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Male-killer symbiont screening reveals novel associations in *Adalia* ladybirds

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Abstract

While male-killing bacteria are known to infect across arthropods, ladybird beetles represent a hotspot for these symbioses. In some host species, there are multiple different symbionts that vary in presence and frequency between populations. To further our understanding of spatial and frequency variation, we tested for the presence of three male-killing bacteria: *Wolbachia*, *Rickettsia* and *Spiroplasma*, in two *Adalia* ladybird species from a previously unexplored UK population. The two-spot ladybird, *A. bipunctata*, is known to harbour all three male-killers, and we identified *Spiroplasma* infection in the Merseyside population for the first time. However, in contrast to previous studies on two-spot ladybirds from continental Europe, evidence from egg-hatch rates indicates the *Spiroplasma* strain present in the Merseyside population does not cause embryonic male-killing. In the related ten-spot ladybird, *A. decempunctata*, there is only one previous record of a male-killing symbiont, a *Rickettsia*, which we did not detect in the Merseyside sample. However, PCR assays indicated the presence of a *Spiroplasma* in a single *A. decempunctata* specimen. Marker sequence indicated that this *Spiroplasma* was divergent from that found in sympatric *A. bipunctata*. Genome sequencing of the *Spiroplasma*-infected *A. decempunctata* additionally revealed the presence of cobionts in the form of a *Centistes* parasitoid wasp and the parasitic fungi *Beauveria*. Further study of *A. decempunctata* from this population is needed to resolve whether it is the ladybird or wasp cobiont that harbours *Spiroplasma*, and to establish the phenotype of this strain. These data indicate first that microbial symbiont phenotype should not be assumed from past studies conducted in different locations, and second that cobiont presence may confound screening studies aimed to detect the frequency of a symbiont in field collected material from a focal host species.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files. Supporting data has been deposited to Figshare with the following DOIs: PCR primer sequences and conditions (https://doi.org/10.6084/m9.figshare.21915558.v1 [1]); individual sample collection and screening data (https://doi.org/10.6084/m9.figshare.21865035 [2]); *Spiroplasma* genome annotations (https://doi.org/10.6084/m9.figshare.21865113.v1 [3]). Four *Spiroplasma* 16S rRNA sequences have been deposited in GenBank (accessions: OQ271402-OQ271404; OQ271406). Further sequencing data has been deposited within BioProject PRJNA921942 to GenBank: whole-genome sequencing data for one *Adalia decempunctata* specimen (SRR23019831), and draft genome assemblies for *Spiroplasma* sp. (SAMN32746590) and *Beauveria* sp. (SAMN32746591).

INTRODUCTION

Arthropods are commonly infected with bacterial endosymbionts that have a profound impact upon their biology; affecting host physiology, development and susceptibility to natural enemies [4]. These effects on individuals can ultimately influence the dynamics of their host population and communities [5], reproductive behaviour [6, 7], and host evolution [8, 9]. The influence of maternally inherited endosymbionts is particularly pronounced as their mode of transmission produces a selective pressure on the symbiont to manipulate host reproduction to favour females. Several reproductive manipulations have been recorded,

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Keywords: endosymbiont; *Spiroplasma*; ladybird; *Adalia*; male-killing; cobiont.
Abbreviations: CDS, coding sequence; CI, cytoplasmic incompatibility; MK, male-killing; PE, paired end; RO, reverse osmosis.

including cytoplasmic incompatibility (CI), parthenogenesis induction (PI), feminization of genetic males, and male-killing (MK) [8, 10].

Male-killing endosymbionts – microbes that kill male host offspring, usually early in development during embryogenesis – are particularly important for two main reasons. First, when they become common, they distort the population sex ratio and this can influence host reproductive ecology. In butterflies, for instance, high prevalence of male-killers in the host population is variously associated with increased female remating rate [7], reduced female capacity to encounter mates [11], and sex role reversal [6]. In ladybirds, the population sex ratio bias associated with a high frequency of MK microbes is sufficient to alter the dynamics of sexually transmitted infections in the host [12]. Where the MK microbe is common, females greatly exceed males in the population, and the mean mating rate of male ladybirds therefore exceeds that of female. This disparity of mating rate is reflected in epidemics of the sexually transmitted mite *Coccipolipus hippodamiae* in which male ladybirds become infected before females [12]. Second, male-killers are parasites, and may engender strong selective pressure upon their host to suppress their action. Indeed, the intense Fisherian selection for restoration of the rare sex make selection to suppress male-killer activity amongst the strongest observed in natural systems [13, 14]. It is also hypothesized that selection for suppression may act on the sex determination system of the host, and thus represent a driver of sex determination system evolution [9].

While MK microbes are known to infect a range of arthropods, members of the family Coccinellidae (ladybird beetles) are particularly commonly infected, albeit with infection usually being at low-mid prevalence [15]. Male-killers in ladybirds derive from diverse microbial groups, including bacteria from the flavobacteria, Mollicutes, ɣ-proteobacteria and α-proteobacteria [16]. Breeding data alongside molecular genetic analysis has demonstrated that the two-spot ladybird *Adalia bipunctata* is infected with three heritable MK bacteria: *Spiroplasma*, *Rickettsia* and *Wolbachia* [17–19]. These vary in presence and frequency between populations. For instance, the *Rickettsia* male-killer has been found broadly in the UK, Germany, The Netherlands, Denmark and Russia, but is commonly at low frequency (<20% of females infected [17, 19, 20]). *Spiroplasma*, by contrast, varies greatly in frequency, being rare in Germany (<20% of females infected) but common in St. Petersburg, Russia (>20% of females infected), and Stockholm, Sweden (>50% of females infected [12, 19, 20]). *Wolbachia* MK infections have been reported solely in the Eastern part of the range, in Moscow [18, 20], although *Wolbachia* of unknown phenotype have also been found in two-spot ladybirds from Stockholm [12]. The causes of variation in the presence and prevalence of different male-killers across *A. bipunctata* populations is poorly understood.

In contrast, there is only one report of a MK infection in the congeneric and often sympatric ten-spot ladybird, *Adalia decempunctata*: a *Rickettsia*. Present in 4–6% of female beetles in two German populations, infected females produced strongly female biassed broods that also carried the *Rickettsia* through vertical transmission [21]. This previous work also noted the absence of *Spiroplasma* and *Wolbachia* in these *A. decempunctata* populations. While *Rickettsia* was also noted in ten-spot ladybirds from Stockholm (4 of 18 females were infected), it is unknown whether this strain also causes MK [22]. Surveys for MK symbionts have been conducted less broadly in *A. decempunctata* than in *A. bipunctata,* making it unclear whether differences between the species reflect intensity of study, breadth of populations studied, or real difference in the diversity and frequency of symbiont infection.

