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Cytoplasmic incompatibility in the parasitic wasp *Encarsia inaron*: disentangling the roles of *Cardinium* and *Wolbachia* symbionts

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Abstract

Many bacterial endosymbionts of insects are capable of manipulating their host's reproduction for their own benefit. The most common strategy of manipulation is cytoplasmic incompatibility (CI), in which embryonic mortality results from matings between uninfected females and infected males. In contrast, embryos develop normally in infected females, whether or not their mate is infected, and infected progeny are produced. In this way, the proportion of infected females increases in the insect population, thereby promoting the spread of the maternally-inherited bacteria. But what happens when multiple endosymbionts inhabit the same host? The parasitoid wasp *Encarsia inaron* is naturally infected with two unrelated endosymbionts, *Cardinium* and *Wolbachia*, both of which have been documented to cause CI in other insects. Doubly-infected wasps show the CI phenotype. We differentially cured *E. inaron* of each endosymbiont, and crossed hosts of different infection status to determine whether either or both bacteria caused the observed CI phenotype in this parasitoid, and whether the two symbionts interacted within their common host. We found that *Wolbachia* caused CI in *E. inaron*, but *Cardinium* did not. We did not find evidence that *Cardinium* was able to modify or rescue *Wolbachia*-induced CI, nor did we find that *Cardinium* caused progeny sex ratio distortion, leaving the role of *Cardinium* in *E. inaron* a mystery.

Keywords

bacterial endosymbionts; multiple infection; reproductive parasites; reproductive manipulators; sex ratio; symbiosis

Introduction

Maternally inherited bacterial endosymbionts are extremely common in arthropods (Douglas, 1989; Duron *et al*, 2008; Hilgenboeker *et al*, 2008), and induce a variety of

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phenotypes in their hosts, ranging from obligate nutritional mutualism to facultative reproductive parasitism (Werren and O'Neill, 1997). Further, it is becoming increasingly evident that many arthropods are infected with multiple lineages of symbionts (e.g., Zchori-Fein and Perlman, 2004; Chiel *et al*, 2007; Weinert *et al*, 2007). Within multiply-infected hosts, different facultative symbionts may exhibit tissue tropism, regulate their density independently or in aggregate, and interact in ways that affect the host phenotype (Ijichi *et al*, 2002; Mouton *et al*, 2004; Kondo *et al*, 2005; Oliver *et al*, 2006). The extent to which multiple infections differ from single infections is as yet poorly understood, and ultimately, requires dissecting the role of each symbiont in isolation, as well as documenting the interactions among them. In the present study, we focus on the reproductive phenotype and interactions of a co-infection of two independent symbiont lineages that are known to promote their own spread by manipulating host reproduction: *Cardinium*, in the Bacteroidetes, and *Wolbachia*, in the α-proteobacteria.

Both *Cardinium* and *Wolbachia* bacteria have been documented to cause cytoplasmic incompatibility (CI) in their hosts (Hoffmann and Turelli, 1997; Hunter *et al*, 2003). CI is an interesting phenomenon because it requires interaction between bacteria in different host individuals for its manifestation. The phenotype can be best described with a "modification/ rescue" model (Werren, 1997). In infected males, the sperm is modified by the symbiont. When uninfected females mate with these infected males, the most common result is embryonic mortality following fertilization. In contrast, the symbiont present in infected females acts to "rescue" the sabotaged sperm, allowing the host to produce infected progeny. Consequently, infected females produce more offspring than uninfected females, causing the proportion of infected females to increase in the host population over time, and thereby promoting the propagation of the maternally-inherited bacteria (Caspari and Watson, 1959; Turelli, 1994; Werren, 1997). Symbionts have also been shown to promote the production of female offspring via parthenogenesis, male-killing, feminization, or other manipulation of offspring sex ratio (O'Neill *et al*, 1997), but CI appears to be the most common reproductive manipulation in arthropods (Stouthamer *et al*, 1999).

The precise mechanisms of CI remain to be elucidated, although recent studies of *Wolbachia*-induced CI have made substantial progress in understanding both the initial sperm modification and the post-zygotic rescue mechanism (e.g., Tram and Sullivan, 2002; Xi *et al*, 2008). It is clear, however, that there is diversity among *Wolbachia* strains in both the modification and rescue function. Through crossing experiments with singly- and multiply-infected hosts, it has been shown that various *Wolbachia* strains evoke CI at different intensities (e.g., Bordenstein and Werren, 2007), are sometimes but not always able to rescue one another (e.g., Mercot and Poinsot, 1998), and that a single strain may have multiple, distinct rescue functions (Zabalou *et al*, 2008), allowing rescue of alternative modification strains in the population. Co-infection by multiple CI-inducing *Wolbachia* strains is also a relatively common phenomenon (e.g., Kondo *et al*, 2002; Mouton *et al*, 2005), allowing multiply-infected females to rescue sperm from males with any subset of their symbionts. Maintenance of co-infection by multiple CI-inducing symbionts is therefore often evolutionarily favored (Frank, 1998; Vautrin *et al*, 2007).

