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Running title: (50 characters) Warm temperature inhibits *Rickettsiella*-induced CI

Title: Warm temperature inhibits cytoplasmic incompatibility induced by endosymbiotic *Rickettsiella* in a spider host

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ABSTRACT

Bacterial endosymbionts manipulate reproduction in arthropods to increase prevalence in the host population. One such manipulation is cytoplasmic incompatibility (CI), wherein the bacteria sabotage sperm in infected males to reduce hatch rate when mated with uninfected females, but zygotes are "rescued" when that male mates with an infected female. In the spider *Mermessus fradeorum* (Linyphiidae), *Rickettsiella* symbionts cause variable levels of CI. We hypothesized that temperature affects CI strength and rescue in *M. fradeorum,* potentially mediated by bacterial titer. We reared *Rickettsiella*-infected spiders in two temperature conditions (26°C vs 20°C) and tested CI induction in males and CI rescue in females. In incompatible crosses between infected males and uninfected females, hatch rate from warm males was doubled (Mean±S.E. = 0.687±0.052) relative to cool males (0.348±0.046), indicating that CI induction is weaker in warm males. In rescue crosses between infected females and infected males, female rearing temperature had a marginal effect on CI rescue, but hatch rate remained high for both warm (0.960±0.023) and cool females (0.994±0.004). Bacterial titer as measured by qPCR was lower in warm than cool spiders, particularly in females, suggesting that bacterial titer may play a role in causing the temperature-mediated changes in CI.

Keywords

bacterial endosymbionts, bacterial titer, climate change, cytoplasmic incompatibility, reproductive manipulation, symbiosis, temperature

INTRODUCTION

Maternally-inherited bacterial symbionts are common in arthropods, some of which can manipulate host reproduction to increase bacterial prevalence within the host population. Cytoplasmic incompatibility (CI) is the most common and well-studied manipulation, sabotaging matings between infected males and uninfected females, preventing the production of uninfected offspring, and increasing the relative proportion of infected offspring in the population over time (Shropshire et al. 2020). CI is typically viewed as a modification/rescue system: The symbiont induces CI by modifying male gametes, which cannot successfully fertilize and develop into progeny unless the egg is also infected and contains an appropriate rescue factor (Shropshire et al. 2020). Numerous bacterial clades have been shown to cause CI,

58 including *Wolbachia* (Alphaproteobacteria; reviewed in Shropshire et al. 2020), *Cardinium*
59 (Bacteroidetes; Hunter et al. 2003), *Mesenet* (Alphaproteobacteria; Takano et al. 2021),
60 *Spiroplasma* (Mollicutes; Pollman et al. 2022), and *Rickettsiella* (Gammaproteobacteria;
61 Rosenwald et al. 2020). The mechanistic underpinnings of CI are only well characterized for
62 *Wolbachia* (Shropshire et al. 2020), but genomic and phenotypic studies with other symbionts
63 suggest novel modes of action may be present (Poinsot et al. 2003, Penz et al. 2012, Pollmann
64 et al. 2022). Consequently, caution should be used when extrapolating inferences from
65 *Wolbachia* CI studies to other bacterial symbionts.

66 There is growing evidence that symbiont-induced host phenotypes, such as CI, can be thermally
67 sensitive but potentially idiosyncratic (Corbin et al. 2017, Doremus et al. 2019, Shropshire et al.
68 2020, Chrostek et al. 2021, Corbin et al. 2021, Hague et al. 2022, Jones et al. 2023, Martins et al.
69 2023). *Wolbachia*-induced phenotypes generally appear to weaken with increased temperature
70 (e.g., Clancy and Hoffmann 1998, Ross et al. 2017), which likely has consequences for the global
71 deployment of this symbiont in vector management programs (Ross et al. 2023). Other
72 symbionts, such as *Spiroplasma*, show a reversed pattern, with weaker effects on their hosts at
73 decreased temperature (Anbutsu et al. 2008, Corbin et al. 2021). The effect of temperature on
74 *Rickettsiella*-induced CI has not previously been investigated, but in a non-CI-inducing
75 *Rickettsiella* strain in aphids, temperature affects *Rickettsiella* titer, host phenotype, and spread
76 in the host population (Gu et al. 2023). More generally, several studies have suggested that
77 thermal conditions might limit the geographic distribution of symbionts, their phenotypes, and
78 consequently even their host species (Zhang et al. 2019, Ross et al. 2020, Hague et al. 2022).
79 Thus, under global climate change, it becomes critical to understand thermal effects on
80 arthropod symbionts, because symbiont-induced phenotypes are likely to change and affect the
81 spread and performance of both pest and beneficial arthropod hosts.