To further investigate the incidence of MK in *Adalia* ladybirds we tested for the presence of *Wolbachia*, *Rickettsia* and *Spiroplasma* in *A. bipunctata* and *A. decempunctata* from a previously un-investigated population in the UK (the Merseyside region). This represents a third population study for the ten-spot ladybird, and a further geographical sample for the two-spot ladybird to that previously studied. We identified *Spiroplasma* in this population of *A. bipunctata* and examined whether infection is associated with embryonic MK activity, as observed elsewhere. We also report an *A. decempunctata* individual that tested positive for *Spiroplasma*, with onward genomic analysis of this association.

METHODS

Sample collection

Thirty-seven adult *A. bipunctata* (15 male, 22 females) and 40 adult *A. decempunctata* (20 males, 20 females) were collected by eye from Liverpool in March 2022 (*A. decempunctata* and *A. bipunctata*), and from Liverpool and the Wirral in May–June 2022 (*A. bipunctata* only). Material collected in March derived from overwintering sites (gravestones in Anfield Cemetery). Material collected in May–June derived from lime trees (*Tilia europea*), rose (*Rosa sp.*) and nettle (*Urtica dioica*). Adults were sexed morphologically following characteristics in Randall *et al.* [23]. Details of beetle collection times and locations can be found in supporting data (https://doi.org/10.6084/m9.figshare.21865035 [2]).

Embryonic male-killing phenotype in *A. bipunctata*

Twenty female *A. bipunctata* from Liverpool and Wirral were individually placed at room temperature in petri dishes along with filter paper on which to lay eggs, supplied with aphids to eat, and a male *A. bipunctata* to maintain fertility. Each day, any egg clutches laid were removed, the eggs counted, and placed in empty labelled petri dishes. At 5–6 days post-oviposition, the number of hatched eggs were scored. From these data, the proportion of eggs that hatched per clutch was calculated

(hatched eggs/total eggs). These data were combined with later infection data of the mothers gained from PCR assays, to indicate whether the symbiont causes embryonic MK.

Estimating endosymbiont presence and prevalence

To test for the presence of bacterial endosymbionts in the two *Adalia* species, DNA was first isolated from either whole adult or leg tissue of ladybirds that had been fed on an artificial diet for 48 h prior to being flash frozen in liquid nitrogen. Tissue was homogenized and DNA template was purified from the homogenate following the Promega Wizard Genomic DNA Purification protocol (www.promega.com). PCR amplification of the host mitochondrial DNA CO1 region (primers HCO/LCO [24]) was carried out to confirm successful DNA extraction. Samples were then tested for *Spiroplasma, Rickettsia* and *Wolbachia* infection *via* PCR assays alongside known positive and negative control samples. Primers (1 µl of 20 pmol μl^{-1}) amplifying *Spiroplasma* (Haln1/MGSO [25]) *Rickettsia* (Ri_Meg17kD_F/ Ri_Meg17kD_R [26]) and *Wolbachia* (81 F/ 691R [27]) were used in separate reactions with the following reagents: 7.5 µl Promega Hotstart GoTaq, 5.5 µl of RO water and 1 µl DNA template. PCR primers and conditions are available at https://doi.org/10.6084/m9.figshare.21915558 [1]. The PCR products were run on a 1.5% agarose gel with 3 µl of Midori Green added to allow visualization of amplicons under UV light.

The resulting amplicons were sequenced from a subset of PCR-positive samples to confirm symbiont identity. To this end, PCR products were purified via the Bioline Isolate II Genomic DNA Kit protocol (www.bioline.com). Each sample was washed using CB buffer, CW buffer and then resuspended in water. Purified samples were sent along with the MGSO primer for *Spiroplasma* identification and the HCO primer for CO1 insect DNA, for onward Sanger sequencing by Eurofins (www.eurofins.co.uk). Similar sequences were searched using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), then aligned and compared in Geneious Prime (v2021.2.2, https://www.geneious.com).

Whole-genome sequencing

Symbiont presence in wild caught specimens can derive from the specimen itself or from cobiont taxa such as parasitic wasps, nematodes, or phoretic mites that are on or in the focal individual (e.g. [28]). Cobionts can be detected from whole-genome-sequencing data, which then additionally allows draft genomes of the symbionts themselves to be constructed. We therefore obtained genomic DNA sequence for the *A. decempunctata* specimen infected with *Spiroplasma*. DNA isolated from the infected male was sent to Novogene (www.novogene.com) for Illumina sequencing. A total of 132.7 million raw reads were produced (150 bp PE, Q20 of 95.4%). Low-quality reads, reads containing Ns, and short reads were removed from the dataset, and adaptors were trimmed from reads, using fastp and default settings (v0.23.2 [29],) to produce a trimmed and filtered dataset (65.8M forward and reverse reads, with Q20 of 96.8 and 94.9%, respectively). *Spiroplasma* presence was confirmed in this sample using Phyloflash (v3.4 [30],) on the trimmed reads.

Trimmed reads were *de novo* assembled using megahit (v1.2.9 [31]), to produce a draft metagenomic assembly containing both eukaryotic and *Spiroplasma* sequences. Trimmed reads were also mapped back to the draft metagenomic assembly using BWA-MEM2 (v2.0pre2 [32]) to obtain coverage information. Using taxonomic assignment data from Blobtools2 (v3.0.0 [33]) and local BLAST +searches (v2.2.29 [34]), the metagenomic assembly was split into separate organism draft genome assemblies. To assess completeness and contamination of the draft genome assemblies, BUSCO (v5.2.2 [35]) scores were calculated, using appropriate databases for the taxonomic group. The raw Illumina sequencing data has been submitted as SRA to NCBI (accession: SRR23019831).

Spiroplasma genome annotation

The draft *Spiroplasma* genome obtained from sequencing the infected *A. decempunctata* individual was annotated using PROKKA (v1.14.6 [36]) and Interproscan5 (v5.59–91.0 [37]). We also specifically examined the genome for the presence of toxin genes that are considered candidates for the mechanism of MK (i.e. ankyrin repeats and OTU-like cysteine proteases, as found in the plasmid-encoded SPAID protein [38]) or protective phenotypes [i.e. genes encoding ribosomal inactivating proteins (RIPs) originally identified in *S. poulsonii*, which damage the ribosomes of parasitic wasps and nematodes of *Drosophila* [39–41]]. We also searched for high-mobility group (HMG) box domain proteins that have been implicated recently in *Spiroplasma*-induced CI [42]. To this end, we used UniProt alignments downloaded from the Pfam database (pfam-legacy.xfam.org) for protein families RIP (PF00161) and OTU (PF02338). These alignments were then used in HMMER (v3.3.2, hmmer.org), with the PROKKA CDS as queries. Coding sequences containing ankyrin repeats or HMG box domains were identified in the Interproscan5 annotation output. SignalP-6.0 (https://services.healthtech.dtu.dk/service.php?SignalP-6.0) was utilized to identify signal peptides within the CDS, particularly in the genes of interest.

