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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 nearly half of all 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 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 are estimated to infect at least a third of 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.

56 In the present study, we provide evidence that CI can be caused by a strain of
57 *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. We fed the spiders fruit flies (*Drosophila*
75 *melanogaster*) at 2-3 day intervals; spiders collected as juveniles were fed collembola (*Sinella*
76 *curviseta*) until maturity. We initiated matrilines from individual field-collected gravid females,
77 or by mating field-collected females to males within the laboratory.
78

79 *Microbiome Survey*

80 To evaluate endosymbiont diversity in the population of *M. fradeorum*, we characterized
81 the microbiome of 23 matrilines that were initiated between August and October 2016. For each
82 matriline, we evaluated the field-collected female specimen when possible; if the field-collected
83 female died of natural causes (n = 8), we instead randomly selected an adult female F1 offspring.
84 We withheld food from each spider for at least five days before preserving the specimen in 95%
85 ethanol. We surface-sterilized each specimen with a series of washes with 0.5% bleach and PCR
86 water. We then extracted total DNA from each individual specimen using DNEasy Blood and
87 Tissue kits (Qiagen, Germantown, MD) according the manufacturer's instructions. Using PCR
88 with dual-indexed 515F/806R primers [21], we amplified the V4 region of bacterial 16S from
89 each specimen and visualized an aliquot of the resulting product on a 1% agarose gel stained
90 with GelRed (Biotium). After verifying the presence of a strong band of the expected product
91 size, we included a 1µl aliquot of the corresponding product in a multiplexed library for
92 sequencing. This library also included samples from aphids and parasitoid wasps that served as
93 controls (see below). After purification using GenCatch PCR Cleanup kit (Epoch Life Sciences,
94 Missouri City, TX) we sequenced the library on an Illumina Miseq 2500 instrument at the
95 University of Kentucky's core sequencing facility.

96 Sequences were demultiplexed, trimmed and quality filtered within BaseSpace (Illumina)
97 then imported into qiime2 (v2017.11) [22] using a manifest. We conducted additional quality
98 control using deblur [23], implemented in qiime2 using default parameters and a trim length of
99 251 bases. To determine the taxonomic placement of each operational taxonomic unit (OTU), we
100 used a naïve Bayes classifier trained on the V4 region of the Greengenes 13_8 99% OTUs
101 reference database [24].

102 The most prevalent OTU in the library, a strain of *Rickettsiella*, constituted 46% of total
103 reads and was associated with every component specimen in the multiplexed sample, albeit at
104 low prevalence for non-*M. fradeorum* samples. We inferred that index swapping may have
105 occured between samples that shared either forward or reverse indices, resulting in reads that
106 were misallocated to the wrong sample [25]. To validate that *Rickettsiella* was genuinely present
107 in all *M. fradeorum* samples, we used *Rickettsiella*-specific primers to diagnostically test for the
108 presence of *Rickettsiella* in the 23 matriline samples, as well as the 8 controls (4 *Aphis*
109 *craccivora* (aphid) specimens and 4 *Habrobracon hebetor* (parasitoid wasp) specimens).
110 Diagnostic primers and protocols followed Duron et al. [19]; samples that tested negative for
111 *Rickettsiella* were rescreened at least two more times, and additionally tested for extraction
112 quality via diagnostic PCR of a segment of the arthropod COI gene [4].
113 To assess vertical transmission of *Rickettsiella*, we included specimens from several
114 generations in the microbiome dataset. In total, 148 mother-offspring pairs from the 23
115 matrilines (representing 83 *Rickettsiella*-infected mothers) were initially included in the dataset.
116 As a quality control measure for the final dataset, we retained only pairs in which both mother
117 and offspring yielded more than 5000 reads (117 pairs, representing 73 different mothers). To be
118 scored positive, the *Rickettsiella* read number for a given microbiome needed to exceed the
119 upper 95% confidence boundary of *Rickettsiella* reads found in the eight control samples (145
120 reads).
121
122 Rickettsiella *Cytoplasmic Incompatibility Assay*
123 To assess the ability of *Rickettsiella* to cause CI, we first created a population of
124 uninfected spiders using antibiotics. Spiderlings originating from seven infected matrilines were

125 treated with a combination of tetracycline (0.1%) and ampicillin (0.1%) by a fine mist spray
126 daily until sub-adulthood [20,26]. Upon maturity, treated spiders were mated to one another, and
127 a subset of those offspring were diagnostically tested for endosymbionts (*Wolbachia, Rickettsia,*
128 *Rickettsiella)* [4,20]. Sibships that were negative for all endosymbionts were used to initiate the
129 uninfected (cured) population for experimental assays. Uninfected spiders used in experiments
130 were 1, 2, or 3 generations removed from antibiotic treatment.