82 Here, we test whether temperature affects the strength of *Rickettsiella*-induced CI and bacterial
83 titer in the linyphiid spider *Mermessus fradeorum* (Berland, 1932). Few studies of manipulative
84 symbionts have been conducted in non-insect arthropods, which can be infected with well-
85 known symbiotic taxa such as *Wolbachia*, and lesser known manipulative clades such as

86 *Rickettsiella*. Populations of *Mermessus fradeorum* are composed of individuals that are
87 naturally co-infected with different combinations of several such maternally inherited
88 symbionts, of which only *Rickettsiella* has been shown to induce CI (Curry et al. 2015,
89 Rosenwald et al. 2020). The mechanism by which *Rickettsiella* induces CI is unknown; however,
90 we have observed the strength of CI to be quite variable. We hypothesized that temperature
91 mediates the penetrance of the phenotype, potentially mediated by bacterial titer. Using
92 laboratory-reared colonies of spiders that were either infected only with *Rickettsiella* or were
93 uninfected because they came from lineages that had been experimentally cured via antibiotics
94 (Rosenwald et al. 2020), we reared spiders under two temperature regimes (26°C or 20°C) and
95 evaluated the ability of infected males to induce CI and infected females to rescue CI as a
96 function of temperature. Because the strength of symbiont-induced phenotypes is often
97 mediated by bacterial quantity (Lopez-Madrigal and Duarte 2019), we also evaluated bacterial
98 titer as a function of temperature.

99 **EXPERIMENTAL PROCEDURES**

100 To test the effect of temperature on *Rickettsiella*-induced CI in *M. fradeorum*, we conducted
101 separate experiments on CI-induction in males versus CI-rescue in females. We assessed
102 induction versus rescue separately to maximize experimental power on informative crosses. We
103 selected two temperature treatments, warm (26˚C) and cool (20˚C) that fall within the range of
104 temperatures the spiders would experience in the field, and at which we knew the spiders
105 could be successfully reared and propagated. *Rickettsiella*-infected (R-infected) spiders were
106 reared under both temperature regimes, while uninfected spiders were only reared under cool
107 conditions because the experiments were designed to test for effects of temperature on CI, not
108 on the spiders *per se*.

109 A cohort of R-infected (n=17) and uninfected (n=13) mother *M. fradeorum* were mated and
110 allowed to lay eggmasses over the span of 2-3 weeks. As each clutch of spiderlings hatched,
111 they were split into individual 4 cm diameter rearing cups with moistened plaster at the bottom
112 for humidity control (Rosenwald et al. 2020). Half of the R-infected spiderlings were randomly
113 assigned to be reared in a 20˚C environmental chamber and half in a 26˚C chamber; all

114 uninfected spiderlings were reared at 20˚C. All were maintained on a diet of *Sinella curviseta*
115 collembola when young and *Drosophila melanogaster* fruit flies when older (Rosenwald et al.
116 2020). After spiders reached maturity, they were randomly assigned to mating crosses in either
117 male CI induction or female CI rescue experiments. All experimental spiders were 7-10 weeks of
118 age at mating; these spiders typically live 6-10 months under laboratory conditions, hence age
119 differences of a couple of weeks are trivial.

