line	11, 49	5.66	< 0.001 ***
	8	'*Spiroplasma*'	20 days	Sub-line	11, 47	10.02	< 0.001 ***
#aphid EF1a	**9**	'*Regiella*'	Both	*Regi.* (0/1)	1, 10.05	1.31	0.278
				Age	1, 106.22	0.25	0.618
				Age × *Regi.*	1, 106.83	4.00	0.048 *
	10	'*Spiroplasma*'	Both	Age	1, 96	0.68	0.412
				Sub-line	11, 96	1.70	0.084 .
				Age × Sub-line	11, 96	1.16	0.325

Figure captions

Figure 1: *Spiroplasma* phylogeny

Phylogeny modified from Mathé-Hubert *et al.* (2018). Strains selected for the phenotyping experiments are followed by an arrow indicating whether they were transfected into pea aphid clone LSR1 containing *R. insecticola* (→ LSR1+Ri) or not (→ LSR1). The clade to which the *Spiroplasma* strain belongs and the other symbionts with which strains of that clade are typically associated (+) or not associated (–) is indicated on the right. Values close to the nodes are bootstrap values. The scale bar indicates the substitution rates.

Figure 2: Mummification rates

Mean proportion of nymphs mummified (±S.E. indicated with error bars) for the three wasp lines and each aphid sub-line. On each panel, S– corresponds to the uninfected subline, and S+ to the mean of all *Spiroplasma* infected sublines. Error bars indicate the standard error.Sub-lines also containing *R. insecticola* are hatched. The significance of the comparisons between the *Spiroplasma* infected sub-lines and the uninfected control sub-lines performed from the 'Spiroplasma' models is indicated below the strains names (●: only significant before adjusting for multiple comparisons; ⦿: still significant after adjusting for multiple comparisons).

Figure 3: *Spiroplasma* effects on aphid fitness

Bar plots depicting the average intrinsic growth rate (A), lifespan (B), lifetime reproduction (C), and mean reproductive age (D) for all aphid sublines. On each panel, S– corresponds to the uninfected subline, and S+ to the mean of all *Spiroplasma* infected sublines. Error bars indicate the standard error. Sub-lines also containing *R. insecticola* are hatched. The significance of the

comparisons between the *Spiroplasma* infected sub-lines and the uninfected control sub-lines performed from the 'Spiroplasma' models is indicated below the strains' names (●: only significant before adjusting for multiple comparisons; ⦿ : still significant after adjusting for multiple comparisons).

Figure 4: *Spiroplasma* density

The number of *Spiroplasma* gene copies #per aphid gene copy (# *Spiroplasma* dnaA / # aphid EF1a) as well as the raw numbers of *Spiroplasma* and aphid gene copies per aphid individual (# *Spiroplasma* dnaA and # aphid EF1a) are shown on panels A-C. Because # *Spiroplasma* dnaA is much higher in 20 days old aphids (dark grey) than in 10 days old aphids (light grey), panels A and B have two y-axes with different scales. To help the comparison, red dots indicate the same values on the left and right axes. Different letters above bars indicate significant pairwise differences in Tukey-HSD tests. Error bars indicate the standard errors.

Figure 5: Phylogenetic signal in the *Spiroplasma* induced phenotypes

A-B: Phylogenetic signal in principal components (PC) 1 and 2, summarizing the phenotypic variation induced by the different *Spiroplasma* strains. The PCA was done on the coefficients of the models fitted to the phenotypic traits of the aphids. On both panels, the horizontal bar plot indicates the correlation between the model coefficients that are the variables of the PCA and the corresponding PCA axis. This informs about the phenotypes of the aphids that are summarized by the PCA axis. Variable names were shortened: S and A refer to # *Spiroplasma* dnaA and #aphid EF1a in 10 and 20 days old aphids; Mum and Eme refer to the mummification and emergences rates respectively in wasps B, D and K and averaged ($_{av}$) over the three wasp lines;

#off refers to lifetime reproduction. The plots on the right side give the score of each *Spiroplasma* strain on the PCA axes and its position in the phylogeny. The scale bar indicates the substitution rate.