Spiroplasma phylogeny

A local BLAST+ search of the draft *Spiroplasma* genome using the Sanger-sequencing short sequence of the *Spiroplasma* 16S rRNA gene as query was used to identify the full sequence of the gene. Nucleotide sequence of 16S rRNA genes from other *Spiroplasma* strains were obtained from (a) *Spiroplasma* 16S deposits in NCBI GenBank, including the full length 16S gene sequence of

Spiroplasma infecting *Adalia bipunctata* (accession: AJ006775) or (b) whole-genome sequence data of *Spiroplasma* (the 16S sequence was extracted using a local BLAST +search of the genomes of *Spiroplasma* infecting *Lariophagus distinguendus* (accession: GCA_023846195) and *Nebria riversi* (accession: GCA_018831625). These were then aligned using MUSCLE (v3.8.425 [43]). The relatedness of strains was estimated using a maximum-likelihood-based (ML) method, based on the GTR+F+I+I+R2 model chosen by ModelFinder [44], using IQTree (v 2.2.0.3 COVID-edition [45], with 1000 Bootstrap replicates calculated to assess internal branch support. The 16S rRNA sequence from *Mycoplasma genitalium* was used to root the tree (accession: OM509887).

RESULTS

Male-killer prevalence in *Adalia* ladybirds

No *Wolbachia* or *Rickettsia* infected specimens were identified in our samples of either *A. bipunctata* or *A. decempunctata*. In contrast, both *A. bipunctata* and *A. decempunctata* were found to be infected at low prevalence with *Spiroplasma* (18 and 5% of individuals, respectively; Table 1; individual data is available at https://doi.org/10.6084/m9.figshare.21865035 [2]). *Spiroplasma* in *A. bipunctata* was detected solely in female individuals (4 of 22 females vs 0 of 15 males), and was recovered from template derived from leg material. However, statistical analysis cannot reject the null hypothesis of equal prevalence between sexes (Fisher's exact test, P=0.13). In *A. decempunctata*, the single *Spiroplasma*-infected individual was male. Sanger sequencing of the *Spiroplasma* 16S rRNA gene from two *A. bipunctata* and the single *A. decempunctata* sample identified as positive for infection from the PCR assay confirmed the presence of an *ixodetis* group *Spiroplasma* strain in both species. The 335 bp of *Spiroplasma* 16S rRNA sequenced from both *Adalia* species were 100% identical, and had a 99.7% sequence similarity to *Spiroplasma ixodetis*, known to infect diverse arthropods and which can cause MK in some species [20, 25, 46, 47]. The three partial *Spiroplasma* 16S rRNA sequences were deposited in GenBank (accessions: OQ271402-OQ271404).

Spiroplasma does not cause embryonic MK in *A. bipunctata*

Of the 20 female *A. bipunctata* that laid eggs, four were later determined by PCR assay to be infected with *Spiroplasma*. Of these four, none had egg-hatch rates that were indicative of embryonic MK (where the males die as embryos, resulting in *c.* 50% egg-hatch rate) (Table 2). Comparisons of the egg-hatch rates from *Spiroplasma*-infected females vs uninfected females did not reject the null hypothesis of equal egg-hatch rate in *Spiroplasma* infected *vs* uninfected females (Mann–Whitney U test: N1=16, N2=4, U=30 P=0.89).

Identification of novel associations in *A. decempunctata*

BUSCO analysis of the initial draft metagenome assembly of the *Spiroplasma* infected *A. decempunctata* sample, using the Insecta database, revealed that a large proportion of the BUSCOs were duplicated (*D*), indicating contamination with DNA from a different insect (BUSCO insecta_odb10: C:98.3%[S:50.8%,D:47.5%],F:1.3%,M:0.4%,n:1367). Taxonomic assignment of the assembly contigs revealed the presence of substantial genomic sequence from two further organisms in addition to the ladybird and *Spiroplasma* bacteria: a hymenopteran and a *Beauveria* fungus. To identify the hymenopteran further, a local BLAST+ search of the assembly using an *A. decempunctata* COI gene sequence (NCBI accession: KU917463) was undertaken and identified the presence of an arthropod non-*Adalia* COI sequence as well as the *A. decempunctata* COI gene. This non-*Adalia* sequence was used as a query against the complete BOLD COI database (www.boldsystems.org), which identified the closest affiliation with a parasitic wasp of the genus *Centistes* (84.6% similarity).

Whilst it was not possible to separate the two insect genomes satisfactorily, as neither the ten-spot ladybird or the *Centistes* wasp have sufficient existing genomic resources to enable this, a draft genome of the *Beauveria* genome could be assembled (accession: SAMN32746591). This assembly consists of 3480 contigs totalling 33539846 bp (33.5 Mb), with an N50 of 24297 and GC content of 52.47%. The draft genome is 92.5% complete (BUSCO hypocreales_odb10: C:92.5%[S:92.1%,D:0.4%],F:3.9%,M:3.6%,n:4494). A genome size of 33.5 Mb is comparable to published *Beauveria* genomes; strains of the entomopathogenic fungus *Beauveria bassiana* have genomes in the range of 33–39 Mb [48].

Table 1. Symbiont infection status of *A. bipunctata* and *A. decempunctata* partitioned by host sex, with infection status determined through PCR assay

Species	Sex	*N*	Uninfected	*Spiroplasma*	*Wolbachia*	*Rickettsia*
Adalia bipunctata	Male	15	15	0	0	0
	Female	22	18	4	0	0
Adalia decempunctata	Male	20	19	1	0	0
	Female	20	20	0	0	0

Table 2. Egg-hatch rates from *A. bipunctata* females partitioned by *Spiroplasma* infection status

Ladybird	*Spiroplasma* infection	Eggs laid	Eggs hatched	HR
AbipLV04	Yes	97	86	0.89
AbipLV17	Yes	18	11	0.61
AbipLV20	Yes	35	34	0.97
AbipGB02	Yes	124	116	0.94
AbipLV02	No	123	122	0.99
AbipLV03	No	88	81	0.92
AbipLV08	No	33	29	0.88
AbipLV09	No	142	115	0.81
AbipLV10	No	30	25	0.83
AbipLV11	No	6	0	0
AbipLV12	No	53	51	0.96
AbipLV16	No	50	41	0.82
AbipLV18	No	13	13	1
AbipLV19	No	14	10	0.71
AbipWL01	No	20	19	0.95
AbipWL02	No	114	98	0.86
AbipWL03	No	116	110	0.95
AbipOX01	No	67	64	0.96
AbipOX02	No	100	86	0.86
AbipGB01	No	49	31	0.63

HR, Egg hatch rate.