CI was once thought to be a unique phenotype of *Wolbachia* (Weeks *et al*, 2002), but *Cardinium* was the second bacterial lineage discovered to induce CI in arthropods (Hunter *et al*, 2003; Gotoh *et al*, 2007a; Perlman *et al*, 2008). Virtually nothing is known about the mechanism of *Cardinium*-induced CI. In much the same way that multiple infection by *Wolbachia* strains has given insight into the mechanisms and evolution of *Wolbachia*-induced CI, interaction of these two distantly related bacteria in a common host may elucidate the similarities or differences in their mode of action. Hosts harboring both *Cardinium* and *Wolbachia* are relatively common (e.g., Weeks *et al*, 2003; Gotoh *et al*, 2007b; Duron *et al*, 2008), but in virtually all cases, the effect of either symbiont is completely unknown. One exception is *Encarsia inaron* (Hymenoptera: Aphelinidae), a parasitic wasp that was introduced from the Middle East and Europe to North America to control the ash whitefly (*Siphoninus phillyreae*) (Pickett and Pitcairn, 1999). Wasps collected in Tucson, Arizona were shown to have both *Cardinium* and *Wolbachia* endosymbionts, and to have a CI phenotype (Perlman *et al*, 2006). Like all Hymenoptera, *E. inaron* is haplodiploid and males develop from unfertilized eggs. CI in haplodiploid systems affects the diploid incipient females, and can result in embryonic lethality, (the “female mortality” type) or conversion of fertilized embryos to haploid males (the “male conversion” type) (Vavre *et al*, 2000). In doubly-infected *E. inaron*, the CI phenotype appeared to be the female mortality type. However, the role of each symbiont remained obscure; initial antibiotic curing experiments removed both symbionts (Perlman *et al*, 2006).

In the present study we examined the CI phenotype in *E. inaron* to determine the relative contributions of *Cardinium* and *Wolbachia*, and to examine potential interactions occurring between the symbionts. Specifically, we sought to 1) determine whether both or one of the bacteria caused the CI phenotype, 2) test whether the bacterial lineages interacted in the expression and rescue of CI, and 3) determine whether the bacteria had any other effects on the sex ratio of *E. inaron*.

Materials and Methods

Cultures

The doubly-infected *Encarsia inaron* culture (“Both”) originated from pupae collected in Tucson, Arizona in 2002 (Perlman *et al*, 2006). *Encarsia inaron* is a solitary endoparasitoid of whiteflies, and is propagated on sweet potato whitefly (*Bemisia tabaci*) in our laboratory as described in Perlman *et al*, (2006). In this species, male and female eggs are laid in first to third instar whitefly nymphs. Single adult wasps emerge approximately two weeks later.

To generate differentially infected cultures, we treated adult wasps with antibiotics. The “Cured” culture received rifampicin-infused honey (50 mg/ml) for 48 hr in three successive generations (Perlman *et al*, 2006). Curing was verified by polymerase chain reaction (PCR, see below). The “*Cardinium*” (Card.) and “*Wolbachia*” (Wol.) lines were generated by treating female wasps with a low dose (1.0 mg/ml) of either ampicillin or doxycycline for 48 hr. *Cardinium* appears to be more susceptible to ampicillin than *Wolbachia*, at least in cell culture, (Stouthamer, 1991; Morimoto *et al*, 2006), and both symbionts are susceptible to doxycycline. At sufficiently low doses, however, the antibiotics did not cure the wasps, but destabilized the infection such that bacterial transmission to offspring was not always complete. Individual treated females were allowed to oviposit in whitefly nymphs on 35 mm leaf disk arenas (Hunter *et al*, 2003). The resulting progeny were isolated as pupae, to prevent mating between siblings of potentially different infection status. Female offspring were mated to cured males in bulk, individually given an opportunity to oviposit on leaf disk arenas, and then sacrificed to determine infection status via PCR (see below). Most F_1 individuals were either cured or still infected with both symbionts, but a small proportion had only one symbiont or the other. Progeny of the singly-infected F_1 females were retained, and the individual propagation and PCR screening procedure was repeated for another generation to ensure stable transmission of the remaining symbiont. In the end, the *Cardinium* and *Wolbachia* lines were each initiated with F_3 individuals descended from at least eight different antibiotic-treated females.