Figure 1

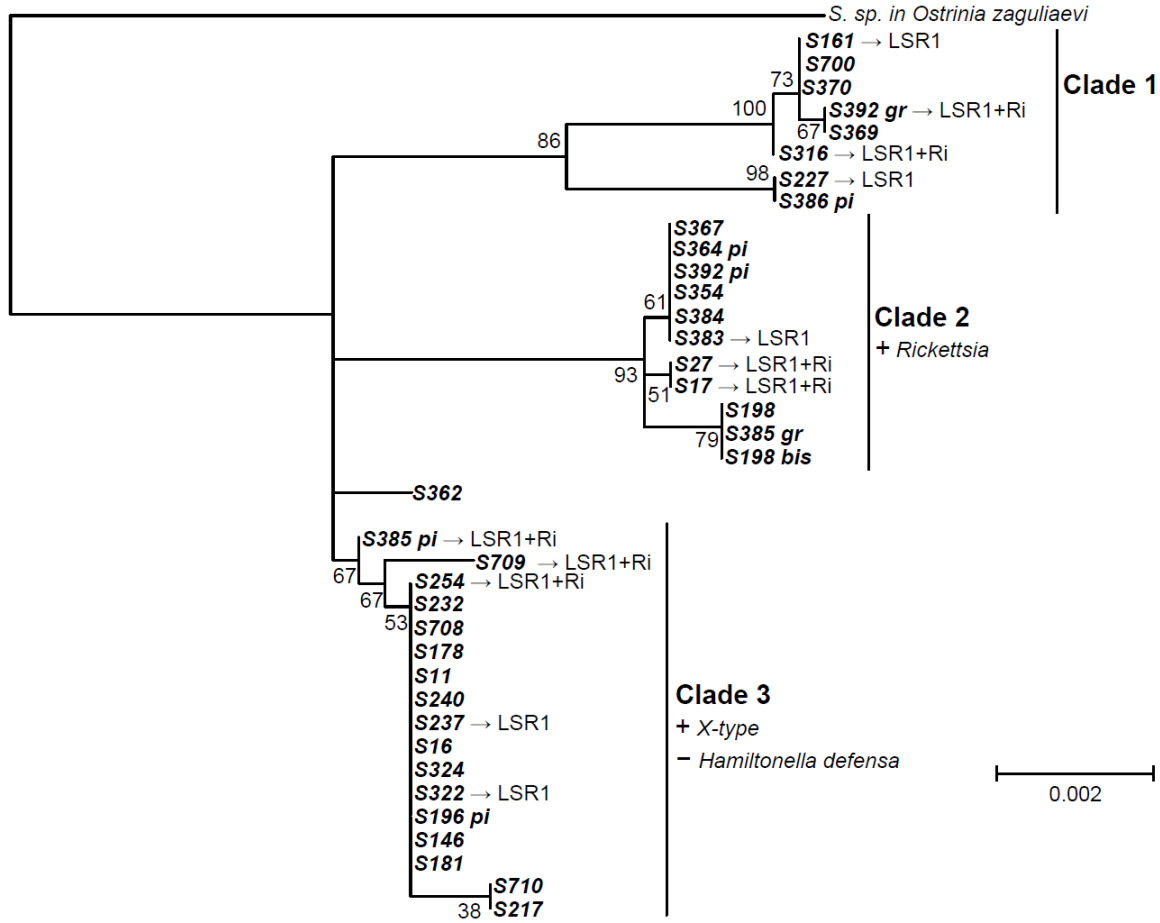

S. sp. in Ostrinia zaguliaevi
S161 → LSR1
S700
S370
S392 gr → LSR1+Ri
S369
S316 → LSR1+Ri
S227 → LSR1
S386 pi
Clade 1
S367
S364 pi
S392 pi
S354
S384
S383 → LSR1
S27 → LSR1+Ri
S17 → LSR1+Ri
S198
S385 gr
S198 bis
Clade 2
+ Rickettsia
S362
S385 pi → LSR1+Ri
S709 → LSR1+Ri
S254 → LSR1+Ri
S232
S708
S178
S11
S240
S237 → LSR1
S16
S324
S322 → LSR1
S196 pi
S146
S181
S710
S217
Clade 3
+ X-type
− Hamiltonella defensa
0.002
73
100
67
86
98
61
93
51
79
67
67
53
38

