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Title: Endosymbiotic *Rickettsiella* causes cytoplasmic incompatibility in a spider host

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Abstract

Many arthropod hosts are infected with bacterial endosymbionts that manipulate host reproduction, but few bacterial taxa have been shown to cause such manipulations. Here we show that a bacterial strain in the genus *Rickettsiella* causes cytoplasmic incompatibility (CI) between infected and uninfected hosts. We first surveyed the bacterial community of the agricultural spider *Mermessus fradeorum* (Linyphiidae) using high throughput sequencing and found that individual spiders can be infected with up to five different strains of maternally-inherited symbiont from the genera *Wolbachia, Rickettsia,* and *Rickettsiella*. The *Rickettsiella* strain was pervasive, found in all 23 tested spider matrilines. We used antibiotic curing to generate uninfected matrilines that we reciprocally crossed with individuals infected only with *Rickettsiella*. We found that only 13% of eggs hatched when uninfected females were mated with *Rickettsiella*-infected males; in contrast, at least 83% of eggs hatched in the other cross types. This is the first documentation of *Rickettsiella*, or any Gammaproteobacteria, causing CI. We speculate that induction of CI may be much more widespread among maternally-inherited bacteria than previously appreciated. Further, our results reinforce the importance of thoroughly characterizing and assessing the inherited microbiome before attributing observed host phenotypes to well-characterized symbionts such as *Wolbachia*.

Keywords: Araneae**,** endosymbiont coinfection, cytoplasmic incompatibility, *Rickettsiella*, *Wolbachia, Rickettsia*

Introduction

Maternally transmitted endosymbiotic bacteria are common in arthropods, and are estimated to infect most arthropod species [1–4]. These inherited endosymbionts frequently manipulate host reproduction to promote symbiont transmission and spread in the host population [5]. The most prevalent and well-studied symbiont-driven manipulation is cytoplasmic incompatibility [5–8]. Cytoplasmic incompatibility (CI) is a conditional sterility phenotype in which crosses between infected males and uninfected females result in offspring mortality, sabotaging the production of uninfected progeny. This phenotype functionally increases the proportion of infected females in the host population over time [5]. Cytoplasmic incompatibility influences host population dynamics and gene flow, acting both as a gene drive mechanism that spreads traits through a population [9] and as a reproductive barrier that can enforce reproductive isolation and contribute toward speciation [10,11]. In application, CI has been used to locally repress pest populations as a form of sterile insect technique [12], and also as a gene drive/defensive mechanism to reduce disease transmission by mosquitoes [13,14].

To date, few bacterial taxa have been shown to cause CI. The most widespread and well-investigated are members of the genus *Wolbachia* (Phylum Proteobacteria, Class Alphaproteobacteria, Order Rickettsiales), which infects many arthropod species [1,8,15,16]. Recently, another unnamed strain in the Rickettsiales has also been suggested to induce CI [17]. Finally, members of the more distantly related *Cardinium* genus (Phylum Bacteroidetes) also induce CI, although using a mechanism that appears to be independently evolved from *Wolbachia* [18]. No other bacterial taxa have been documented to cause CI to date.

In the present study, we provide evidence that CI can be caused by a strain of *Rickettsiella* (Phylum Proteobacteria, Class Gammaproteobacteria, Order Legionellales), a

58 bacterium that is not closely related to either *Wolbachia* or *Cardinium* [19]. We began by
59 characterizing the microbiome of *Mermessus fradeorum* (Araneae: Linyphiidae), an agricultural
60 spider that had been previously shown to be infected by multiple *Wolbachia* and *Rickettsia*
61 (Phylum Proteobacteria, Class Alphaproteobacteria, Order Rickettsiales) symbionts, and to
62 exhibit CI [20]. Upon discovery of an unexpected *Rickettsiella* symbiont that was pervasive
63 throughout our sampled population, we proceeded to experimentally document that *Rickettsiella*
64 causes CI in *M. fradeorum*. This is the first demonstration of CI by a Gammaproteobacteria,
65 which suggests that the taxonomic distribution of bacteria that cause CI may be wider than
66 previously appreciated, and could have important implications for the population genetics,
67 biodiversity, and evolution of arthropod hosts.
68
69 Methods
70 *Study System*
71 We initially collected *Mermessus fradeorum* from alfalfa at the University of Kentucky's
72 Spindletop Research Farm (38.127, -84.508). We placed individual spiders into deli cups (6cm
73 diam.) with hydrated plaster in the bottom for humidity control, and maintained them in the
74 laboratory at 22°C 24hr dark conditions. Spiders collected as juveniles were fed symbiont-free
75 collembola (*Sinella curviseta*) until maturity. Adult spiders were fed fruit flies (*Drosophila*
76 *melanogaster*) at 2-3 day intervals; these flies were infected with a distinct strain of *Wolbachia*
77 (MLST ST1) [21] that was never detected in our microbiome survey or diagnostic assays. We
78 specifically tested whether infected prey could result in false-positive infection diagnoses in the
79 spiders, and found that even an hour after feeding on *Wolbachia*-infected flies, uninfected *M.*
80 *fradeorum* did not test positive for *Wolbachia* (Electronic supplemental material figure S1). We