Verification of symbiont infection

We used diagnostic PCR to assess infection status during the initiation of the laboratory cultures, as well as for verification that experimental wasps contained the expected symbionts. Previous work by Perlman *et al* (2006) found no other symbionts in *E. inaron* except *Cardinium* and *Wolbachia*. DNA was extracted by grinding individual wasps in 3 µL of 20 mg/ml proteinase K, incubating the samples at 37 °C in 50 µl 10% w/v Chelex (Sigma-Aldrich, St. Louis, MO) in purified water for one hour with periodic vortexing, followed by 8 min at 96 °C for enzyme denaturation (T. Groot, personal communication). Extracted samples were stored at −20 °C. For *Cardinium* amplification, we used 10 µL reactions (4.9 µL purified water, 1 µL Invitrogen 10× buffer, 0.8 µL of 10 mM dNTPs, 0.2 µL of 50 mM $MgCl_2$, 0.5 µL each of 5 pmol μL^{-1} forward and reverse primer, 0.1 µL of 5 U µL−1 Invitrogen *Taq* polymerase and 2 µL DNA sample). We used *Cardinium*-specific primers (Ch-F 5′ - TACTGTAAGAATAAGCACCGGC - 3′, Ch-R 5′ - GTGGATCACTTAACGCTTTCG - 3′) that amplify a 394 bp product (Zchori-Fein and Perlman, 2004). Each PCR was run for one cycle of 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, 51 °C for 30 sec, 72 °C for 30 sec and a final extension of 5 min at 72 °C. *Wolbachia* amplification was similar, except the volume of 50 mM $MgCl_2$ was increased to 0.8 µL, the volume of purified water was decreased to 4.3 µL, and *Wolbachia*-specific *ftsZ* primers were used that amplify a 775 bp product (ftsZunif 5′ - GG(CT)AA(AG)GGTGC(AG)GCAGAAGA - 3′, ftsZunir 5′ - ATC(AG)AT(AG)CCAGTTGCAAG - 3′) (Lo *et al*, 2002). The PCR program was one cycle of 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec, 72 °C for 45 sec and a final extension of 6 min at 72 °C. All PCRs were accompanied by positive and negative DNA controls. *Encarsia pergandiella* wasps served as positive controls for *Cardinium*, and *Encarsia formosa* wasps served as positive controls for *Wolbachia*. To visualize the reaction products, we added 1.6 µL 20X SYBR Green to each reaction, and visualized them on a 1.125% agarose gel in a UV transilluminator.

Experimental crosses

To test for CI among the differentially infected *E. inaron* cultures, we conducted a full factorial experiment, in which males of each of the four cultures were crossed with females of each culture, resulting in 16 treatments. We isolated pupae from each culture into individual 1.2 ml vials that contained a small droplet of honey, and plugged the vials with cotton. Upon emergence, wasps within each culture were sexed by visual inspection, and randomly assigned to mate with the opposite sex from one of the four cultures. Approximately 30 males and 30 females were assigned to each of the 16 treatments, and allowed to mate for 4 d in 3.8 L mating jars with free access to water and honey. Ten to 15 female wasps were selected at random from each mating jar, and individually placed on cowpea (*Vigna ungulata*) leaf disks on 1% agar medium in a 35 mm petri dish. Each leaf disk had ~50 first to third instar *B. tabaci* hosts for oviposition (range = 30–75 whiteflies per disk). The petri dishes were covered with modified screen-top lids, and placed in an environmental chamber at 27 °C, 60 %RH, 16:8 h photoperiod. After 24 h, the wasps were transferred to a second leaf disk and allowed to oviposit for an additional 24 h to maximize progeny production per wasp. The wasps were then frozen at −20 °C for later analysis. The leaf disks were maintained on agar for two wk for parasitoid development, and the parasitoid progeny that emerged were quantified and sexed. If a mother produced no female offspring, it was possible that she was either affected by CI or unmated (because haplodiploid wasps can produce haploid male offspring without mating). To distinguish between these possibilities, we dissected out the spermatheca from each female that produced only male offspring, cleared the spermatheca in a lactophenol solution (1 part carbolic acid, 1 part lactic acid, 2 parts glycerine, 1 part distilled water), and examined it at 200–400X magnification for the presence of sperm. Unmated females were excluded from the dataset. To confirm that each mother had the expected symbionts, we extracted the DNA either from her or one of her offspring (if the mother had been dissected), followed by diagnostic PCR. We also tested the infection status of a sample of males from each mating jar.

In addition to the experiment described above, we performed three other preliminary experiments that involved only partial sets of crosses. We occasionally draw on these in the following sections for comparison with results from the full experiment. These earlier experiments were conducted in the same fashion as described above, but, for brevity, are not described in detail here.