120 For the male CI induction experiment, the experimental test cross was to compare CI induced
121 by R-infected males reared under warm versus cool conditions. These males (n=30 per
122 treatment) were mated to uninfected females to evaluate CI induction. To ensure that hatch
123 rate reductions observed in the CI crosses were not due to innate male infertility, these same
124 males were mated one week later to cool-reared R-infected females (compatible cross). Male
125 *M. fradeorum* are capable of mating multiple times and we have not detected decreases in
126 fertility or CI strength with subsequent matings (J. White, unpublished data). To control for
127 potential differences in innate female fertility, additional sets of cool-reared R-infected (n=15)
128 and uninfected (n=15) females were mated with uninfected males.

129 For the female CI rescue experiment, the experimental test cross of interest was to compare CI
130 rescue by R-infected females reared under warm versus cool conditions. These females (n=30
131 per treatment) were mated to cool-reared R-infected males to evaluate CI rescue. Because we
132 could not use the same re-mating technique with females as males, we mated separate sets of
133 warm and cool R-infected females (n=15 per treatment) to uninfected males, as controls for
134 female fertility. We included an additional control cross between cool R-infected males and
135 uninfected females (n=15), to ensure that males used in this experiment were capable of
136 causing CI. Finally, an additional set of warm-reared R-infected males (n=15) was also crossed
137 with uninfected females to aid in comparison across the two experiments.

138 After matings for each experiment were complete, male spiders were placed in 1.5mL
139 microcentrifuge tubes and stored at their rearing temperature without food for five to seven
140 days to ensure that gut contents had been emptied. Specimens were then preserved in 95%
141 ethanol and stored at -20˚C for further analysis. Female spiders were maintained under

142 standard rearing conditions at their assigned temperature until they deposited three
143 eggmasses. Then, females were moved to microcentrifuge tubes and underwent the same food
144 deprivation and preservation sequence as the males. To validate expected infection status, we
145 extracted DNA from a subset of 20 spiders using DNeasy Blood & Tissue Kit (Qiagen) following
146 the manufacturer's protocol. We performed diagnostic PCR for *Rickettsiella*, and two other
147 symbiont genera (*Tisiphia* and *Wolbachia*) known to be present in some *M. fradeorum* using
148 previously published protocols (wsp primers for *Wolbachia*, Baldo et al. 2006; Ricklong primers
149 for *Tisiphia*, Curry et al. 2015; RLA primers for *Rickettsiella*, Rosenwald et al. 2020). All tested
150 spiders were positive or negative for *Rickettsiella* as expected, and all spiders were negative for
151 the other symbionts.

152 To evaluate the hatch rate, we dissected each eggmass after spiderlings emerged, and counted
153 the remaining unhatched eggs as in Rosenwald et al. (2020). Total hatched and unhatched eggs
154 per female were used for a series of logistical regression contrasts, using Williams' correction
155 for overdispersion (Arc v 1.06; Williams 1982). In the CI induction experiment, we conducted
156 three statistical contrasts. First, to ensure CI was in evidence in the experiment, we compared
157 hatch rates in expected CI treatments (warm and cool R-infected males mated to uninfected
158 females) to control matings between uninfected males and uninfected females. Second, to
159 ensure that neither temperature nor *Rickettsiella* infection negatively affected male fertility, we
160 then contrasted hatch rates when these same males were mated to cool-reared *R*-infected
161 females, relative to uninfected males mated to R-infected females. Finally, we directly
162 contrasted the hatch rates of the warm and cool CI treatments to one another, to evaluate the
163 effect of rearing temperature on CI induction. In the CI rescue experiment we likewise
164 conducted three statistical contrasts. First, to ensure rearing temperature didn't directly affect
165 hatch rate, we contrasted hatch rates from warm versus cool R-infected females when mated
166 to uninfected males. Second, to ensure CI was in evidence in the experiment, we contrasted
167 cool R-infected females mated to cool R-infected males (cool rescue treatment), relative to cool
168 uninfected females mated to cool R-infected males (CI control). Finally, we directly contrasted
169 hatch rates of the warm and cool CI rescue treatments to one another, to evaluate the effect of
170 rearing temperature on CI rescue.