81 initiated matrilines from individual field-collected gravid females, or by mating field-collected
82 females to males within the laboratory.
83
84 *Microbiome Survey*
85 To evaluate endosymbiont diversity in the population of *M. fradeorum*, we characterized
86 the microbiome of 23 matrilines that were initiated between August and October 2016. For each
87 matriline, we evaluated the field-collected female specimen when possible; if the field-collected
88 female died of natural causes (n = 8), we instead randomly selected an adult female F1 offspring.
89 We withheld food from each spider for at least five days before preserving the specimen in 95%
90 ethanol. We surface-sterilized each specimen with a series of washes with 0.5% bleach and PCR
91 water. We then extracted total DNA from each individual specimen using DNEasy Blood and
92 Tissue kits (Qiagen, Germantown, MD) according the manufacturer's instructions. Using PCR
93 with dual-indexed 515F/806R primers [22], we amplified the V4 region of bacterial 16S from
94 each specimen and visualized an aliquot of the resulting product on a 1% agarose gel stained
95 with GelRed (Biotium). After verifying the presence of a strong band of the expected product
96 size, we included a 1µl aliquot of the corresponding product in a multiplexed library for
97 sequencing. This library also included samples from aphids and parasitoid wasps that served as
98 controls (see below). After purification using GenCatch PCR Cleanup kit (Epoch Life Sciences,
99 Missouri City, TX) we sequenced the library on an Illumina Miseq 2500 instrument at the
100 University of Kentucky's core sequencing facility.
101 Sequences were demultiplexed, trimmed and quality filtered within BaseSpace (Illumina)
102 then imported into qiime2 (v2017.11) [23] using a manifest. We conducted additional quality
103 control using deblur [24], implemented in qiime2 using default parameters and a trim length of

104 251 bases. To determine the taxonomic placement of each operational taxonomic unit (OTU), we
105 used a naïve Bayes classifier trained on the V4 region of the Greengenes 13_8 99% OTUs
106 reference database [25].

107 The most prevalent OTU in the library, a strain of *Rickettsiella*, constituted 46% of total
108 reads and was associated with every component specimen in the multiplexed sample, albeit at
109 low prevalence for non-*M. fradeorum* samples. We inferred that index swapping may have
110 occured between samples that shared either forward or reverse indices, resulting in reads that
111 were misallocated to the wrong sample [26]. To validate that *Rickettsiella* was genuinely present
112 in all *M. fradeorum* samples, we used *Rickettsiella*-specific primers to diagnostically test for the
113 presence of *Rickettsiella* in the 23 matriline samples, as well as the 8 controls (4 *Aphis*
114 *craccivora* (aphid) specimens and 4 *Habrobracon hebetor* (parasitoid wasp) specimens).
115 Diagnostic primers and protocols followed Duron et al. [19]; samples that tested negative for
116 *Rickettsiella* were rescreened at least two more times, and additionally tested for extraction
117 quality via diagnostic PCR of a segment of the arthropod COI gene [4].

118 To assess vertical transmission of *Rickettsiella*, we included specimens from several
119 generations in the microbiome dataset. In total, 148 mother-offspring pairs from the 23
120 matrilines (representing 83 *Rickettsiella*-infected mothers) were initially included in the dataset.
121 As a quality control measure for the final dataset, we retained only pairs in which both mother
122 and offspring yielded more than 5000 reads (117 pairs, representing 73 different mothers,
123 sometimes with multiple offspring). To be scored positive, the *Rickettsiella* read number for a
124 given microbiome needed to exceed the upper 95% confidence boundary of *Rickettsiella* reads
125 found in the eight control samples (145 reads).
126