131 To determine if *Rickettsiella* was causing CI in *M. fradeorum*, we reciprocally mated
132 uninfected spiders with spiders that were infected with only *Rickettsiella* (8 matrilines). We
133 randomly assigned virgin spiders to mate with either a *Rickettsiella*-infected or uninfected
134 partner, ensuring that siblings and cousins were not paired with one another. Males and females
135 were paired for 8 hours, and were observed at approximately 15 minute intervals for the first two
136 hours and periodically thereafter. If the pair was not observed to mate, they were excluded from
137 the trial. Females were then allowed up to six weeks to lay three egg masses; females that failed
138 to lay three egg masses within this time period (n=2) were excluded from the sample set. Egg
139 masses were checked daily for spiderlings. Once spiderlings were observed, we allowed 24 hours
140 for emergence, then dissected the egg mass to quantify unhatched eggs. Egg masses hatched
141 within a mean ± SE of 14.1 ± 0.3 days. If no spiderlings were observed within 20 days, the entire
142 egg mass was scored as unhatched, and dissected to count the number of unhatched eggs.
143 Throughout the experiment, we found a small number of spiderlings (n=9) that hatched but died
144 prior to exiting the egg mass. These unemerged spiderlings were excluded from the data set, as
145 they accounted for only 2% of all eggs.

146 The infection status of all parents in the experiment was validated using diagnostic PCR
147 for *Wolbachia, Rickettsia,* and *Rickettsiella* [4,19,20]. Final classification of all matings was

148 based on these diagnostic results; in several instances spiders from "cured" matrilines were
149 found to be symbiont infected, presumably due to cryptic low titer infections that rebounded in
150 subsequent generations. In such cases, matings were reclassified or excluded as necessary,
151 reflecting the actual infection status of both partners. The final distribution of mating cross types
152 across four trial dates included 18 pairings in which both females and males were infected with
153 only *Rickettsiella* (+/+), 14 in which the female was *Rickettsiella*-infected and the male was
154 uninfected (+/-), 12 in which the female was uninfected and the male was *Rickettsiella*-infected
155 (-/+), and 8 in which both female and male were uninfected (-/-).

156 We compared total eggs laid across treatments using ANOVA (JMP) after verifying that
157 model assumptions of normality and homoscedacity were not violated. We compared egg hatch
158 across treatments using logistic regression (Arc v. 1.06) with Williams correction [27] to account
159 for moderate overdispersion. To verify that generations removed from curing (1, 2, or 3
160 generations) did not influence experimental outcome, we considered a separate model that
161 incorporated this factor, but found no significant effect (Wald = -0.93, p = 0.35). Generation
162 from curing was therefore not included in the final model.

163

164 Results

165 *Microbiome Survey*

166 The most notable feature of the *M. fradeorum* microbiome was the pervasiveness of a
167 single strain of *Rickettsiella*, present in all 23 matrilines (figure 1). Mean ± SE reads per sample
168 was 22,054 ± 2,739, of which 16,172 ± 2,109 (80 ± 6%) were *Rickettsiella*. In most specimens
169 (20/23), *Rickettsiella* constituted the majority of reads, often representing >95% of reads (figure
170 1, Electronic supplementary material tables S1-S2). Proportional representation of *Rickettsiella*

