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, often manipulating 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). Numerous bacterial clades have been shown to cause CI, including *Wolbachia*, *Cardinium*, *Spiroplasma*, and *Rickettsiella* (Hunter et al. 2003, Rosenwald et al. 2020, Shropshire et al. 2020, Pollmann et al. 2022). The mechanistic underpinnings of CI are only well characterized for *Wolbachia* (Shropshire et al. 2020), but genomic and phenotypic studies with other symbionts suggest novel modes of action may be present (Poinsot et al. 2003, Penz et al. 2012, Pollmann et al. 2022). Consequently, caution should be used when extrapolating inferences from *Wolbachia* CI studies to other bacterial symbionts.

There is growing evidence that symbiont-induced host phenotypes, such as CI, can be thermally sensitive but potentially idiosyncratic (Corbin et al. 2017, Doremus et al. 2019, Shropshire et al. 2020, Chrostek et al. 2021, Corbin et al. 2021, Hague et al. 2022, Jones et al. 2023, Martins et al. 2023). The effect of temperature on *Rickettsiella*-induced CI has not previously been investigated, but in a non-CI-inducing *Rickettsiella* strain in aphids, temperature affects *Rickettsiella* titer, host phenotype, and spread in the host population (Gu et al. PNAS 2023). More generally, several studies have suggested that thermal conditions might limit the geographic distribution of symbionts, their phenotypes, and consequently even their host species (Zhang et al 2019, Ross et al. 2020, Hague et al. 2022). Thus, under global climate

change, it becomes critical to understand thermal effects on arthropod symbionts, because symbiont-induced phenotypes are likely to change and affect the spread and performance of both pest and beneficial arthropod hosts.

Here, we test whether temperature affects the strength of *Rickettsiella*-induced CI and bacterial titer in *Mermessus fradeorum* (Berland, 1932). This linyphiid spider is infected with several maternally inherited symbionts, of which only *Rickettsiella* has been shown to induce CI (Curry et al. 2015, Rosenwald et al. 2020). Using laboratory-reared colonies of spiders that were either uninfected or infected only with *Rickettsiella* (Rosenwald et al. 2020), we reared spiders under two temperature regimes (26°C or 20°C) and evaluated the ability of infected males to induce CI and infected females to rescue CI as a function of temperature. We also evaluated bacterial titer as a function of temperature because the strength of symbiont-induced phenotypes is often mediated by bacterial quantity (Lopez-Madrigal and Duarte 2019).

EXPERIMENTAL PROCEDURES

Clutches of newly hatched *M. fradeorum* spiderlings infected with *Rickettsiella* were split into individual 4cm diameter rearing cups with moistened plaster at the bottom for humidity control (Rosenwald et al. 2020). Half of the spiderlings were randomly assigned to be reared in a 20˚C environmental chamber and half to a 26˚C chamber. All were maintained on a diet of *Sinella curviseta* collembola and *Drosophila melanogaster* fruit flies later on (Rosenwald et al. 2020). Uninfected spiders were treated identically, except all were reared at 20˚C. After spiders reached maturity, they were randomly assigned to mating crosses in either male CI induction or female CI rescue experiments. In the CI induction experiment, *Rickettsiella* (R)-infected male spiders were mated twice, first to uninfected females (the CI cross) and second to *R*-infected females to verify the fertility of each male spider. Male spiders were maintained under standard rearing conditions (fed twice a week with *D. melanogaster*) at their assigned temperature between matings. In the female CI rescue experiment, all spiders were mated only once. After matings were complete, male spiders were placed in 1.5mL microcentrifuge tubes and stored at their rearing temperature without food for five to seven days to ensure that gut contents had been emptied. Specimens were then preserved in 95% ethanol and stored at -20˚C for further analysis. Female spiders were maintained under standard rearing conditions at their assigned temperature until they deposited three eggmasses. Then, females were moved to microcentrifuge tubes and underwent the same food deprivation and preservation sequence as the males.

To evaluate the hatch rate, we dissected each eggmass after spiderlings emerged, and counted the remaining unhatched eggs as in Rosenwald et al. (2020). Total hatched and unhatched eggs per female were used for a series of logistical regression contrasts, using Williams' correction for overdispersion (Arc v 1.06; Williams 1982). In the CI induction experiment, we first compared both expected CI treatments, warm and cool R-infected males mated to uninfected females, relative to control matings between uninfected males and uninfected females, to ensure that CI was in evidence in the experiment. We then contrasted when these same males were mated to cool-reared *R*-infected females, relative to uninfected males and *R*-infected