127 Rickettsiella *Cytoplasmic Incompatibility Assay*

128 To assess the ability of *Rickettsiella* to cause CI, we first created a population of
129 uninfected spiders using antibiotics. Spiderlings originating from seven infected matrilines were
130 treated with a combination of tetracycline (0.1%) and ampicillin (0.1%) by a fine mist spray
131 daily until sub-adulthood [20,27]. Upon maturity, treated spiders were mated to one another, and
132 a subset of those offspring were diagnostically tested for endosymbionts (*Wolbachia, Rickettsia,*
133 *Rickettsiella)* [4,20]. Sibships that appeared negative for all endosymbionts were used to initiate
134 the putatively uninfected population for experimental assays, with the understanding that not all
135 spiders within this group would be truly cleared of infection. Antibiotic treatment often
136 destabilizes symbiont transmission to offspring, resulting in inconsistent infection status among
137 sibships and/or low titer infections that rebound in subsequent generations [28,29]. It is common
138 practice to treat hosts with antibiotics for several generations to ensure symbiont clearance, but
139 due to the labor intensive rearing of cannibalistic spiders, here we chose instead to diagnostically
140 test all parental spiders to properly classify mating cross types, as described below. Spiders used
141 in experiments were 1, 2, or 3 generations removed from antibiotic treatment.

142 To determine if *Rickettsiella* was causing CI in *M. fradeorum*, we reciprocally mated
143 uninfected spiders with spiders that were infected with only *Rickettsiella*. We randomly assigned
144 virgin spiders to mate with a partner that we expected to be either *Rickettsiella*-infected or
145 uninfected, ensuring that siblings and cousins were not paired with one another. Males and
146 females were paired for 8 hours, and were observed at approximately 15 minute intervals for the
147 first two hours and periodically thereafter. If the pair was not observed to mate, they were
148 excluded from the trial. Females were then allowed up to six weeks to lay three egg masses;
149 females that failed to lay three egg masses within this time period (n=2) were excluded from the

150 sample set. Egg masses were checked daily for spiderlings. Once spiderlings were observed, we
151 allowed 24 hours for emergence, then dissected the egg mass to quantify unhatched eggs. Egg
152 masses hatched within a mean ± SE of 14.1 ± 0.3 days. If no spiderlings were observed within 20
153 days, the entire egg mass was scored as unhatched, and dissected to count the number of
154 unhatched eggs. Throughout the experiment, we found a small number of spiderlings (n=30) that
155 hatched but died prior to exiting the egg mass. These unemerged spiderlings were excluded from
156 the data set, as they accounted for only 2% of all eggs.

157 The infection status of all parents in the experiment was validated using diagnostic PCR
158 for *Wolbachia, Rickettsia,* and *Rickettsiella* [4,19,20]. Final classification of all matings was
159 based on these diagnostic results, rather than initial expectations based on sibling or parental
160 infection status. Matings were reclassified if *Rickettsiella* presence/absence in either parent
161 deviated from expectation, and were excluded altogether if either parent tested positive for a
162 symbiont other than *Rickettsiella* (Electronic supplementary material table S1). The final
163 distribution of mating cross types across four trial dates included 18 pairings in which both
164 female and male were infected with only *Rickettsiella* (+/+), 14 in which the female was
165 *Rickettsiella*-infected and the male was uninfected (+/-), 12 in which the female was uninfected
166 and the male was *Rickettsiella*-infected (-/+), and 8 in which both female and male were
167 uninfected (-/-).

168 We compared total eggs laid among treatments using ANOVA (JMP) after verifying that
169 model assumptions of normality and homoscedacity were not violated. We compared egg hatch
170 among treatments using logistic regression (Arc v. 1.06) with Williams correction [30] to
171 account for moderate overdispersion. To verify that generations removed from antibiotic
172 treatment (1, 2, or 3 generations) did not influence experimental outcome, we considered a

separate model that incorporated this factor, but found no significant effect (Wald = -0.93, p = 0.35). Generation from antibiotic treatment was therefore not included in the final model. We additionally tested whether our diagnostic reclassification and exclusion criteria affected our conclusions by running the statistical tests on alternative datasets in which we 1) completely ignored our diagnostic results and had no reclassification, 2) reclassified according to *Rickettsiella* infection but ignored the presence of other symbionts (no exclusions), or 3) excluded all matings that did not fit with their initial expectations (exclusions instead of reclassifications). All of these variant analyses yielded statistically significant results that concur with the analysis of the original dataset presented below.