Figure 2

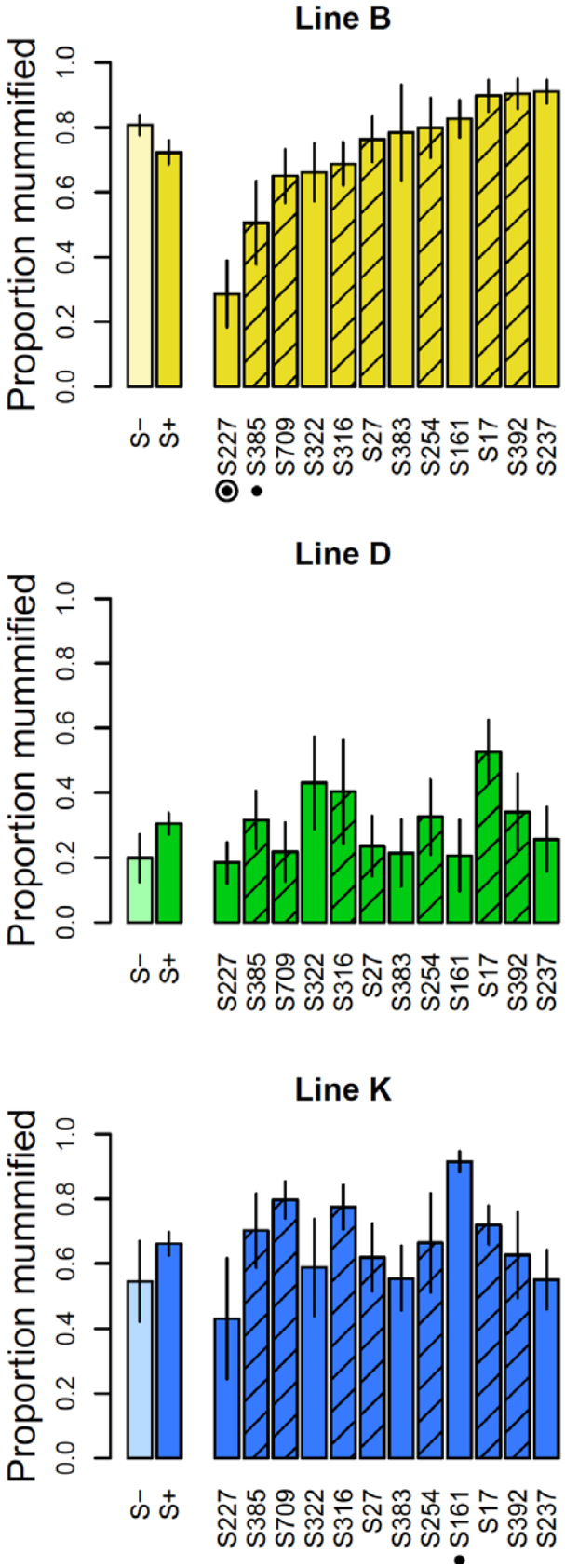

Line B
Proportion mummified
0.0
0.2
0.4
0.6
0.8
1.0
S-
S+
S227
S385
S709
S322
S316
S27
S383
S254
S161
S17
S392
S237
Line D
Proportion mummified
0.0
0.2
0.4
0.6
0.8
1.0
S-
S+
S227
S385
S709
S322
S316
S27
S383
S254
S161
S17
S392
S237
Line K
Proportion mummified
0.0
0.2
0.4
0.6
0.8
1.0
S-
S+
S227
S385
S709
S322
S316
S27
S383
S254
S161
S17
S392
S237