Spiroplasma genome assembly and annotation

The draft genome of the *Spiroplasma* infecting *A. decempunctata* consists of 55 contigs, including six contigs that are putative plasmids or partial plasmids (accession: SAMN32746590). Total size is 965424 bp with a GC (%) of 26.77 and N50 of 23759 bp. Genome completeness is estimated to be 96.7% according to BUSCO, with three missing BUSCOs (alanine–tRNA ligase, rRNA (cytidine-2′-O-)-methyltransferase and 50S ribosomal protein L7/L12). However, PROKKA identified all three among the CDS annotated, and so the draft genome is near complete. In all, annotation using PROKKA revealed there to be 937 CDS (380 annotated), 27 tRNAs, three rRNAs (5S, 16S and 23S) and one tmRNA within the main *Spiroplasma* contigs, and 65 CDS (three annotated) within the six putative plasmid contigs.

No genes carrying RIP toxin domains were identified in the *Spiroplasma* genome, however two CDS contain OTU-like cysteine protease domains. Interestingly, both sequences are closely related to *Spiroplasma* homologues including that recently published from a CI-inducing *Spiroplasma* infecting the parasitic wasp *Lariophagus distinguendus* [42]. Unlike for the *Spiroplasma* MK candidate gene, SPAID, the CDS identified here containing OTU-like cysteine protease domains do not additionally contain ankyrin repeats. However, several other CDS-containing ankyrin repeats were detected; there were ankyrin repeat domains in 11 CDS in the main genome, and a further two among the plasmid CDS. Also of interest is that the *Spiroplasma* genome plus plasmids encode a total of five proteins containing HMG box domains that are very rare in bacteria, though found in several ixodetis group *Spiroplasma*. SignalP analysis reveals that none of the genes of interest contained signal peptides. Annotations are available at https://doi.org/10.6084/m9.figshare.21865113 [3].

Spiroplasma phylogeny

Analysis of the complete 16S rRNA gene obtained from Illumina sequencing of the *A. decempunctata* specimen revealed that this strain of *Spiroplasma* resided within the *ixodetis* clade. However, the *A. decempunctata* derived *Spiroplasma* 16S rRNA was distinct to that of the *Spiroplasma* infecting *A. bipunctata* (Fig. 1). The full 16S rRNA sequence of the *A. decempunctata Spiroplasma* was deposited in GenBank (accession: OQ271406).

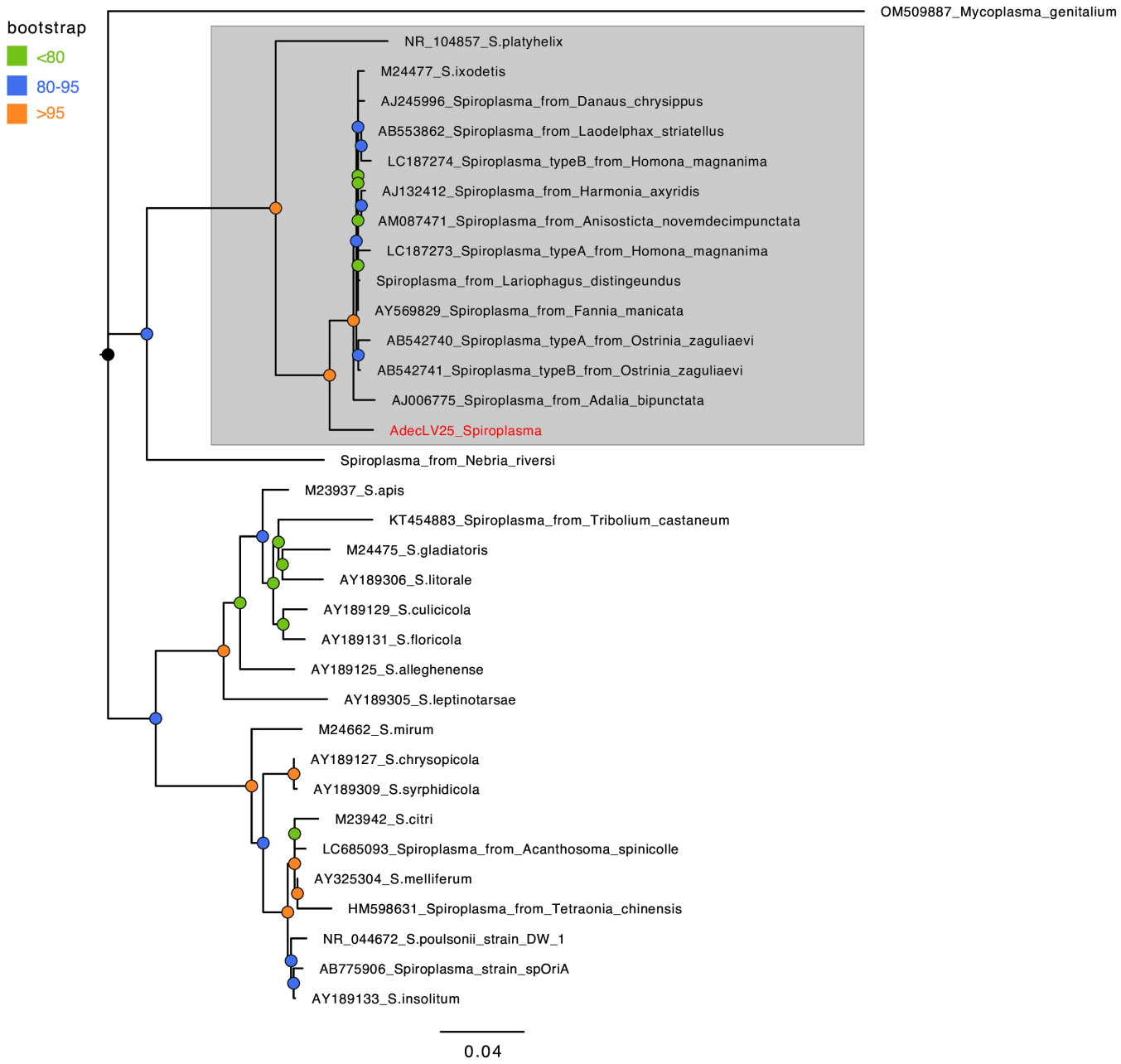

Fig. 1. Phylogenetic tree of *Spiroplasma* constructed using full-length 16S rRNA gene sequences as estimated in IQTree. The *Spiroplasma* 16S rRNA sequence obtained from sequencing *Adalia decempunctata* is highlighted in red. Bootstrap support is given by node colour. Scale bar indicates nucleotide divergence along branches. The *ixodetis* clade, to which the *Spiroplasma* from *A. decempunctata* belongs, is indicated by the grey shaded region. The 16S rRNA gene sequence of *Mycoplasma genitalium* was used to root the tree.