171 We evaluated symbiont titer via qPCR for a subset of 10-15 R-infected spiders from each sex
172 and temperature treatment. For the sake of consistency, all evaluated spiders originated from
173 the same experiment, the CI rescue experiment. We placed specimens into 50% PBS (EtOH:
174 PBS) for 30 min, followed by 75% PBS (EtOH: PBS) for 30 min, and finally kept in 100% PBS
175 overnight. We crushed these specimens in cryogenic vials containing solid-glass beads using
176 FastPrep® Tissue Homogenizer (MP Biomedicals), then extracted DNA using DNeasy Blood &
177 Tissue Kit (Qiagen) following the manufacturer's protocol. We designed short primers specific
178 to the *Rickettsiella recA* gene and *M. fradeorum 18S rRNA* gene using Geneious Prime Version
179 2021.2 (Table S1). Primer specificity was tested *in-silico* and using diagnostic PCR (Table S2). We
180 cloned these short specific sequences using a vector from CloneJET™ PCR Cloning Kit and
181 JM109 Mix & Go Competent Cells. Serial dilutions with serial factor 1:10 for the symbiont gene
182 and 1:3 for the spider gene were used for a standard calibration curve. qPCR was performed
183 using the Step One Plus real-time PCR system (Applied Biosystems, CA, USA) and Fast SYBR
184 green master mix (Thermo Fisher Scientific, MA, USA). For each 20 µL reaction mixture, we used
185 30 ng of gDNA, and each sample was measured in three technical triplicates. Cycling conditions
186 are listed in Table S2. We used output Ct, Intercept, and Slope values to calculate the amount
187 (ng) of the symbiont's gene and the spider's gene from each sample ($ng = 10^{(\frac{Ct-Intercept}{Slope})}$). We
188 then used these weights to calculate the gene copy number (gcn) ($gcn =$
189 $\frac{ng \times gcn/mole}{bp \times ng/g \times g/mole\ of\ bp}$), followed by data normalization ($normalized\ symbiont\ titer =$
190 $\frac{symbiont\ recA\ gcn}{spider\ 18S\ gcn}$). We log-transformed the normalized titer values to reduce heteroscedasticity,
191 and compared means using a general linear model using Type III sums of squares in IBM SPSS v
192 28.0., with sex, temperature, and their interaction included as fixed factors.

193 **RESULTS**

194 When we reared R-infected spiders at either 26°C or 20°C and then evaluated the strength of CI
195 induction (Figure 1A), we found that both warm and cool R-infected males caused reduced
196 hatch rates when mated to uninfected females, relative to control matings between uninfected