171 was lower in samples that were also infected with *Rickettsia* (7 samples) and/or up to 3 strains of
172 *Wolbachia* (8 samples), but still constituted half the reads returned for this subset of samples (50
173 ± 9%). In contrast, aphid and parasitoid wasp control samples that were included in the same
174 sequencing run had few reads of this *Rickettsiella* strain (115 ± 15 reads/sample, constituting 0.3
175 ± 0.05% of reads/sample). Diagnostic validation confirmed the presence of *Rickettsiella* from all
176 23 matriline samples, and the absence of *Rickettsiella* from the aphids and parasitoid wasps. We
177 observed 100% vertical transmission efficiency in the unmanipulated mother-offspring pairs in
178 the dataset (Electronic supplementary material table S3). Across 117 pairs, all offspring of
179 *Rickettsiella*-infected mothers were themselves infected with *Rickettsiella*.
180
181 *Reproductive Manipulation Assay: Cytoplasmic Incompatibility*
182 When we reciprocally mated spiders bearing only *Rickettsiella* with those from matrilines
183 that had been cured of all facultative symbionts, we found a large difference in the number of
184 hatchlings produced. Total egg number was consistent and not significantly different across
185 treatments (figure 2, Electronic supplementary material table S4; ANOVA: $F_{3,48} = 0.56$, $p =$
186 0.644), but the odds that these eggs hatched was significantly lower for uninfected females mated
187 to *Rickettsiella*-infected males, relative to all three other treatments, ($\chi^2 = 111$, d.f. = 3, $p <$
188 0.001). Only 12.5 ± 3.6% of eggs hatched in the incompatible cross between uninfected females
189 and *Rickettsiella*-infected males, compared to 98.4 ± 1.0% of eggs when both parents were
190 infected, 89.5 ± 4.5% from *Ricketsiella*-infected females mated with uninfected males, and 83.1
191 ± 12.2% when both parents were uninfected.
192
193 Discussion

194 We found that the spider *Mermessus fradeorum* is infected by up to five strains of
195 bacterial symbiont, including a strain of *Rickettsiella* that was present in 100% of our field-
196 collected matrilines. We then generated uninfected matrilines, and used crossing experiments to
197 document that this *Rickettsiella* strain induced cytoplasmic incompatibility in the spider host.
198 Previous examples of CI-causing bacteria have been restricted to *Wolbachia* and closely-allied
199 bacteria in the Alphaproteobacteria, and *Cardinium* in the Bacteroidetes [6,7,17,28]. Ours is the
200 first demonstration of a Gammaproteobacteria, *Rickettsiella*, causing CI.

201 Previous studies with this spider species diagnostically documented three endosymbiotic
202 bacterial strains (two *Wolbachia* and one *Rickettsia*) associated with bacterially-induced
203 reproductive manipulations, but could not attribute causality to individual bacterial strains [20].
204 The present microbiome study confirmed the presence of these same three symbiont strains at
205 similar field frequencies as the previous work, and additionally revealed a third strain of
206 *Wolbachia* along with the ubiquitous *Rickettsiella*. We were able to screen archived specimens
207 from the previous study [20] and found that the CI-inducing matriline from that study was in fact
208 co-infected with *Rickettsiella* and one strain of *Wolbachia* (L. Rosenwald, unpublished results).
209 It is therefore likely that the CI observed in this previous study was at least partially attributable
210 to *Rickettsiella*, although both co-infecting symbionts may have contributed to the CI phenotype
211 [29]. Focus on taxa that are well-established as reproductive manipulators, such as *Wolbachia*,
212 may lead researchers to overlook co-infecting bacteria that cause or contribute to manipulated
213 host reproductive phenotypes, as co-infections are common in arthropods [1,4].

214 The mechanism by which *Rickettsiella* induces CI is unknown. In *Wolbachia,* CI is
215 induced by phage-encoded operons in which one gene modifies the sperm of infected males and
216 the other rescues the modification in the cytoplasm of the egg of infected females [30,31]. In

217 *Cardinium,* the mechanism to induce CI appears to be independently evolved from *Wolbachia,*
218 and is not phage associated [18]. No previously described *Rickettsiella* strains have been
219 documented to cause CI, and genetic resources for making mechanistic inferences are lacking.
220 However, strains of *Rickettsiella* infect a wide range of arthropods [19,32], sometimes as a
221 pathogen [32], sometimes a mutualist [33], and sometimes as a potential reproductive
222 manipulator [34]. Speculatively, it is possible that horizontal gene transfer among co-infecting
223 symbionts may be responsible for *Rickettsiella's* ability to induce CI [35,36], particularly given
224 the high frequency of symbiotic co-infection in spiders in general [1,3,4,37] and *M. fradeorum* in
225 particular.