Figure 3

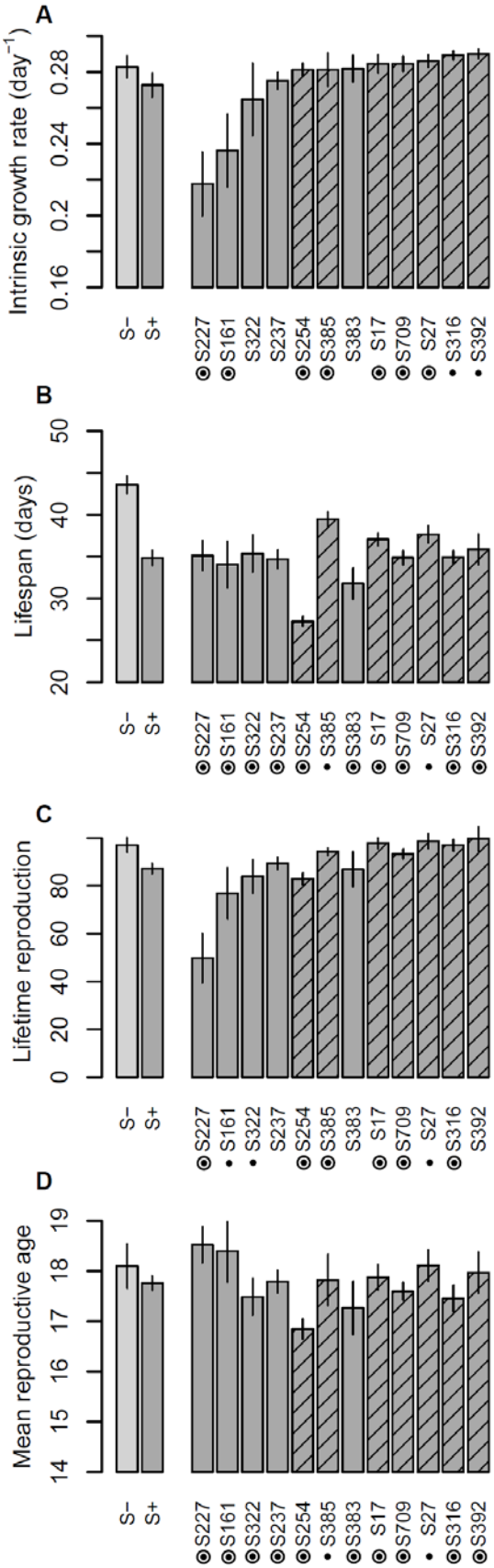

A
Intrinsic growth rate (day^{-1})
0.16
0.2
0.24
0.28
B
Lifespan (days)
20
30
40
50
C
Lifetime reproduction
0
20
40
60
80
D
Mean reproductive age
14
15
16
17
18
19
S−
S+
S227
S161
S322
S237
S254
S385
S383
S17
S709
S27
S316
S392