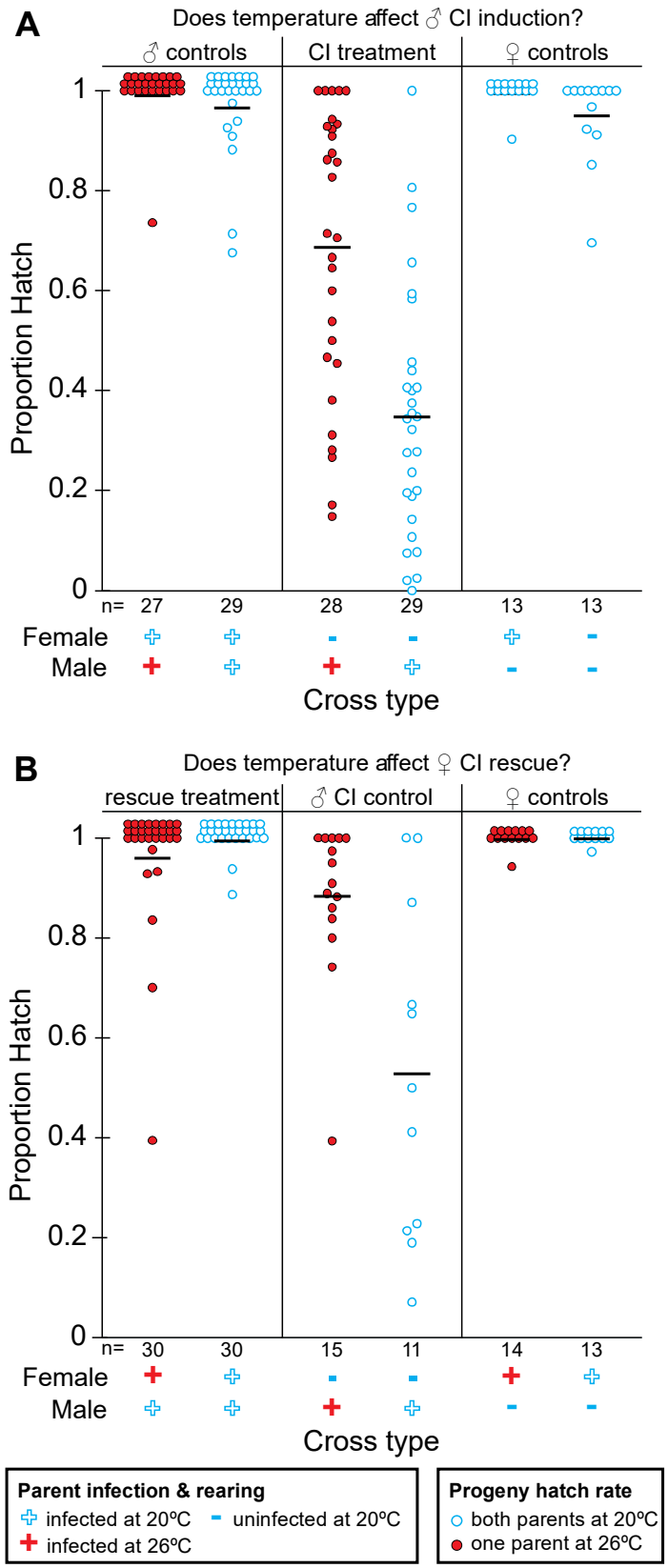

Figure 1. Proportion hatch of eggs from crosses between *Rickettsiella*-infected *Mermessus fradeorum* spiders that were individually reared at 26°C or 20°C, and subsequently mated to either R-infected or uninfected spiders reared at 20°C as indicated on the x-axes. Realized sample sizes per cross (excluding failed matings) are indicated below each column. Crosses to evaluate male CI induction (Panel A) showed a significantly higher hatch rate (weaker CI) for offspring of warm-reared than cool-reared infected males when mated to uninfected females. All control crosses had high hatch rates. Crosses to evaluate female CI rescue (Panel B) showed marginally lower hatch rate for warm-reared than cool-reared infected females mated to infected males, but high hatch rates overall indicate temperature had relatively little effect on CI rescue. Incompatible crosses between infected males and uninfected females were included as controls to aid comparison across the two experiments and validate that infected males used within this experiment were capable of inducing CI.

225 males and females (ΔDeviance = 58.95, d.f. = 2, P<0.001). When these same R-infected males
226 were mated with R-infected females, hatch rates were nearly perfect and did not differ from
227 control crosses between uninfected males and R-infected females (ΔDeviance = 2.87, d.f. = 2,
228 P=0.238), indicating that neither rearing temperature nor *Rickettsiella* infection intrinsically
229 decreased fertility of males. When compared directly, the hatch rate of incompatible crosses
230 between uninfected females and warm males was twice as high (0.687±0.052) as with cool
231 males (0.348±0.046; ΔDeviance = 21.30, d.f. = 1, P<0.001), indicating that warmer rearing
232 temperatures weakened CI induction.