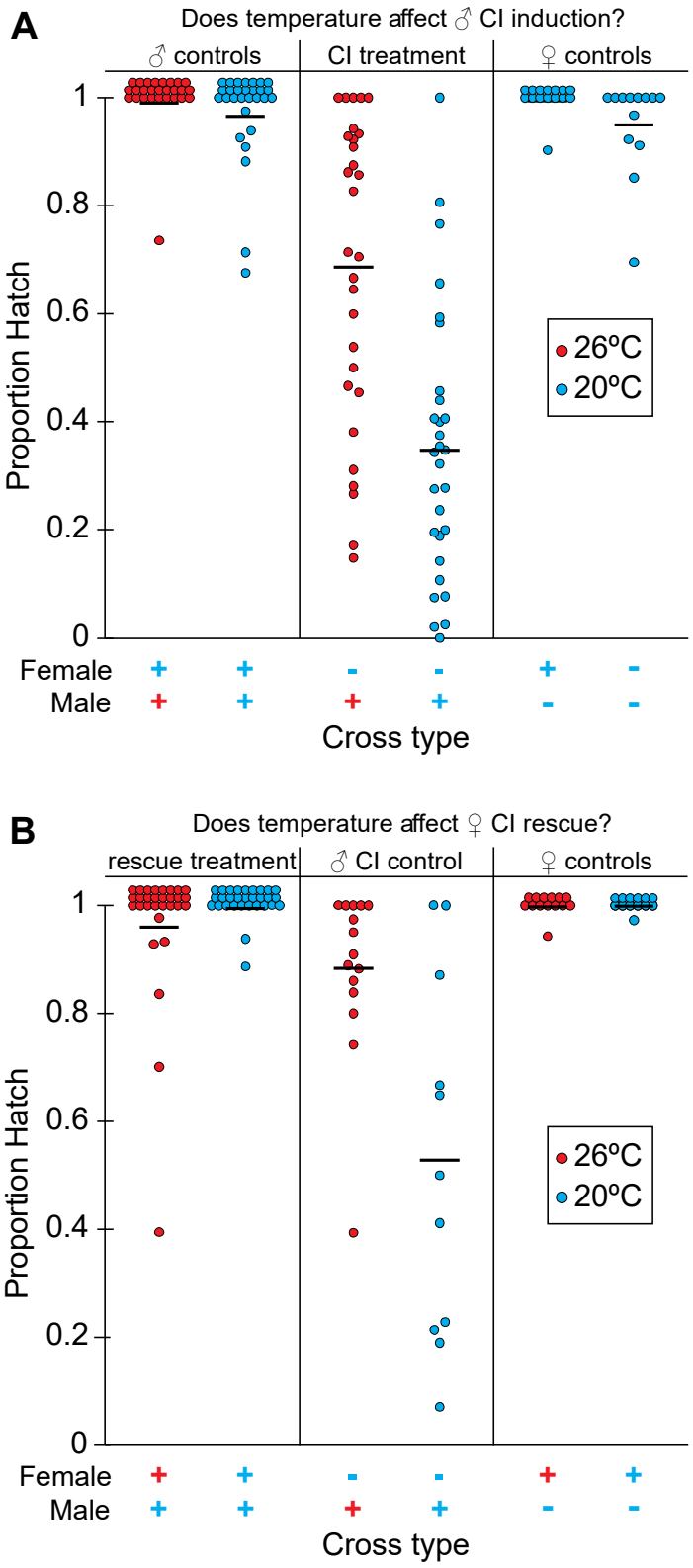
110 females. This contrast ensured that neither temperature nor *Rickettsiella* infection negatively
111 affected male fertility. Finally, we directly contrasted the hatch rates of the warm and cool CI
112 treatments to one another, to evaluate the effect of rearing temperature on CI induction. In the
113 CI rescue experiment, we first contrasted warm versus cool R-infected females mated to
114 uninfected males, to ensure rearing temperature didn't directly affect hatch rate. Next, we
115 contrasted cool R-infected females mated to cool R-infected males, relative to cool uninfected
116 females mated to cool R-infected males, to ensure that this group of males was capable of
117 inducing CI. Finally, we directly contrasted hatch rates of the warm and cool CI rescue
118 treatments to one another, to evaluate the effect of rearing temperature on CI rescue.

119 We evaluated symbiont titer via qPCR for a subset of 10-15 *R*-infected spiders from each sex
120 and temperature treatment. For the sake of consistency, all evaluated spiders originated from
121 the same experiment, the female CI rescue experiment. We placed specimens into 50% PBS
122 (EtOH: PBS) for 30 min, followed by 75% PBS (EtOH: PBS) for 30 min, and finally, kept in 100%
123 PBS overnight. We crushed these specimens in cryogenic vials containing solid-glass beads using
124 FastPrep® Tissue Homogenizer (MP Biomedicals), then extracted DNA using DNeasy Blood &
125 Tissue Kit (Qiagen) following the manufacturer's protocol. We designed short primers specific
126 to the *Rickettsiella recA* gene and *M. fradeorum 18S rRNA* gene using Geneious Prime Version
127 2021.2. Primer sequences, reaction mixtures, cycling conditions, and titer calculation
128 methodology are provided in Supplementary Document 1.