Results

Microbiome Survey

The most notable feature of the *M. fradeorum* microbiome was the pervasiveness of a single strain of *Rickettsiella*, present in all 23 matrilines (figure 1). Mean ± SE reads per sample was 22,054 ± 2,739, of which 16,172 ± 2,109 (80 ± 6%) were *Rickettsiella*. In most specimens (20/23), *Rickettsiella* constituted the majority of reads, often representing >95% of reads (figure 1, Electronic supplementary material tables S2-S3). Proportional representation of *Rickettsiella* was lower in samples that were also infected with *Rickettsia* (7 samples) and/or up to 3 strains of *Wolbachia* (8 samples), but still constituted half the reads returned for this subset of samples (50 ± 9%). In contrast, aphid and parasitoid wasp control samples that were included in the same sequencing run had few reads of this *Rickettsiella* strain (115 ± 15 reads/sample, constituting 0.3 ± 0.05% of reads/sample). Diagnostic validation confirmed the presence of *Rickettsiella* from all 23 matriline samples, and the absence of *Rickettsiella* from the aphids and parasitoid wasps. We observed 100% vertical transmission efficiency in the unmanipulated mother-offspring pairs in

196 the dataset (Electronic supplementary material table S4). Across 117 pairs, all offspring of
197 *Rickettsiella*-infected mothers were themselves infected with *Rickettsiella*.
198
199 *Reproductive Manipulation Assay: Cytoplasmic Incompatibility*
200 When we reciprocally mated spiders bearing only *Rickettsiella* with those from matrilines
201 that had been cured of all facultative symbionts, we found a large difference in the number of
202 hatchlings produced. Total egg number was consistent and not significantly different across
203 treatments (figure 2, Electronic supplementary material table S1; ANOVA: $F_{3,48} = 0.56$, $p =$
204 0.644), but the odds that these eggs hatched was significantly lower for uninfected females mated
205 to *Rickettsiella*-infected males, relative to all three other treatments, which did not differ
206 significantly from one another ($\chi^2 = 119$, d.f. = 3, $p < 0.001$). Only 13.2 ± 3.8% of eggs hatched
207 in the incompatible cross between uninfected females and *Rickettsiella*-infected males, compared
208 to 99.7 ± 0.2% of eggs when both parents were infected, 91.3 ± 4.6% from *Rickettsiella*-infected
209 females mated with uninfected males, and 83.9 ± 12.2% when both parents were uninfected.
210
211 Discussion
212 We found that the spider *Mermessus fradeorum* is infected by up to five strains of
213 bacterial symbiont, including a strain of *Rickettsiella* that was present in 100% of our field-
214 collected matrilines. We then generated uninfected matrilines, and used crossing experiments to
215 document that this *Rickettsiella* strain induced cytoplasmic incompatibility in the spider host.
216 Previous examples of CI-causing bacteria have been restricted to *Wolbachia* and closely-allied
217 bacteria in the Alphaproteobacteria, and *Cardinium* in the Bacteroidetes [6,7,17,31]. Ours is the
218 first demonstration of a Gammaproteobacteria, *Rickettsiella*, causing CI.

219 Previous studies with this spider species diagnostically documented three endosymbiotic
220 bacterial strains (two *Wolbachia* and one *Rickettsia*) associated with bacterially-induced
221 reproductive manipulations, but could not attribute causality to individual bacterial strains [20].
222 The present microbiome study confirmed the presence of these same three symbiont strains at
223 similar field frequencies as the previous work, and additionally revealed a third strain of
224 *Wolbachia* along with the ubiquitous *Rickettsiella*. We were able to screen archived specimens
225 from the previous study [20] and found that the CI-inducing matriline from that study was in fact
226 co-infected with *Rickettsiella* and one strain of *Wolbachia* (L. Rosenwald, unpublished results).
227 It is therefore likely that the CI observed in this previous study was at least partially attributable
228 to *Rickettsiella*, although both co-infecting symbionts may have contributed to the CI phenotype
229 [32]. Focus on taxa that are well-established as reproductive manipulators, such as *Wolbachia*,
230 may lead researchers to overlook co-infecting bacteria that cause or contribute to manipulated
231 host reproductive phenotypes, as co-infections are common in arthropods [1,3].
232 The mechanism by which *Rickettsiella* induces CI is unknown. In *Wolbachia,* CI is
233 induced by phage-encoded operons in which one gene modifies the sperm of infected males and
234 the other rescues the modification in the cytoplasm of the egg of infected females [33,34]. In
235 *Cardinium,* the mechanism to induce CI appears to be independently evolved from *Wolbachia,*
236 and is not phage associated [18]. No previously described *Rickettsiella* strains have been
237 documented to cause CI, but CI-induction has been hypothesized based on genetic analysis of a
238 *Rickettsiella* strain in fleas [35]. Strains of *Rickettsiella* infect a wide range of arthropods [19,36],
239 sometimes as a pathogen [36], sometimes a mutualist [37], and sometimes as a potential
240 reproductive manipulator [38]. Speculatively, it is possible that horizontal gene transfer among
241 co-infecting symbionts may be responsible for *Rickettsiella's* ability to induce CI [39,40],

242 particularly given the high frequency of symbiotic co-infection in spiders in general [1,3,4,41]
243 and *M. fradeorum* in particular.