Statistics and contrasts

We used logistic regression (Arc v. 1.06) to compare offspring sex distribution among the treatments. We used Williams' correction (Williams, 1982) to correct for moderate overdispersion in the data. When the overall model was significant, rather than multiple comparisons of all treatment pairs (120 pairs) we tested the significance of specific contrasts of interest using Wald statistics.

Question 1: Which symbiont(s) cause(s) CI?—We first verified that doubly-infected *E. inaron* retained a CI phenotype by comparing the progeny sex ratio of doubly-infected males mated to cured females (test cross: Both ♂ × Cured ♀) relative to a control cross of cured males mated to cured females (Cured ♂ × Cured ♀) and also relative to a control cross of doubly-infected males mated to doubly-infected females (Both ♂ × Both ♀). In this way we were able to control for both female type and male type. Significantly reduced female production in the test cross relative to controls indicates CI. If CI was detected, we followed up with t-tests comparing total offspring production and male offspring production between the test cross and the cured by cured control, to determine whether CI was the "female mortality" (female embryos die) or "male replacement" (incipient female embryos develop as males) type (Vavre *et al*, 2000). Female mortality CI would be characterized by lower total offspring production in the test cross than control, but similar male offspring production. Male replacement CI would be characterized by similar total offspring production between the test cross and control, but male offspring production would be higher in the test cross.

To test whether *Cardinium* or *Wolbachia* causes CI, we used parallel contrasts to those described for the doubly-infected line, except using the *Cardinium* only line (test cross = Card. ♂ × Cured ♀, contrasted with Cured ♂ × Cured ♀ and Card. ♂ × Card. ♀ controls) or the *Wolbachia* only line (test cross = Wol. ♂ × Cured ♀, contrasted with Cured ♂ × Cured ♀ and Wol. ♂ × Wol. ♀ controls). If CI was detected from either symbiont, we again used t-tests of total progeny and male progeny production to distinguish between the male replacement and female mortality type.

Question 2: Do the symbionts interact in the expression and rescue of CI?— To test whether the presence of one symbiont in any way modifies the CI induced by the other symbiont, we investigated several contrasts, each designed to address a specific type of interaction (Table 1). Table 1 includes the contrasts used to test for the effect *Cardinium* might have on *Wolbachia*-induced CI; we did not conduct the analogous set of contrasts to test for the effect *Wolbachia* might have on *Cardinium*-induced CI because we didn't detect *Cardinium*-induced CI.

Question 3: Does either symbiont manipulate progeny sex ratio?—Symbionts may also promote their own spread by directly manipulating the progeny sex ratio in favor of female offspring. To test this possibility, we compared the progeny sex ratio of each infected female type mated to cured males relative to that of cured females mated to cured males (Cured ♂ × Both ♀, Cured ♂× Card. ♀, and Cured ♂× Wol. ♀ contrasted with Cured ♂× Cured ♀). In this way we were able to eliminate the potential influence of symbiont-altered sperm on progeny production.

Results

Question 1: Which symbiont(s) cause(s) CI?

Consistent with Perlman *et al* (2006), we found evidence for CI in doubly-infected *E. inaron*. Cured females mated to doubly-infected males (the test cross) had a male bias in their offspring (2 ♂: 1 ♀; Figure 1). In contrast, cured females mated to cured males had a female-biased sex ratio in their offspring (1 ♂: 1.7 ♀). This significant difference (Wald = 3.521, $P < 0.001$) indicates that low female offspring production in the predicted CI cross was not due to male bias in cured females. Likewise we found that doubly-infected females mated to doubly-infected males also had a female bias in their offspring (1 ♂: 1.5 ♀). This ratio was again significantly different than what was observed in the test cross (Wald = 2.698, $P = 0.007$), indicating that sperm from doubly-infected males is not intrinsically low quality; it is only when mated to cured females that incompatibility occurs, just as one would expect in symbiont-induced CI.

On the basis of this experiment alone, it was not clear whether CI was of the male replacement or female mortality type. We found that total offspring production was not significantly different between the test cross (22.3 ± 3.0) and the cured × cured control (29.0 ± 2.8; $t = 1.589$, *d.f.* = 15, $P = 0.133$), but we also found no difference in male production between the two crosses (test cross = 12.1 ± 1.0, control = 9.3 ± 1.5; $t = 1.483$, *d.f.* = 15, $P = 0.159$). However, we also found a small proportion of uninfected males in the test cross: 2/20 males checked were uninfected, rather than doubly-infected. The origin of these uninfected males is unclear, but may have resulted from incomplete vertical transmission. Note that this was the only case where the parasitoids did not have the expected infection status: all experimental females, and all other males sampled were of the expected infection status. When we inspected the data, we found that three females in the test cross showed no evidence of CI, and may have mated with the uninfected males. These females had the highest total offspring production; the other females, which had male biased offspring, also had reduced total offspring production (15.5 ± 1.7), suggesting female mortality CI consistent with the findings of Perlman *et al* (2006) and other results from our laboratory (JAW unpublished data).