233 In the CI rescue experiment (Figure 1B), hatch rates were high and equivalent for warm and
234 cool R-infected females mated to uninfected males (ΔDeviance=0.20, d.f.=1, P=0.654),
235 indicating that rearing temperatures did not directly affect hatch rate. Cool R-infected females
236 also exhibited nearly perfect hatch rates when mated to cool R-infected males, much higher
237 than cool uninfected females mated to cool R-infected males (ΔDeviance=84.49, d.f.=1,
238 P<0.001). This control contrast establishes both that the R-infected males in this experiment
239 induced CI, and that cool R-infected females were capable of rescue. When we contrasted
240 rescue between the two female rearing temperatures, we found that maternal rearing
241 temperature had a marginally significant effect on hatch rate (ΔDeviance = 3.825, d.f. =1,
242 P=0.0505), suggesting that CI rescue ability can be weakened in at least some R-infected
243 females. Nevertheless, most females in these rescue crosses exhibited perfect hatch rates,
244 indicating that the CI rescue ability of *Rickettsiella* usually remains intact regardless of rearing
245 temperature.

246 When we evaluated bacterial titer in a subset of warm-reared versus cool-reared spiders
247 (Figure 2), we found an interactive effect between sex and temperature (Sex $F_{1,44}$ =0.329,
248 P=0.529, Temperature $F_{1,44}$ =6.026, P=0.018, Sex × Temp interaction $F_{1,44}$ =5.22, P=0.027). Titer
249 was lower in the warm treatments, but this lower titer was associated with females more than
250 males, even though temperature effects on symbiont phenotype were more evident in males
251 (CI induction) than females (CI rescue).

252

253 **DISCUSSION**

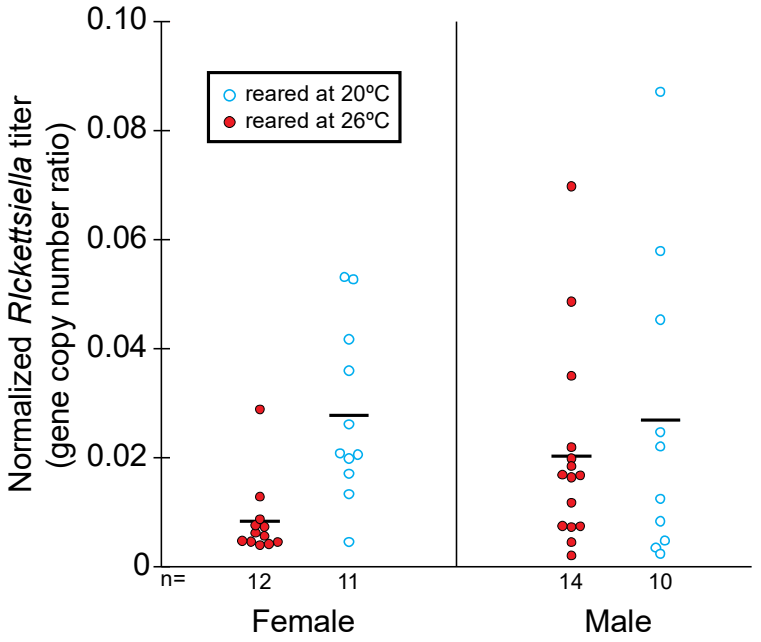

Figure 2. *Rickettsiella* titer in a subset of warm (26°C) and cool (20°C) reared spiders from the CI rescue experiment. Log transformed titer (normalized against spider 18S quantity) was significantly affected by the interaction between temperature and sex, primarily driven by lower titer in warm reared females.