244 Our data also reinforce the perception that spiders can harbor exceptionally diverse
245 communities of co-infecting symbionts, rivaling the most symbiont-rich inherited communities
246 among insects [4,42,43]. The discovery of an unexpected reproductive manipulator within such a
247 community highlights the hazard of focusing only on well-characterized manipulative taxa (e.g.
248 *Wolbachia*) when reproductive anomalies are observed in a host population. Our data emphasize
249 the need for careful characterization of the entire inherited microbiome before attributing
250 reproductive phenotypes to particular bacterial strains.

251 Reproductive manipulation by endosymbionts, through mechanisms such as CI, can
252 influence arthropod behavioral ecology [44], population dynamics [45], and evolutionary
253 trajectory [46]. As the known roster of symbionts capable of undertaking these manipulations
254 increases, the potential for understanding and potentially exploiting their effects on the biology
255 of their hosts increases as well. CI is currently being used as part of a symbiont-based strategy to
256 decrease disease transmission in mosquitoes [12]; newly discovered CI-inducing symbionts, such
257 as described in the present work, have similar potential in a wider range of arthropod hosts. In
258 particular, *Wolbachia* is rare or absent in ticks (Class Arachnida, Order Acari), but strains of
259 *Rickettsiella* are routine [19,38,47,48]. It would be worthwhile to evaluate the function of
260 *Rickettsiella* and other members of tick microbiomes, similarly to what we have done with
261 *Rickettsiella* in the spider *M. fradeorum*, as the potential opportunities for symbiont-based pest
262 control and vector management are substantial.

263

264 Data accessibility

The datasets supporting this article have been uploaded as part of the electronic supplementary material (Electronic supplementary material figure S1, tables S1-S4). High throughput sequencing data is available in the NCBI Sequence Read Archive under accession number PRJNA604923.

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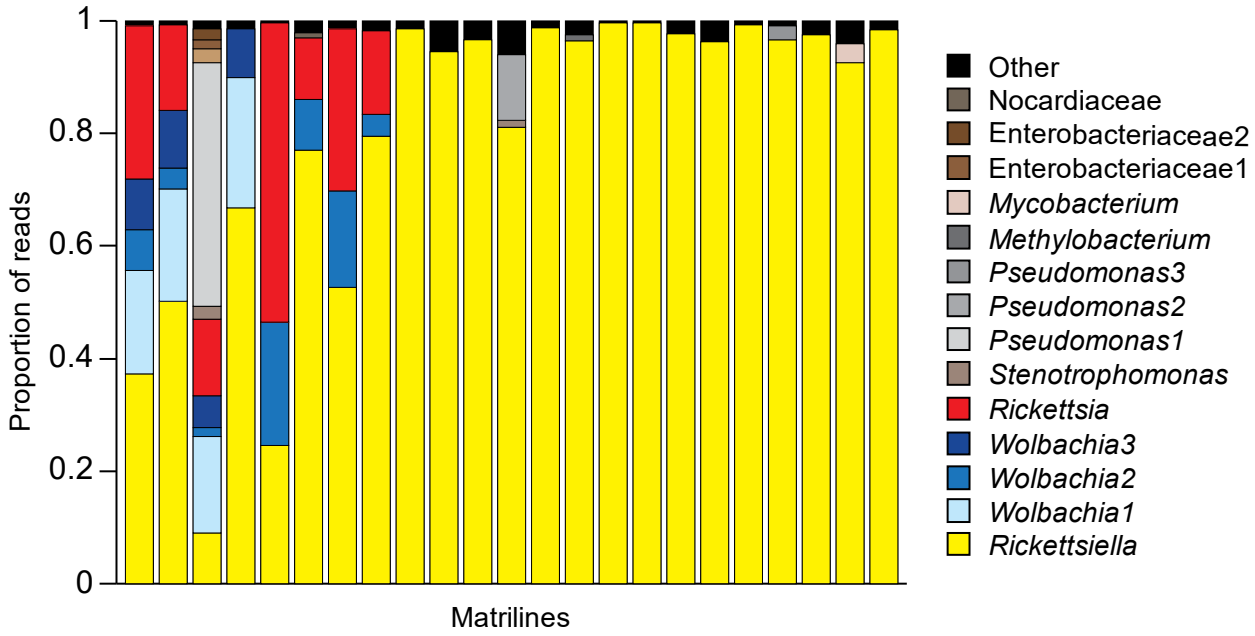