We found strong evidence that *Wolbachia* induces CI in *E. inaron*. Cured females mated to *Wolbachia*-infected males had a very strong male bias in their offspring (4.4 ♂: 1 ♀; Figure 1), strongly contrasting with the female bias of cured females mated to cured males (1 ♂: 1.7 ♀; Wald = 4.547, $P < 0.001$), or *Wolbachia*-infected females mated to *Wolbachia*-infected males (1 ♂: 1.46 ♀; Wald = 3.565, $P < 0.001$). Total offspring production was halved in the test cross relative to the control (test cross = 14.9 ± 1.5, control = 29.0 ± 2.8; $t = 4.178$, *d.f.* = 16, $P = 0.001$), whereas male production did not differ significantly (test cross = 10.5 ± 1.3, control = 9.3 ± 1.5; $t = 0.605$, *d.f.* = 16, $P = 0.554$), indicating that *Wolbachia* induced the female mortality type of CI.

We did not find evidence for *Cardinium*-induced CI in this experiment, but the results are equivocal. The test cross of cured females mated to *Cardinium*-infected males produced nearly equal proportions of male and female offspring (1.05 ♂: 1 ♀; Figure 1). This value was not significantly more male-biased than the offspring of cured females mated to cured males (1 ♂: 1.7 ♀, Wald = 1.809, $P = 0.071$) or *Cardinium*-infected females mated to *Cardinium*-infected males (1 ♂: 1.6 ♀, Wald = 1.606, $P = 0.108$), but in both contrasts there was a trend toward significance. In two previous experiments, however, we found no evidence of *Cardinium*-induced CI. In each previous experiment, the test cross showed female-biased offspring production (expt. 1 = 1 ♂: 1.4 ♀ $n = 11$; expt. 2 = 1 ♂: 1.6 ♀, $n = 33$) that did not differ significantly from the cured × cured treatment (expt. 1 Wald = 0.518, $P = 0.605$; expt. 2 Wald = 0.159, $P = 0.874$) or the Card. × Card. treatment (expt. 1 Wald = 0.987, $P = 0.324$; expt. 2 Wald = 0.922, $P = 0.357$). The preponderance of evidence thus suggests that *Cardinium* does not cause CI in *E. inaron*.

Question 2: Do the symbionts interact in the expression and rescue of CI?

We did not find evidence that *Cardinium* modifies *Wolbachia*-induced CI. For potential interaction A (Table 1), we found that *Cardinium*-infected females cannot “rescue” *Wolbachia* modified sperm. *Cardinium*-infected females mated to *Wolbachia*-infected males produce 2.7 ♂: 1 ♀ offspring (Figure 1), which is statistically equivalent to the 4.4 ♂: 1 ♀ produced by cured females mated to *Wolbachia*-infected males (Wald = 1.003, P = 0.316).

The additional presence of *Cardinium* in a male does not alter the strength of *Wolbachia*-induced CI. For interaction B (Table 1), we found that cured females produced male-biased offspring whether mated to doubly-infected males (2.3 ♂: 1♀) or *Wolbachia*-infected males (4.4 ♂: 1♀; Wald = 1.267, P = 0.205). Likewise, for potential interaction C (Table 1), we found that *Cardinium*-infected females produced male-biased offspring whether mated to doubly-infected males (5.7 ♂: 1♀) or *Wolbachia*-infected males (2.7 ♂: 1 ♀; Wald = 1.697, P = 0.090). There is an apparent trend toward reduced male bias in *Cardinium*-infected females mated with *Wolbachia*-infected males, but note that a previous experiment had found a very strong male bias in this treatment (6.7 ♂: 1 ♀, n = 10 mothers), supporting the lack of significance in this contrast.

The presence of *Cardinium* in both male and female of a cross does not appear to change CI expression. For potential interaction D (Table 1), we found that the sex ratio produced by *Cardinium*-infected females mated to doubly-infected males (5.7 ♂: 1 ♀) is similar to that of cured females mated to *Wolbachia*-infected males (4.4 ♂: 1 ♀; Wald = 0.542, P = 0.588).

Similarly, the additional presence of *Cardinium* in either *Wolbachia*-infected males or females does not affect the ability of *Wolbachia*-infected females to rescue *Wolbachia*-induced CI. For potential interaction E (Table 1) we found that doubly-infected females mated to *Wolbachia*-infected males produced 1 ♂: 1.2 ♀, which did not differ significantly from the 1 ♂: 1.5 ♀ produced by *Wolbachia*-infected females mated to *Wolbachia*-infected males (Wald = 0.368, P = 0.713). Also, for potential interaction F (Table 1), we found that *Wolbachia*-infected females mated to doubly-infected males produced 1 ♂: 1.1 ♀, which did not differ significantly from the sex ratio produced by *Wolbachia*-infected females mated to *Wolbachia*-infected males (Wald = 0.497, P = 0.619), indicating that the additional presence of *Cardinium* in the male does not interfere with sperm modification in such a way that it cannot be rescued.