226 Our data also reinforce the perception that spiders can harbor exceptionally diverse
227 communities of co-infecting symbionts, rivaling the most symbiont-rich inherited communities
228 among insects [4,38,39]. The discovery of an unexpected reproductive manipulator within such a
229 community highlights the hazard of focusing only on well-characterized manipulative taxa (e.g.
230 *Wolbachia*) when reproductive anomalies are observed in a host population. Our data emphasize
231 the need for careful characterization of the entire inherited microbiome before attributing
232 reproductive phenotypes to particular bacterial strains.

233 Reproductive manipulation by endosymbionts, through mechanisms such as CI, can
234 influence arthropod behavioral ecology [40], population dynamics [41], and evolutionary
235 trajectory [42]. As the known roster of symbionts capable of undertaking these manipulations
236 increases, the potential for understanding and potentially exploiting their effects on the biology
237 of their hosts increases as well. CI is currently being used as a gene drive mechanism to decrease
238 disease transmission in mosquitoes [12]; newly discovered CI-inducing symbionts, such as
239 described in the present work, have similar potential in a wider range of arthropod hosts. In

240 particular, *Wolbachia* is rare or absent in ticks (Class Arachnida, Order Acari), but strains of
241 *Rickettsiella* are routine [19,34,43,44]. It would be worthwhile to evaluate the function of
242 *Rickettsiella* and other members of tick microbiomes, similarly to what we have done with
243 *Rickettsiella* in the spider *M. fradeorum*, as the potential opportunities for symbiont-based pest
244 control and vector management are substantial.
245
246 Data accessibility
247 The datasets supporting this article have been uploaded as part of the electronic supplementary
248 material (Electronic supplementary material, tables S1-S4). High throughput sequencing data is
249 available in the NCBI Sequence Read Archive under accession number PRJNA604923.
250
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262

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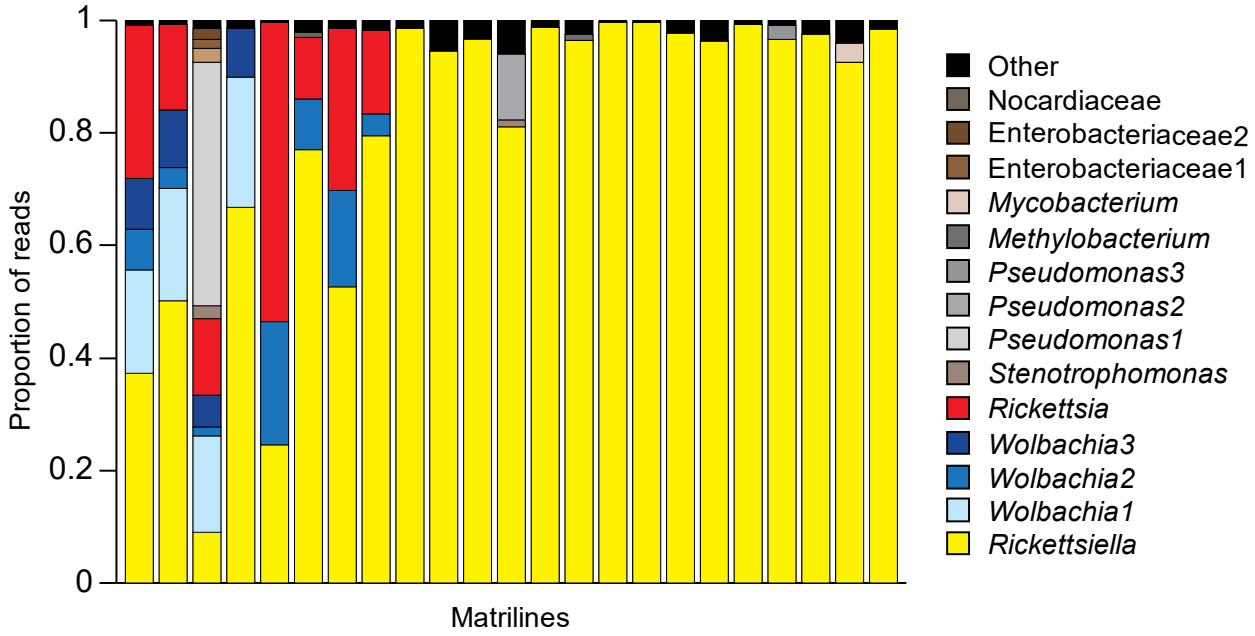
386 Figures