129 **RESULTS**

130 When we reared *R*-infected spiders at either 26°C or 20°C and then evaluated the strength of CI
131 induction (Figure 1A), we found that both warm and cool R-infected males caused reduced
132 hatch rates when mated to uninfected females, relative to control matings between uninfected
133 males and females (ΔDeviance = 58.95, d.f. = 2, P<0.001). When these same R-infected males
134 were mated with *R*-infected females, hatch rates were nearly perfect and did not differ from
135 control crosses between uninfected males and *R*-infected females (ΔDeviance = 2.87, d.f. = 2,
136 P=0.238), indicating that neither rearing temperature nor *Rickettsiella* infection intrinsically
137 decreased fertility of males. When compared directly, the hatch rate of incompatible crosses
138 between uninfected females and warm males was twice as high (0.687±0.052) as with cool
139 males (0.348±0.046; ΔDeviance = 21.30, d.f. = 1, P<0.001), indicating that warmer rearing
140 temperatures weakened CI induction.

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 infected (+) or uninfected (-) spiders reared at 20°C as indicated on the x-axes. Crosses to evaluate male CI induction (Panel A) showed a significantly higher hatch rate (weaker CI) for offspring of warm (+) than cool (+) 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 (+) than cool (+) 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.



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

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

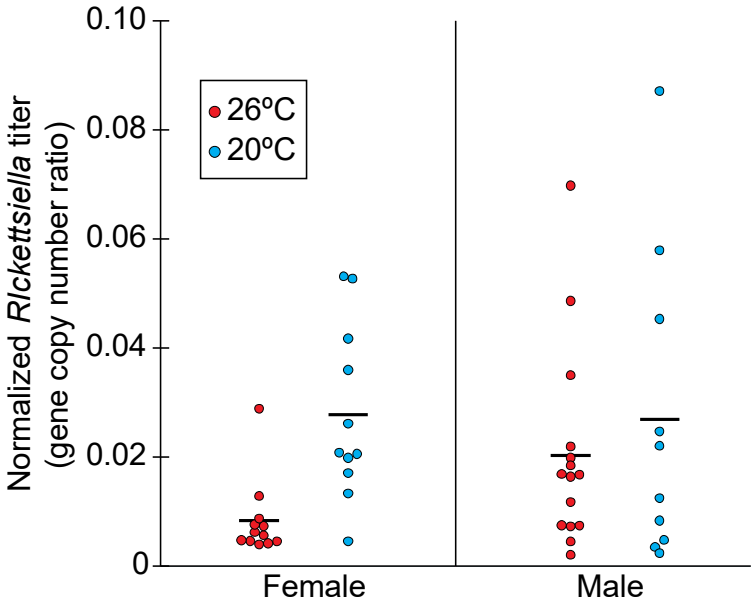


Figure 2. *Rickettsiella* titer in a subset of warm (26°C) and cool (20°C) spiders from the CI rescue experiment. Log transformed titer (normalized against spider 18S quantity) was significantly affected by the interaction between temperature and sex, primarily driven by lower titer in warm females.

DISCUSSION

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

Warmer rearing temperature also decreased *Rickettsiella* titer, particularly in female spiders, even though temperature effects on symbiont phenotype were more apparent in males than females. This result is somewhat surprising because gravid females are usually expected to carry higher loads of maternally transmitted symbionts than males; perhaps the use of post-oviposition females in the present study depleted the bacterial loads of females. Associations between temperature and symbiont titers have previously been demonstrated with other endosymbionts (Breeuwer and Warren 1993, Lopez-Madrigal and Duarte 2019, Ross et al. 2020), and thus our results further support the potential links between temperature, titer, and phenotypic outcome.

Environmental effects on symbiont phenotypic penetrance may have important implications in both managed and natural ecosystems. *Wolbachia* is already in use in vector management in several areas of the globe, with mixed results (Hoffmann et al. 2011, Dos Santos et al. 2022, Hoffmann et al. 2024). Symbiont thermal tolerance is one factor that appears to limit the success of this technique in some regions (Ross et al. 2020, 2023). As the use of symbionts to control pests and vectors comes under consideration in a range of other systems (e.g., Gong et al. 2020, Mateos et al. 2020) it becomes critical to characterize the environmental constraints that might influence efficacy. Even in less-managed systems, the thermal optima of symbionts might play a critical role in determining the distribution of pests and the ability of natural enemies to control them (Zhang et al. 2019, Hague et al. 2022). Our study contributes to a growing body of literature that suggests environmental contingency may be the rule for interactions between hosts and reproductive manipulators, not the exception.

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