Question 3: Does either symbiont manipulate progeny sex ratio?

We found no significant differences in the offspring sex ratios among females of the different lines mated to cured males, although there was a trend toward reduced female bias in the *Cardinium* line. *Cardinium*-infected females mated to cured males produced 1 ♂:1 ♀, whereas cured females mated to cured males produced 1 ♂:1.7 ♀ (Wald = 1.692, P = 0.091; Figure 1). Females carrying *Wolbachia* alone produced female-biased offspring and did not differ from cured females (1 ♂:1.5 ♀, Wald = 0.364, P = 0.716). Likewise, doubly-infected females mated to cured males produced female biased offspring and did not differ from cured females (1 ♂: 2 ♀; Wald = 0.457, P = 0.646).

Discussion

In doubly-infected *E. inaron*, *Wolbachia*, and not *Cardinium* causes cytoplasmic incompatibility of the female mortality type. CI is the most prevalent phenotype induced by *Wolbachia* (Stouthamer *et al*, 1999), and multiple infections of *Wolbachia* and other symbionts are common, but to our knowledge, this is the first record of differential curing being used to dissect the relative contributions of *Wolbachia* and another symbiont to CI modification and rescue in a doubly-infected host. It is also interesting that it is *Wolbachia* and not *Cardinium* that causes CI in *E. inaron. Wolbachia* is prevalent in this family of parasitoids, the Aphelinidae in the Chalcidoidea (e.g., Weeks *et al*, 2003) but to date has been associated only with the induction of parthenogenesis (Gottlieb *et al*, 1998). The only other documented instance of CI in the Aphelinidae, in Encarsia pergandiella, is caused by Cardinium (Hunter et al, 2003).

Presuming that CI induction by *Cardinium* follows a modification/rescue model similar to that proposed for *Wolbachia* (Werren, 1997), our results suggest that *Cardinium* in *E. inaron* has a *mod*$^-$ phenotype. However, host background has also been shown to be important in the expression of CI (Veneti *et al*, 2003), and it is possible that *E. inaron* has evolved a phenotype that is not permissive of *Cardinium*-induced CI, yet permissive of *Wolbachia*-induced CI. Horizontal transfer experiments of *E. inaron Cardinium* into other host backgrounds would therefore be necessary to absolutely verify a *mod*$^-$ phenotype. Similarly, even though our experiment found that *Cardinium* in *E. inaron* is *resc*$^-$ with respect to *Wolbachia*-induced CI, it is possibly *resc*$^+$ for *Cardinium*-induced CI. Such a dichotomy would be particularly likely if the mechanisms for *Cardinium*- and *Wolbachia*-induced CI are very different. It would be most informative to investigate CI mechanisms and interactions in a system in which both *Cardinium* and *Wolbachia* cause CI within the same host. Unfortunately, all doubly-infected arthropods that have been investigated to date have either had CI caused by only one symbiont (present study, Ros and Breeuwer, submitted) or a CI phenotype was not present (Gotoh *et al*, 2007a).

Since *Cardinium* doesn't appear to cause or influence CI in *E. inaron*, it remains to be determined what, if anything, *Cardinium* does. We did not find that *Cardinium* promotes its own existence in *E. inaron* by encouraging a female bias in progeny: if anything, the *Cardinium* line showed a trend toward fewer female progeny than the other lines (but the effect was not significant). It is possible that *Cardinium* provides *E. inaron* with some sort of fitness benefit, such as increased fecundity (Weeks and Stouthamer, 2004). Alternatively, *Cardinium* could be effectively neutral within *E. inaron*, but is maintained in the population via perfect maternal transmission (Hoffmann *et al*, 1996). It is also possible that maintenance of *Cardinium* within *E. inaron* is directly attributable to co-infection with *Wolbachia*. Perfect co-transmission with *Wolbachia* would confer the same CI transmission advantage to *Cardinium* as its CI-inducing partner. Recent theoretical studies have suggested that parameters for symbiont invasion and maintenance within a population can be altered by co-infection (Vautrin *et al*, 2007). Finally, studies of co-infecting symbiont taxa have found complementarity between their genomes (e.g., McCutcheon and Moran, 2007) suggesting that species may lose redundant portions of their genomes. Symbiont interactions appear to be more dynamic over relatively short periods of time than previously appreciated (Riegler and O'Neill, 2007; Weeks *et al*, 2007), and it is possible that *Cardinium* has lost an historical ability to cause CI because co-infection and co-transmission with *Wolbachia* rendered it unnecessary. To test such speculations, however, more will need to be known about the history of the *Cardinium*/*Wolbachia* association within *E. inaron*.