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388 Figure 1. Proportional composition of 16S bacterial reads from 23 adult female *Mermessus*
389 *fradeorum* spiders. All spiders were either field collected or adult offspring of different field
390 collected mothers. Each shade represents a different bacterial strain; different strains from the
391 same genus are designated by different numbers in the legend. Bacteria known to be maternally
392 transmitted are brightly colored (yellow, blue, red), other strains are shades of gray and brown.
393 Strain sequences, read numbers, and composition of the "Other" category available in Electronic
394 supplemental material Tables S1 and S2.

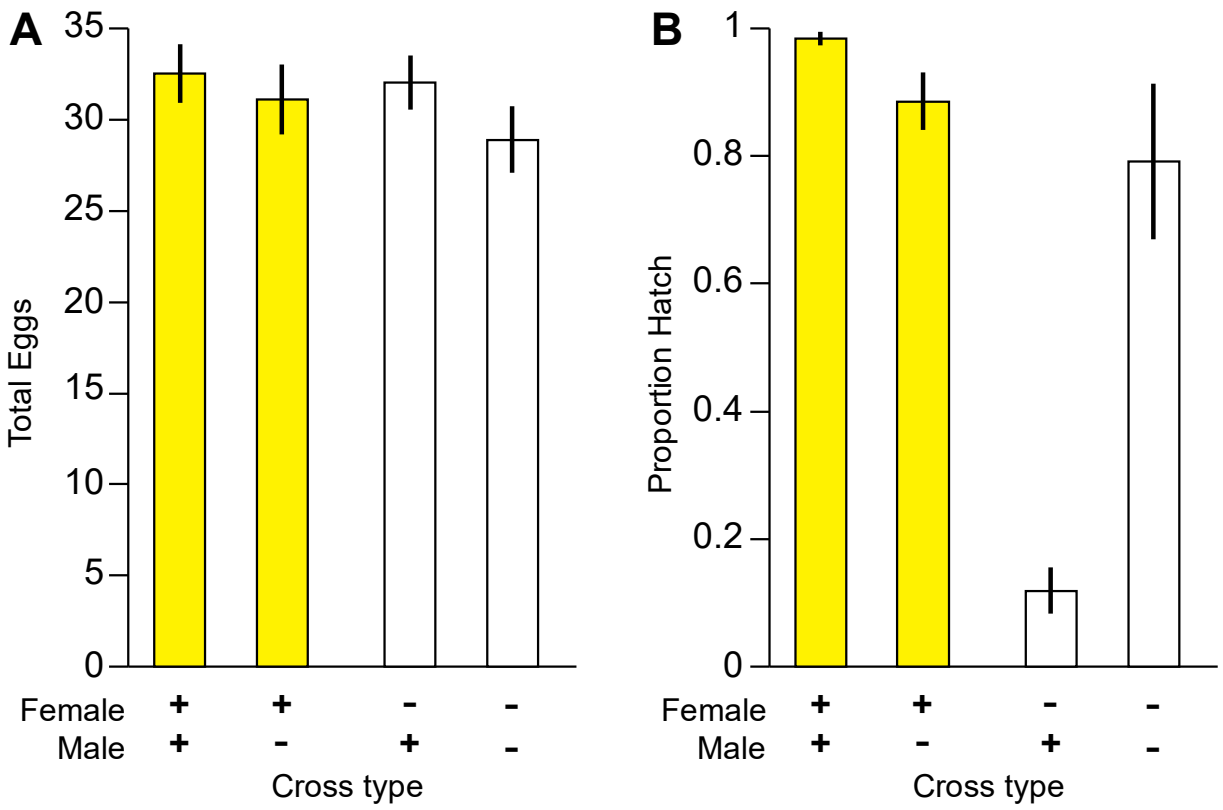

Figure 2. Mean ± SE total eggs laid (A) and proportion eggs that hatched (B) in reciprocal crosses between *Mermessus fradeorum* females and males that varied in *Rickettsiella* infection status. Column color indicates expected offspring infection status: Yellow columns reflect offspring of *Rickettsiella*+ mothers, white columns reflect offspring of *Rickettsiella*– mothers.