DISCUSSION

Spatial variation in heritable symbiont presence is well known in insects [49] such that a full account of symbionts within a species requires analysis of multiple populations. In this paper, we examined the Merseyside population of two ladybirds, *A. bipunctata* (known in other populations studied to be infected with *Wolbachia, Rickettsia* or *Spiroplasma* depending on population sampled, e.g. [20]) and *A. decempunctata* (known to carry MK *Rickettsia* in Germany [21]).

In *A. bipunctata*, a *Spiroplasma ixodetis* relative was found in four female individuals. The *Spiroplasma* was identical in 16S rRNA sequence to the previously reported maternally inherited strain found in *A. bipunctata* [12, 19]. Because of this prior work, it is most parsimonious to presume the strain is an associate of the ladybird rather than a cobiont. However, in contrast to previous

laboratory studies, embryonic MK was not observed in infected females from this population, with infected females producing clutches with high egg-hatch rates. This observation, alongside previous PCR screens indicating *Spiroplasma* infection in both male and female *A. bipunctata* from Scotland [50] indicate *Spiroplasma* is not an early male-killer in UK *A. bipunctata* (our data), and that at least some male hosts can survive *Spiroplasma* infection. To determine whether the strain of *Spiroplasma* present is alternatively a late male-killer (as observed in planthoppers [47]), clutches would need to be reared through to maturity. Additionally, transinfecting the *Spiroplasma* onto a Scandinavian genetic background known to support MK would allow us to establish whether phenotype differences are associated with host genetic background (as found for other MK symbiont/host interactions [14, 51]) or symbiont differences (as found in tea tortrix moths [52]). It would also be interesting to determine whether the strain expresses an alternative phenotype such as parasite protection. It is notable that *Rickettsia* infection was not observed in Merseyside *A. bipunctata*, despite being reported in populations from both Southern UK and Scotland [50]. However, *Rickettsia* in *A. bipunctata* commonly exists at low prevalence (5–10% of females [19]) and may be present in Merseyside but not in our sample of beetles.

Previous work on ten-spot ladybirds revealed male-killing *Rickettsia* in 5–10% of female beetles collected from Germany [21]. We did not find evidence for *Rickettsia* in Merseyside ten-spot ladybirds, but again the relatively small number of females collected makes it impossible to exclude presence as a rare associate. More surprisingly, *Spiroplasma* bacteria were detected for the first time in *A. decempunctata*, from a single male individual. To investigate whether it is the same strain as the MK *Spiroplasma* carried by *A. bipunctata*, and whether the field caught specimen carried cobionts that might be an alternative source of the infection, genomic sequence was obtained from this individual. Comparison of the full *Spiroplasma* 16S rRNA gene from the *A. decempunctata Spiroplasma* with those previously sequenced indicated that it is not a sister strain to that previously recorded in *A. bipunctata* but is a novel strain in coccinellids. Assembly of the genome of the *Spiroplasma* identified in *A. decempunctata* revealed that it has a genome at the smaller end of the range (965 kbp) relative to other *Spiroplasma* (780 to 2220,220 kbp); more similar in size to *Spiroplasma platyhelix* infecting the dragonfly *Pachydiplax longipennis* [53, 54].

Unexpectedly, the genome data indicated that the *Spiroplasma* infected *A. decempunctata* was also infected with a parasitoid wasp from the Braconidae genus *Centistes*, representing a new natural enemy record for *A. decempunctata*. Ladybirds are host to a number of parasitoids, the best documented of which is another braconid wasp – *Dinocampus coccinellae* [55–57]. While *Centistes* wasps are known to parasitize beetles including ladybirds [58], their interaction with ladybirds remains poorly characterized. The presence of the wasp within the ladybird host raises the question of whether the *Spiroplasma* infects the wasp or the ladybird. *Spiroplasma* have been recorded as symbionts of parasitoid wasps, including the recent discovery of a novel CI-inducing *Spiroplasma* strain in the wasp *Lariophagus distinguendus* [42]. Future work would establish the host for the *Spiroplasma* through a broader collection of wasps and ladybirds, and fluorescence *in situ* hybridization (FISH) analysis to establish the site of symbiont infection.

The phenotype of the *Spiroplasma* identified in the *A. decempunctata* metagenome remains unknown. Should it be a symbiont of the ladybird, its presence in a male suggests either incomplete MK or a non-MK *Spiroplasma* strain. Annotation of the *Spiroplasma* genome reveals that it encodes two OTU-like cysteine proteases closely related to a homologue found in the CI-inducing *Spiroplasma* of the parasitic wasp *Lariophagus distinguendus* [42]. Similarly, the genome also contains multiple high-mobility group (HMG) box proteins as does the *Spiroplasma* in *Lariophagus distinguendus*. HMG box domain containing genes are common in eukaryotes, but very rare in bacteria. Indeed, all records to date are from *ixodetis* group *Spiroplasma*. In addition, 13 CDS were identified to contain ankyrin-repeat domains, which commonly mediate interactions with host eukaryotic proteins and are important components of establishing symbiosis and symbiont phenotype [59]. This genome therefore provides several candidates for further investigation.

This case study highlights a general issue of the accuracy of screening programmes where field collected material is analysed solely on the basis of PCR assays – namely that an individual of a species may return PCR positive, but this is a false positive for the specimen and/or species because it arises from a cobiont. Our study complements others, with recent cases identifying *Arsenophonus* and *Wolbachia* amplifying from cobiont material rather than the identified collected arthropod species [28, 60]. The problem of false-positive reports from cobionts is likely greatest for infections that are apparently rare within a species, because cobionts will tend to be in a fraction of individuals rather than all individuals. Whilst rare symbiont infections certainly exist in insects, PCR screening data alone should therefore not be used to establish their presence. Rather, low prevalence infections require corroboration either from whole specimen genome sequence (excluding a cobiont from being in the material), laboratory breeding work using individuals uninfected with the cobiont, or additional FISH studies (localizing symbiont to host).

Genome sequencing of the *A. decempunctata* individual also revealed the presence of a parasitic fungus from the genus *Beauveria*. These fungi are facultative pathogens commonly known to infect ladybirds via exposure in leaf matter and soil during overwintering [61, 62]. *Beauvaria* are known to be able to infect and cause mortality in *Adalia* in laboratory exposure trials [63]. However, despite being documented as a major cause of mortality in *Coccinellidae* [64], mycosed *Adalia* have not been reported in the field, leading to the widespread belief that their above ground overwintering site makes exposure unlikely. Our infected individual was an individual collected from an above ground overwintering site (gravestone) and indicates *Beauvaria* does infect this species in the field despite being spatially separate from leaf litter sources of infection.