Figure 4

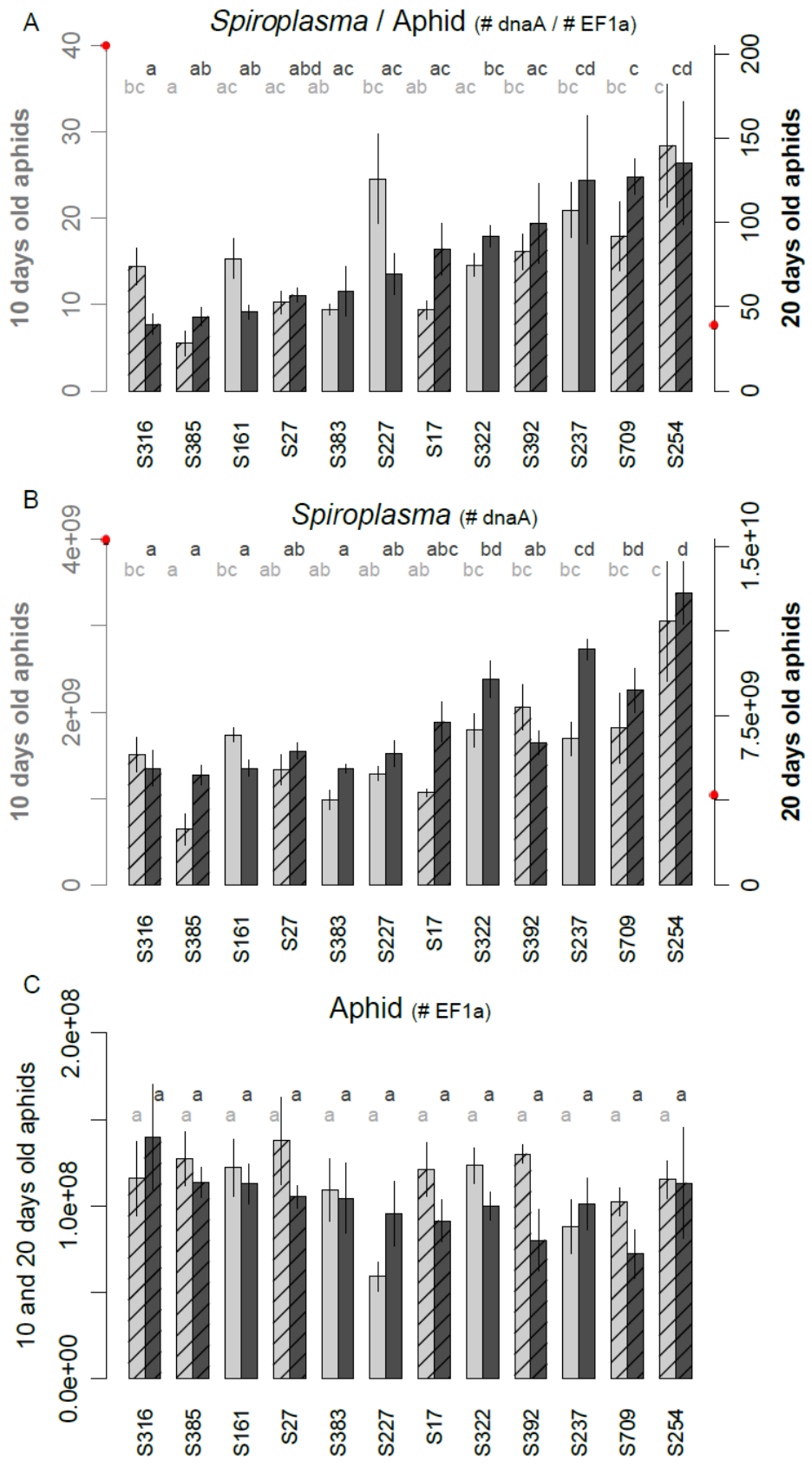
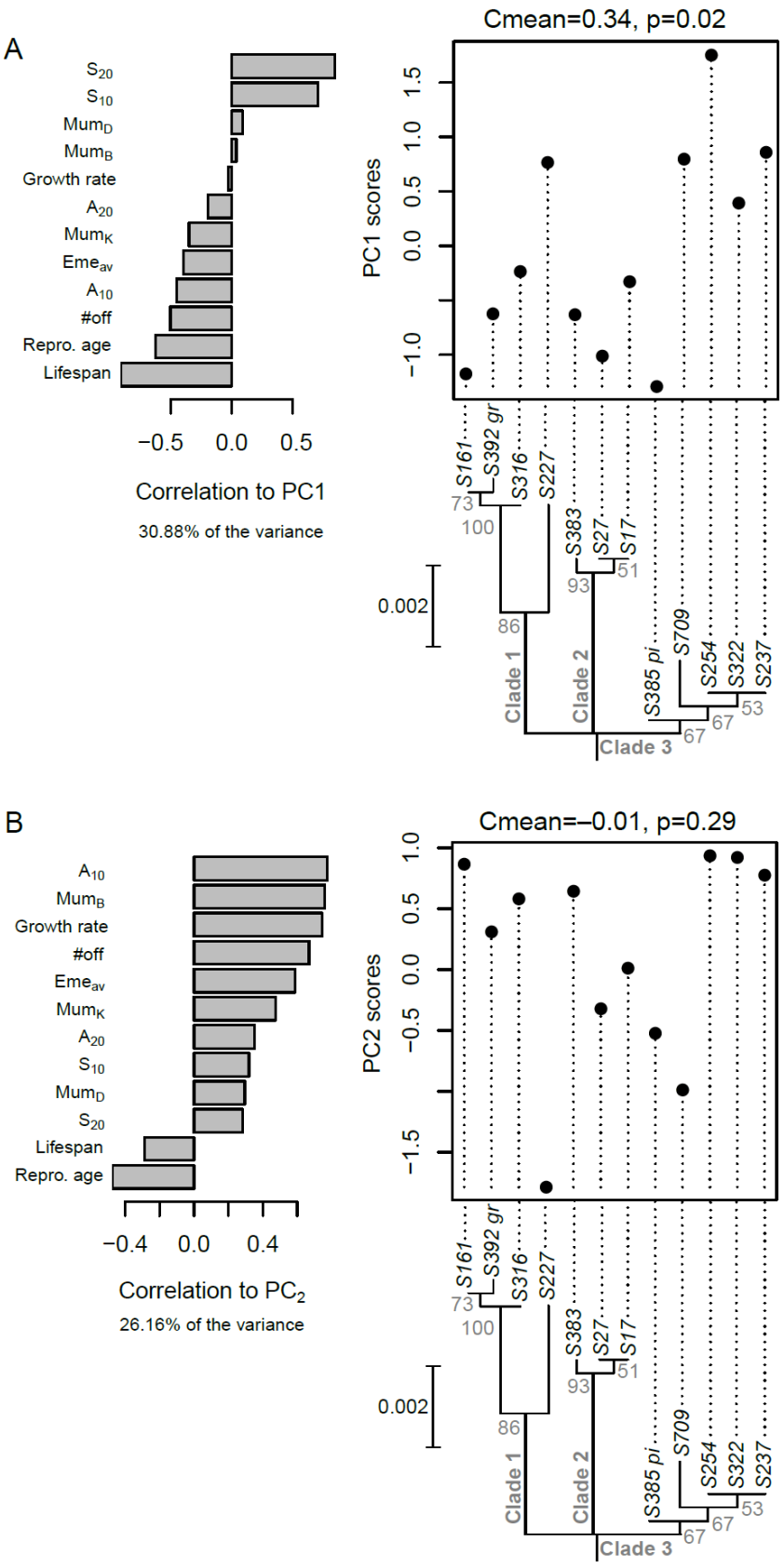