Figure 1. Proportional composition of 16S bacterial reads from 23 adult female *Mermessus fradeorum* spiders. All spiders were either field collected or adult offspring of different field collected mothers. Each shade represents a different bacterial strain; different strains from the same genus are designated by different numbers in the legend. Bacteria known to be maternally transmitted are brightly colored (yellow, blue, red), other strains are shades of gray and brown. Strain sequences, read numbers, and composition of the "Other" category available in Electronic supplemental material Tables S2 and S3.

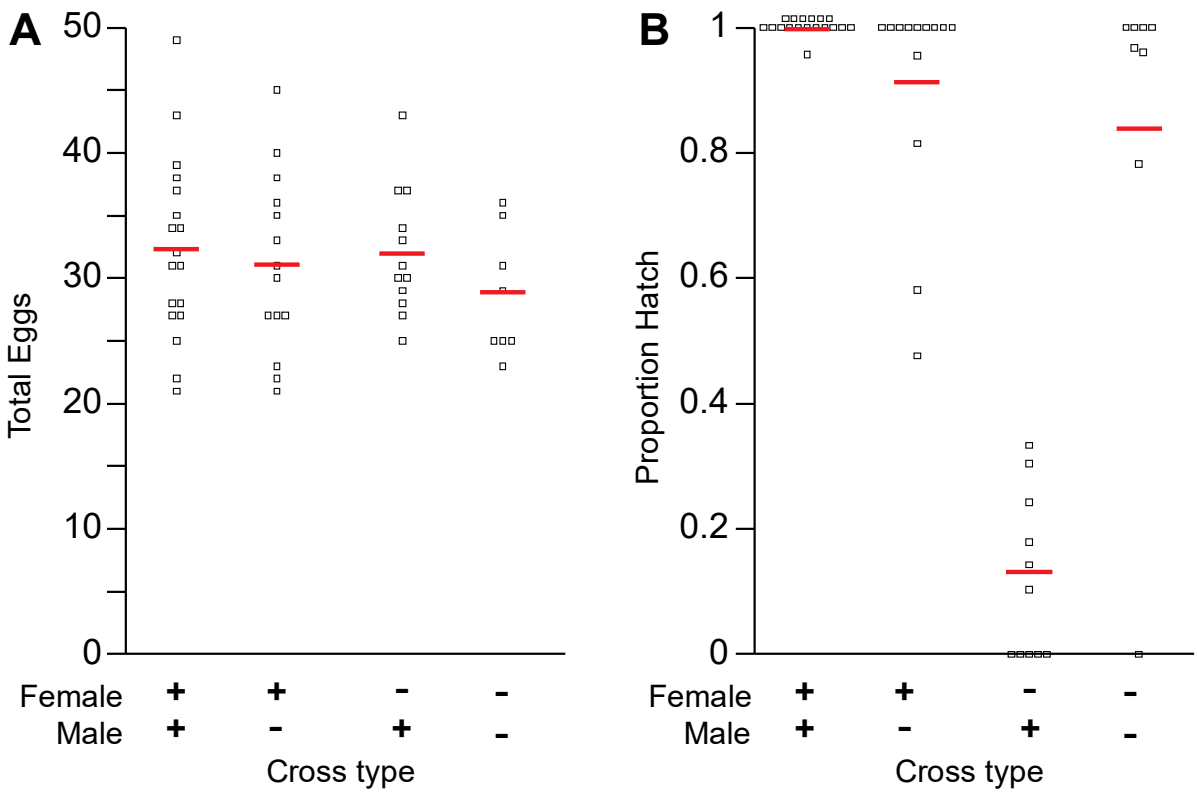

Figure 2. Total eggs laid (A) and proportion eggs that hatched (B) in reciprocal crosses between *Mermessus fradeorum* females and males that varied in *Rickettsiella* infection status. Each point represents the outcome from a single cross, red horizontal bars represent mean value per treatment. Points above 1.0 in panel B are 1.0.