In conclusion, we screened for the presence of three common male-killers in two *Adalia* ladybirds. While our data extends previous work in *A. bipunctata* in describing *Spiroplasma* presence in a new population, we also show that this strain does not cause embryonic male-killing, contrasting to its phenotype in *A. bipunctata* from Continental European and Scandinavian populations. We also present the discovery of a novel strain of *Spiroplasma* in *A. decempunctata*, quite unlike those previously sequenced from ladybird beetles. Genomic analysis of the metagenome of *A. decempunctata* unexpectedly revealed the presence of two further organisms infecting the ladybird: a *Centistes* parasitoid wasp, and the entomopathogenic fungus *Beauveria*. These cobiont records highlight the complexity of ascertaining the infection status of field-collected individuals. Moreover, it emphasizes that organisms rarely, if ever, develop, live, or evolve without direct interaction with a multitude of others.

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Author contributions
Conceptualization: G.H., E.H. Data curation: E.H., J.A. Formal analysis: E.H., J.A., G.H. Funding acquisition: G.H. Investigation: J.A., E.H. Methodology: E.H., G.H. Project administration: E.H., G.H. Resources: G.H., E.H., J.A. Software: E.H. Supervision: E.H., G.H. Validation: G.H., E.H., J.A. Visualization: E.H., J.A., G.H. Writing – original draft: J.A., E.H. Writing – review and editing: E.H., G.H., J.A. Field collections: E.H., G.H. and J.A.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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Peer review history

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000585.v2.1

Jana Katharina Schniete; Leibniz Universitat Hannover Naturwissenschaftliche Fakultat, Institut fuer Mikrobiologie, Herrenhaeuser Strasse 2, Geb 4104, GERMANY, Hannover

Date report received: 18 May 2023
Recommendation: Accept

Comments: The revised version of the paper has addressed all concerns raised by the reviewers.

Author response to reviewers to Version 1

Response to Reviewers

Editor comments:

The reviewers have highlighted major concerns with the work presented. Please ensure that you address their comments.

Most importantly, a more elaborate introduction is needed as raised by reviewer 1 as well as reviewer 2 raises an important concern: details are lacking in the methods section- in particular around how and where the samples were taken and how they were fed in the lab.

We have made the introduction longer and more thorough, emphasising the state of the art of the field and why male-killing bacteria are interesting. The online supplementary material now has full details of collection dates and places. Feeding details are supplied. We thank the editor and reviewers for their constructive comments, and we especially feel the more in-depth introduction has benefitted the paper.

Reviewers' comments and responses to custom questions:

Reviewier 1

Please rate the manuscript for methodological rigour

Reviewer 1: Very good

Please rate the quality of the presentation and structure of the manuscript

Reviewer 1: Satisfactory

To what extent are the conclusions supported by the data?

Reviewer 1: Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 1: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 1: Yes:

Reviewer 1 Comments to Author: Dear authors and editors,

Thank you for the interesting paper, I enjoyed reading it. It presented novel research well with some intriguing conclusions and an abundance of future possibilities of study, including a very thorough experimental setup and write-up. My main criticisms of the article are mainly on style and the briefness of the introduction leading to a lack of background to the study, so I suggest minor revisions before accepting and publishing.

1. Methodological rigour, reproducibility and availability of underlying data

The methodology of this article is very thorough and should make it easy to duplicate the entirety of the experiment for any researcher. The researchers not only screened the caught ladybirds for infections, they also investigated other strains of DNA found alongside the bacteria, leading to some of the more interesting conclusions: a braconid wasp's DNA was found, as well as an infectious fungus (Beauveria) which has not yet been described to infect Adalia spp. All the data was very easy to find, as it all has been submitted to databases online and have been directly linked in the article itself.

2. Presentation of results

The results of the experiments have been presented clearly and succinctly. Some results can not be interpreted as statistically significant, which is stated upfront by the authors. As the incidence of these bacteria is low in these ladybirds, a weak statistical power is to be expected due to a relatively small number of data points (i.e. four infected females). However, this is typical for studies investigating these interactions (eg. von der Schulenberg et al., 2000) and still allows for comparison between populations. I am curious why "it was not possible to separate the two insect genomes satisfactorily" (line 276), as this can have a variety of reasons, but is not expanded upon. While the numbers of caught Adalia are small, the authors make no mention of the sex ratios of the wild-caught population which in a glance seem evenly divided - it might be worth mentioning as sex ratios of A. bipunctata can be extremely skewed (eg. Zakharov et al., 2006), as a result of male-killing endosymbionts.

Population sex ratios from field caught material should be treated with caution in ladybirds – these are known to depend on time of year of the collection. For instance, male ladybirds die disproportionately overwinter, and early in the spring reproductive period, such that population sex ratios become highly skewed towards May/June even in the absence of male-killers. Thus, we don't make inferences between populations sex ratio and male-killer presence, as the latter is just one contributor to the overall pattern. We do now note case studies of strongly biased sex ratios from the field that are caused by male-killers in the introduction, including the suggested reference.

Separation of the genomes of the two species is hard because there is no reference genome for either the 10-spot ladybird or *Centistes*, and the use of Illumina reads means there are many 1000s of insect contigs that would need partitioning. Isolating these insect genomes is also not the main driver of this paper – the sequence was to isolate symbiont genomes and identify cobionts.

3. How the style and organization of the paper communicates and represents key findings

The paper has an odd balance of its organization: the introduction is extremely brief and fairly confusing, while the discussion is very thorough and intriguing. I was put off from reading the rest of the article from its introduction, which is a shame considering the results and discussion are well worth your time. The introduction would do well to introduce a few of the concepts outlined as results in the discussion: for example, the Scandinavian, Central European, and Scottish populations of Adalia spp. and their infection rates should be mentioned as they come in play later. Additionally, the urgency of this research is somewhat lost from its introduction as almost every citation is over 15 years old and some key points are argued in a confusing way. For example, line 73 is an odd sentence talking about a two-fold cost for the Adalia, which suddenly talks about a high reproductive success for the males without explaining why this is detrimental. Some other background mentioned in the discussion, such as the other associations (wasps, fungi), are not mentioned in the introduction at all. This also ties into the next point:

Thank you – these are useful comments. We have expanded the literature review in the introduction to include more current insight into why male-killers are important for host ecology and evolution, and also more detail on patterns in both two-spot and ten-spot ladybirds. We haven't considered cobionts in the introduction as these were not the focus of the paper, but emerged during the study, and we have left our introduction as our 'original intentions' for the study. Cobionts remain extensively covered in discussion as an outcome of the study.