Intriguingly, other populations of *E. inaron* appear to harbor only *Cardinium* (JAW, unpublished data), raising questions about the origin of the *Wolbachia* infection, and whether *Cardinium* causes CI when it is alone in the host.

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References

Bordenstein SR, Werren JH. Bidirectional incompatibility among divergent *Wolbachia* and incompatibility level differences among closely related *Wolbachia* in *Nasonia*. Heredity. 2007; 99:278–287. [PubMed: 17519968]

Caspari E, Watson GS. On the evolutionary importance of cytoplasmic sterility in mosquitoes. Evolution. 1959; 13:568–570.

Chiel E, Gottlieb Y, Zchori-Fein E, Mozes-Daube N, Katzir N, Inbar M, Ghanim M. Biotype-dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. Bull Entomol Res. 2007; 97:407–413. [PubMed: 17645822]

Douglas AE. Mycetocyte symbiosis in insects. Biol Rev Cambridge Phil Soc. 1989; 64:409–434.

Duron O, Hurst GDD, Hornett EA, Josling JA, Engelstadter J. High incidence of the maternally inherited bacterium *Cardinium* in spiders. Mol Ecol. 2008; 17:1427–1437. [PubMed: 18266629]

Frank SA. Dynamics of cytoplasmic incompatibility with multiple *Wolbachia* infections. J Theor Biol. 1998; 192:213–218. [PubMed: 9735249]

Gotoh T, Noda H, Ito S. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity. 2007a; 98:13–20. [PubMed: 17035954]

Gotoh T, Sugasawa J, Noda H, Kitashima Y. *Wolbachia*-induced cytoplasmic incompatibility in Japanese populations of *Tetranychus urticae* (Acari : Tetranychidae). Expl Appl Acarol. 2007b; 42:1–16.

Gottlieb Y, Zchori-Fein E, Faktor O, Rosen D. Phylogenetic analysis of parthenogenesis-inducing *Wolbachia* in the genus *Aphytis* (Hymenoptera : Aphelinidae). Insect Mol Biol. 1998; 7:393–396. [PubMed: 9723877]

Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*? - a statistical analysis of current data. FEMS Microbiol Let. 2008; 281:215–220. [PubMed: 18312577]

Hoffmann AA, Clancy D, Duncan J. Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility. Heredity. 1996; 76:1–8. [PubMed: 8575931]

Hoffmann, AA.; Turelli, M. Cytoplasmic incompatibility in insects. In: O'Neill, SL.; Hoffmann, AA.; Werren, JH., editors. Influential passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford University Press; New York: 1997. p. 42-80.

Hunter MS, Perlman SJ, Kelly SE. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc R Soc Lond Series B. 2003; 270:2185–2190.

Ijichi N, Kondo N, Matsumoto R, Shimada M, Ishikawa H, Fukatsu T. Internal spatiotemporal population dynamics of infection with three *Wolbachia* strains in the adzuki bean beetle, *Callosobruchus chinensis* (Coleoptera : Bruchidae). Appl Environl Microbiol. 2002; 68:4074–4080.

Kondo N, Ijichi N, Shimada M, Fukatsu T. Prevailing triple infection with *Wolbachia* in *Callosobruchus chinensis* (Coleoptera : Bruchidae). Mol Ecol. 2002; 11 :167–180. [PubMed: 11856419]

Kondo N, Shimada M, Fukatsu T. Infection density of *Wolbachia* endosymbiont affected by co-infection and host genotype. Biol Lett. 2005; 1:488–491. [PubMed: 17148240]

Lo N, Casiraghi M, Salati E, Bazzocchi C, Bandi C. How many *Wolbachia* supergroups exist? Mol Biol Evol. 2002; 19:341–346. [PubMed: 11861893]

McCutcheon JP, Moran NA. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci USA. 2007; 104:19392–19397. [PubMed: 18048332]

Mercot H, Poinsot D. Rescuing *Wolbachia* have been overlooked and discovered on Mount Kilimanjaro. Nature. 1998; 391:853–853. [PubMed: 9495338]

Morimoto S, Kurtti TJ, Noda H. *In vitro* cultivation and antibiotic susceptibility of a *Cytophaga*-like intracellular symbiote isolated from the tick *Ixodes scapularis*. Curr Microbiol. 2006; 52:324–329. [PubMed: 16550461]