254 Warmer rearing temperatures
255 strongly decreased CI induction by
256 *Rickettsiella* in male *M. fradeorum*
257 spiders, but only mildly decreased
258 CI rescue in female spiders.
259 Thermal sensitivity for *Rickettsiella*-
260 induced phenotypes is consistent
261 with results for a different strain of
262 *Rickettsiella* in an aphid host, which
263 exhibits a different set of host
264 phenotypic effects that don't
265 include CI (Gu et al. 2023). In the
266 present study, CI strength overall
267 was notably weaker than in prior
268 studies by our group (Rosenwald et al. 2020), which were conducted at room temperature, but
269 in a laboratory that was cool throughout the duration of the experiments (J. White, pers. obs.).
270 Highly variable hatch rates across all CI crosses (Fig 1A), suggest that other factors may
271 additionally be driving CI strength in this system (e.g., Shropshire et al. 2021). More generally,
272 bacterial symbionts often show reduced phenotypic penetrance at warmer temperatures
273 (Martins et al. 2023), although the phenomenon is not universal, and instances where
274 symbiont-induced phenotypes increase with warmer temperatures also exist (e.g., Corbin et al.
275 2021).

276 Warmer rearing temperature also decreased *Rickettsiella* titer, particularly in female spiders,
277 which also tended to have lower titer than males. The latter was surprising, because gravid
278 females are usually expected to carry higher loads of maternally transmitted symbionts than
279 males (e.g., Noda et al., 2001). However, it is also possible that relatively high loads of bacteria
280 would be allocated to eggs, and these post-ovipositional females, which had laid 3 eggmasses

281 prior to preservation and extraction, may have exhibited substantially reduced bacterial titer
282 relative to their pre-ovipositional state. It is also worth noting that our standard laboratory
283 protocol calls for imposing several days of food deprivation to clear gut contents (and
284 associated prey symbionts) before spider specimen preservation. This protocol likely
285 diminished the utility of the qPCR assay, by temporally disassociating the titer of the preserved
286 specimen from its titer at biologically relevant timepoints (fertilization, egg deposition). Despite
287 these caveats, we nevertheless did detect a signal of temperature treatment on bacterial titer,
288 suggesting that stronger CI penetrance may be associated with higher bacterial titer. We will be
289 following up with assays more specifically designed to probe the mechanism and timing of CI
290 modification and rescue in this novel host/symbiont system. Associations between
291 temperature and symbiont titers have previously been demonstrated with other
292 endosymbionts (Breeuwer and Warren 1993, Lopez-Madrigal and Duarte 2019, Ross et al.
293 2020), and thus our results further support the potential links between temperature, titer, and
294 phenotypic outcome.

295 Our study demonstrates the thermal sensitivity of CI in a novel arthropod/symbiont system,
296 implying that temperature may be modulating host/symbiont interactions quite broadly in
297 nature (e.g., Kreisner et al. 2016, Hague et al. 2022). However, much additional study will be
298 needed before we can predict how temperature affects CI dynamics in the field. For *M.*
299 *fradeorum*, we selected only two relatively moderate temperatures to test, which the spider
300 likely experiences routinely in the field. We also kept each temperature constant, because we
301 do not yet know the mechanism or timing of *Rickettsiella*-induced CI modification and rescue.
302 Future studies will explore variations of timing, duration, and extent of thermal exposures on CI
303 in this system, which will inform our understanding of spider/symbiont biogeography for this
304 widespread introduced species within the context of global climate change.

305 More broadly, environmental effects on symbiont phenotypic penetrance may have important
306 implications in both managed and natural ecosystems. *Wolbachia* is already in use in vector
307 management in several areas of the globe, with mixed results (Hoffmann et al. 2011, Dos
308 Santos et al. 2022, Hoffmann et al. 2024). Symbiont thermal tolerance is one factor that

309 appears to limit the success of this technique in some regions (Ross et al. 2020, 2023). As the
310 use of symbionts to control pests and vectors comes under consideration in a range of other
311 systems (e.g., Gong et al. 2020, Mateos et al. 2020), it becomes critical to characterize the
312 environmental constraints that might influence efficacy. Even in less-managed systems, the
313 thermal optima of symbionts might play a critical role in determining the distribution of pests
314 and the ability of natural enemies to control them (Zhang et al. 2019, Hague et al. 2022). Our
315 study contributes to a growing body of literature that suggests environmental contingency may
316 be the rule for interactions between hosts and reproductive manipulators, not the exception.

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