4. Literature analysis or discussion

The background sketched in this article of the research subject is fairly poor. Most articles cited in the introduction are not recent, even though there is a wealth of recent research on Adalia spp. and other coccinellids with regards to their symbionts and how this affects their ecology. The introduction is much too brief and sometimes does not cite sources for important statements; for example, line 85 mentions "The disparity in the number, and prevalence, of different male-killers in A. bipunctata vs. A. decempunctata is poorly understood", but it is unclear if there have been investigations of both species and the sex ratios of their populations at all. There has been one mention in a German study of infections of A. decempunctata, but a lack of reports is not enough to state that there is such a disparity. The introduction could also expand on putting the trophic interactions

between Adalia and their symbionts into a broader ecological context and their place in the variety of interactions of parasites in other coccinellids. Taking some more time to explain the background more in depth would significantly improve this paper. In comparison, the discussion is excellent, and raises several questions gained from the results of the study, as well as multiple avenues of interesting future research. It is worth mentioning with regards to novel Beauveria infection finding that infections of one species of parasite can sometimes result in susceptibility to other parasites; for example, in Harmonia axyridis, parasitic fungi, mites, and bacteria can affect other parasites, sometimes as competition, sometimes as enabler.

We now expand on the introduction in terms of both wider background and the past patterns of symbiont presence in the two species. We don't cover other associates as these were not an original aim, but do cover these in the discussion.

5. Any other relevant comments

There are a few other minor comments or criticisms of the writing itself. Line 67 has a reference to Jaenike et al., but this is a very specific paper on defensive symbionts; a more general review on the effects of endosymbionts in insects would be better here. Line 70 mentions "selection", where selective pressure is meant. Line 78 has "Coccinelidae" in italics, as do the other mentions of families in rest of the paper; family names should not be italicized.

We have taken these comments into consideration in revision, thank you.

Reviewer 2

Please rate the manuscript for methodological rigour

Reviewer 2: Poor

Please rate the quality of the presentation and structure of the manuscript

Reviewer 2: Satisfactory

To what extent are the conclusions supported by the data?

Reviewer 2: Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?

Reviewer 2: No:

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?

Reviewer 2: Yes:

Reviewer 2 Comments to Author:

The manuscript addresses an interesting topic. However, there are methodological ambiguities that need to be clarified. Otherwise, the replicability of the study would be unapproachable. Furthermore, the contribution of the study to the spatial component of the relationship between MK symbionts and Adalia species is not clear, if any.

The description of insect sampling needs to be greatly improved. Where exactly are the sampling sites? Please provide complete information. How exactly was the sampling done? What method was used? Sweeping net, fall traps or any other? Are these localities representative of the male/female ratio of these species? Once again, the description of the sample collection is very poor. To what extent is this sample size representative of the biology of both Adalia species?

We now provide these details for completeness. Sample collection details are precisely given in the supplementary online data (collection times, locations), but we would argue they are not as important as the referee believes, simply because *Adalia*is a very motile species, and will have moved from summer sites on plants, to overwinter sites away from plants, and back to plants during their life and so are 'well mixed.

The samples are not representative – 10 spots were easy to find and sampled only in March from overwintering, 2 spots are rare because of *Harmonia*(predatory) and were sampled over a longer period. This is now noted in the paper.

In the section "Embryonic phenotype that kills males in A. bipunctata" it is mentioned that 20 females of A. bipunctata, after collection, were placed individually in Petri dishes together with filter paper on which they deposited eggs, provided with aphids to eat and a male of A. bipunctata to maintain fertility. It is not fully established in the manuscript if the presence of symbionts

in the sampled individuals derives from other possible organisms (cobionts). Regarding this, it seems that the WGS detection was made only in specimens of A. decempunctata infected with Spiroplasma. Why was a female infected with A. bipunctata not sequenced? It seems to me that the study focused on two species of ladybugs, but that whole section of genomic sequence was apparently done on only one of the species.

The reason is cost compared to the benefit of a WGS confirmation, which varies for the two species.

The two spot ladybird is a known host of heritable *Spiroplasma*symbionts from past studies that have demonstrated intergeneration transmission of the microbe and male-killing, i.e. heritable *Spiroplasma*presence in non-cobiont infected laboratory reared individuals is firmly established. The PCR positive individuals were detected in template from leg samples (see online supplementary data), and this itself reduces the chance of false positive from gut and cobionts. The PCR amplicon was the same sequence as that found in the previous studies of *A. bipuncatata*, so it is most parsimonious to conclude the same situation applies, making WGS less important evidentially.

The finding in the 10 spot, on the other hand, was novel, so lacked a historical context for heritable symbiont presence. Further, the positive material was from whole body template producing a higher risk of gut contamination/cobionts. Lacking any form of confirmation that this was a 10 spot symbiont made the evidential benefit of WGS to establish presence/absence of cobionts much greater.

What aphids were used for food? where did the latter came from? From rearing stocks colonies or directly from the field? In this regard, it should be considered that aphids harbor several facultative bacterial endosymbionts, including Spiroplasma. How was the possibility that the presence of aphid-derived symbionts could be transferred to Adalia individuals excluded? The aphids used to feed the aphids may have eaten differently by Adalia individuals. Aphids free of facultative endosymbionts should have been used. That would needed a specific detection of endosymbionts in aphids used for food.

As can be observed in the online data, the four individuals which tested *Spiroplasma*positive were all originally tested from leg material. This effectively excludes gut contents. Also, the ladybirds were starved of aphids for 48 hours before being tested for symbiont presence, such that we can be confident any PCR assay is detecting *Spiroplasma*in the ladybird. Echoing above, this is a species known to have heritable Spiroplasma from experimental study with the same 16S rRNA sequence as found and thus it is most parsimonious to believe it represents the same *Spiroplasma*.

On the other hand, in its first lines the manuscript highlighted the spatial variation in the distributions of symbionts, but the study itself does not shed new light on this issue. Instead, the manuscript attempts to link the presence of MK symbionts in two Adalia species, and that's it. Is the Merseyside region particularly relevant to these Adalia-MK symbiotes?

A core finding is that the *Spiroplasma*strain in Merseyside two-spot ladybirds does not kill males – a finding that is different from the rest of the species range. So yes, this study does produce 'new light' on spatial variation. The second finding is the presence of *Spiroplasma*in 10-spots, which was not previously known. This is further new light on the host-symbiont association derived from studying a different population to that previously studied.