A
Spiroplasma / Aphid (# dnaA / # EF1a)
10 days old aphids
20 days old aphids
S316 S385 S161 S27 S383 S227 S17 S322 S392 S237 S709 S254
B
Spiroplasma (# dnaA)
10 days old aphids
20 days old aphids
S316 S385 S161 S27 S383 S227 S17 S322 S392 S237 S709 S254
C
Aphid (# EF1a)
10 and 20 days old aphids
S316 S385 S161 S27 S383 S227 S17 S322 S392 S237 S709 S254

Figure 5


A
S20
S10
MumD
MumB
Growth rate
A20
MumK
Emeav
A10
#off
Repro. age
Lifespan
−0.5
0.0
0.5
Correlation to PC1
30.88% of the variance
Cmean=0.34, p=0.02
PC1 scores
−1.0
0.0
0.5
1.0
1.5
S161
S392 gr
S316
S227
S383
S27
S17
S385 pi
S709
S254
S322
S237
73
100
93
51
86
53
67
67
0.002
Clade 1
Clade 2
Clade 3
B
A10
MumB
Growth rate
#off
Emeav
MumK
A20
S10
MumD
S20
Lifespan
Repro. age
−0.4
0.0
0.4
Correlation to PC2
26.16% of the variance
Cmean=−0.01, p=0.29
PC2 scores
−1.5
−0.5
0.0
0.5
1.0
S161
S392 gr
S316
S227
S383
S27
S17
S385 pi
S709
S254
S322
S237
73
100
93
51
86
53
67
67
0.002
Clade 1
Clade 2
Clade 3