There is no special reason to choose Merseyside aside proximity – in spatial studies any population that has been unstudied represents novel data, which in this case does provide novel insights. You don't know until you look.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000585.v1.5

Jana Katharina Schniete; Leibniz Universitat Hannover Naturwissenschaftliche Fakultat, Institut fuer Mikrobiologie, Herrenhaeuser Strasse 2, Geb 4104, GERMANY, Hannover

Date report received: 24 March 2023
Recommendation: Major Revision

Comments: Please make sure you address all the concerns raised by the reviewers, most importantly a more elaborate introduction is needed as raised by reviewer 1 and reviewer 2 raises an important concern, that details are lacking in the methods section- in particular around how and where the samples were taken and how they were fed in the lab.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000585.v1.4

Claudio Ramirez; Universidad de Talca, CHILE

Date report received: 19 March 2023
Recommendation: Major Revision

Comments: The manuscript addresses an interesting topic. However, there are methodological ambiguities that need to be clarified. Otherwise, the replicability of the study would be unapproachable. Furthermore, the contribution of the study to the spatial component of the relationship between MK symbionts and Adalia species is not clear, if any. The description of insect sampling needs to be greatly improved. Where exactly are the sampling sites? Please provide complete information. How exactly was the sampling done? What method was used? Sweeping net, fall traps or any other? Are these localities representative of the male/female ratio of these species? Once again, the description of the sample collection is very poor. To what extent is this sample size representative of the biology of both Adalia species? In the section "Embryonic phenotype that kills males in A. bipunctata" it is mentioned that 20 females of A. bipunctata, after collection, were placed individually in Petri dishes together with filter paper on which they deposited eggs, provided with aphids to eat and a male of A. bipunctata to maintain fertility. It is not fully established in the manuscript if the presence of symbionts in the sampled individuals derives from other possible organisms (cobionts). Regarding this, it seems that the WGS detection was made only in specimens of A. decempunctata infected with Spiroplasma. Why was a female infected with A. bipunctata not sequenced? It seems to me that the study focused on two species of ladybugs, but that whole section of genomic sequence was apparently done on only one of the species. What aphids were used for food? where did the latter came from? From rearing stocks colonies or directly from the field? In this regard, it should be considered that aphids harbor several facultative bacterial endosymbionts, including Spiroplasma. How was the possibility that the presence of aphid-derived symbionts could be transferred to Adalia individuals excluded? The aphids used to feed the aphids may have eaten differently by Adalia individuals. Aphids free of facultative endosymbionts should have been used. That would needed a specific detection of endosymbionts in aphids used for food. On the other hand, in its first lines the manuscript highlighted the spatial variation in the distributions of symbionts, but the study itself does not shed new light on this issue. Instead, the manuscript attempts to link the presence of MK symbionts in two Adalia species, and that's it. Is the Merseyside region particularly relevant to these Adalia-MK symbiotes?

Please rate the manuscript for methodological rigour
Poor

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000585.v1.3

Michiel de Groot; Ghent University: Universiteit Gent, BELGIUM
https://orcid.org/0000-0002-0406-1076

Date report received: 03 March 2023
Recommendation: Minor Amendment

Comments: Dear authors and editors, Thank you for the interesting paper, I enjoyed reading it. It presented novel research well with some intriguing conclusions and an abundance of future possibilities of study, including a very thorough experimental setup and write-up. My main criticisms of the article are mainly on style and the briefness of the introduction leading to a lack of background to the study, so I suggest minor revisions before accepting and publishing. 1. Methodological rigour, reproducibility and availability of underlying data The methodology of this article is very thorough and should make it easy to duplicate the entirety of the experiment for any researcher. The researchers not only screened the caught ladybirds for infections, they also investigated other strains of DNA found alongside the bacteria, leading to some of the more interesting conclusions: a braconid wasp's DNA was found, as well as an infectious fungus (Beauveria) which has not yet been described to infect Adalia spp. All the data was very easy to find, as it all has been submitted to databases online and have been directly linked in the article itself. 2. Presentation of results The results of the experiments have been presented clearly and succinctly. Some results can not be interpreted as statistically significant, which is stated upfront by the authors. As the incidence of these bacteria is low in these ladybirds, a weak statistical power is to be expected due to a relatively small number of data points (i.e. four infected females). However, this is typical for studies investigating these interactions (eg. von der Schulenberg et al., 2000) and still allows for comparison between populations. I am curious why "it was not possible to separate the two insect genomes satisfactorily" (line 276), as this can have a variety of reasons, but is not expanded upon. While the numbers of caught Adalia are small, the authors make no mention of the sex ratios of the wild-caught population which in a glance seem evenly divided - it might be worth mentioning as sex ratios of A. bipunctata can be extremely skewed (eg. Zakharov et al., 2006), as a result of male-killing endosymbionts. 3. How the style and organization of the paper communicates and represents key findings The paper has an odd balance of its organization: the introduction is extremely brief and fairly confusing, while the discussion is very thorough and intriguing. I was put off from reading the rest of the article from its introduction, which is a shame considering the results and discussion are well worth your time. The introduction would do well to introduce a few of the concepts outlined as results in the discussion: for example, the Scandinavian, Central European, and Scottish populations of Adalia spp. and their infection rates should be mentioned as they come in play later. Additionally, the urgency of this research is somewhat lost from its introduction as almost every citation is over 15 years old and some key points are argued in a confusing way. For example, line 73 is an odd sentence talking about a two-fold cost for the Adalia, which suddenly talks about a high reproductive success for the males without explaining why this is detrimental. Some other background mentioned in the discussion, such as the other associations (wasps, fungi), are not mentioned in the introduction at all. This also ties into the next point: 4. Literature analysis or discussion The background sketched in this article of the research subject is fairly poor. Most articles cited in the introduction are not recent, even though there is a wealth of recent research on Adalia spp. and other coccinellids with regards to their symbionts and how this affects their ecology. The introduction is much too brief and sometimes does not cite sources for important statements; for example, line 85 mentions "The disparity in the number, and prevalence, of different male-killers in A. bipunctata vs. A. decempunctata is poorly understood", but it is unclear if there have been investigations of both species and the sex ratios of their populations at all. There has been one mention in a German study of infections of A. decempunctata, but a lack of reports is not enough to state that there is such a disparity. The introduction could also expand on putting the trophic interactions between Adalia and their symbionts into a broader ecological context and their place in the variety of interactions of parasites in other coccinellids. Taking some more time to explain the background more in depth would significantly improve this paper. In comparison, the discussion is excellent, and raises several questions gained from the results of the study, as well as multiple avenues of interesting future research. It is worth mentioning with regards to novel Beauveria infection finding that infections of one species of parasite can sometimes result in susceptibility to other parasites; for example, in Harmonia axyridis, parasitic fungi, mites, and bacteria can affect other parasites, sometimes as competition, sometimes as enabler. 5. Any other relevant comments There are a few other minor comments or criticisms of the writing itself. Line 67 has a reference to Jaenike et al., but this is a very specific paper on defensive symbionts; a more general review on the effects of endosymbionts in insects would be better here. Line 70 mentions "selection", where selective pressure is meant. Line 78 has "Coccinelidae" in italics, as do the other mentions of families in rest of the paper; family names should not be italicized.

Please rate the manuscript for methodological rigour
Very good

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Strongly support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000585.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000585.v1.2