Mouton L, Dedeine F, Henri H, Bouletreau M, Profizi N, Vavre F. Virulence, multiple infections and regulation of symbiotic populations in the *Wolbachia-Asobara tabida* symbiosis. Genetics. 2004; 168:181–189. [PubMed: 15454536]

Mouton L, Henri H, Bouletreau M, Vavre F. Multiple infections and diversity of cytoplasmic incompatibility in a haplodiploid species. Heredity. 2005; 94 :187–192. [PubMed: 15562287]

O'Neill, SL.; Hoffmann, AA.; Werren, JH., editors. Influential Passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford University Press; New York: 1997. p. 214

Oliver KM, Moran NA, Hunter MS. Costs and benefits of a superinfection of facultative symbionts in aphids. Proc R Soc Lond Series B. 2006; 273:1273–1280.

Perlman SJ, Kelly SE, Zchori-Fein E, Hunter MS. Cytoplasmic incompatibility and multiple symbiont infection in the ash whitefly parasitoid, *Encarsia inaron*. Biol Cont. 2006; 39:474–480.

Perlman SJ, Kelly SE, Hunter MS. Population biology of cytoplasmic incompatibility: maintenance and spread of *Cardinium* symbionts in a parasitic wasp. Genetics. 2008; 178:1003–1011. [PubMed: 18245338]

Pickett CH, Pitcairn MJ. Classical biological control of ash whitefly: factors contributing to its success in California. Biocontrol. 1999; 44:143–158.

Riegler M, O'Neill SL. Evolutionary dynamics of insect symbiont associations. Trends Ecol Evol. 2007; 22:625–627. [PubMed: 17981362]

Ros VID, Breeuwer JAJ. The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobia sarothamni*. (submitted to Heredity).

Stouthamer R. Effectiveness of several antibiotics in reverting thelytoky to arrhenotoky in *Trichogramma* spp. Colloques de l'INRA. 1991; 56:119–122.

Stouthamer R, Breeuwer JAJ, Hurst GDD. *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. Annu Rev Microbiol. 1999; 53:71–102. [PubMed: 10547686]

Tram U, Sullivan W. Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*-induced cytoplasmic incompatibility. Science. 2002; 296:1124–1126. [PubMed: 12004132]

Turelli M. Evolution of incompatibility-inducing microbes and their hosts. Evolution. 1994; 48:1500–1513.

Vautrin E, Charles S, Genieys S, Vavre F. Evolution and invasion dynamics of multiple infections with *Wolbachia* investigated using matrix based models. J Theor Biol. 2007; 245:197–209. [PubMed: 17112544]

Vavre F, Fleury F, Varaldi J, Fouillet P, Bouletreau M. Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: Epidemiologic and evolutionary consequences. Evolution. 2000; 54:191–200. [PubMed: 10937195]

Veneti Z, Clark ME, Zabalou S, Karr TL, Savakis C, Bourtzis K. Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila-Wolbachia* associations. Genetics. 2003; 164:545–552. [PubMed: 12807775]

Weeks AR, Reynolds KT, Hoffmann AA, Mann H. *Wolbachia* dynamics and host effects: what has (and has not) been demonstrated? Trends Ecol Evol. 2002; 17:257–262.

Weeks AR, Stouthamer R. Increased fecundity associated with infection by a *Cytophaga*-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. Proc R Soc Lond Series B. 2004; 271:S193–S195.

Weeks AR, Velten R, Stouthamer R. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc R Soc Lond Series B. 2003; 270:1857–1865.

Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biology. 2007; 5:997–1005.

Weinert LA, Tinsley MC, Temperley M, Jiggins FM. Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles. Biol Lett. 2007; 3:678–681. [PubMed: 17878145]

Werren JH. Biology of *Wolbachia*. Annu Rev Entomol. 1997; 42:587–609. [PubMed: 15012323]

Werren, JH.; O'Neill, SL. The evolution of heritable symbionts. In: O'Neill, SL.; Hoffmann, AA.; Werren, JH., editors. Influential passengers: Inherited Microorganisms and Arthropod Reproduction. Oxford University Press; New York: 1997. p. 1-41.

Williams DA. Extra-binomial variation in logistic linear models. Appl Stat. 1982; 31 :144–148.

Xi ZY, Gavotte L, Xie Y, Dobson SL. Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its *Drosophila* host. BMC Genomics. 2008; 9:Article 1.

Zabalou S, Apostolaki A, Pattas S, Veneti Z, Paraskevopoulos C, Livadaras I, Markakis G, Brissac T, Mercot H, Bourtzis K. Multiple rescue factors within a *Wolbachia* strain. Genetics. 2008; 178:2145–2160. [PubMed: 18430940]

Zchori-Fein E, Perlman SJ. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol Ecol. 2004; 13:2009–2016. [PubMed: 15189221]

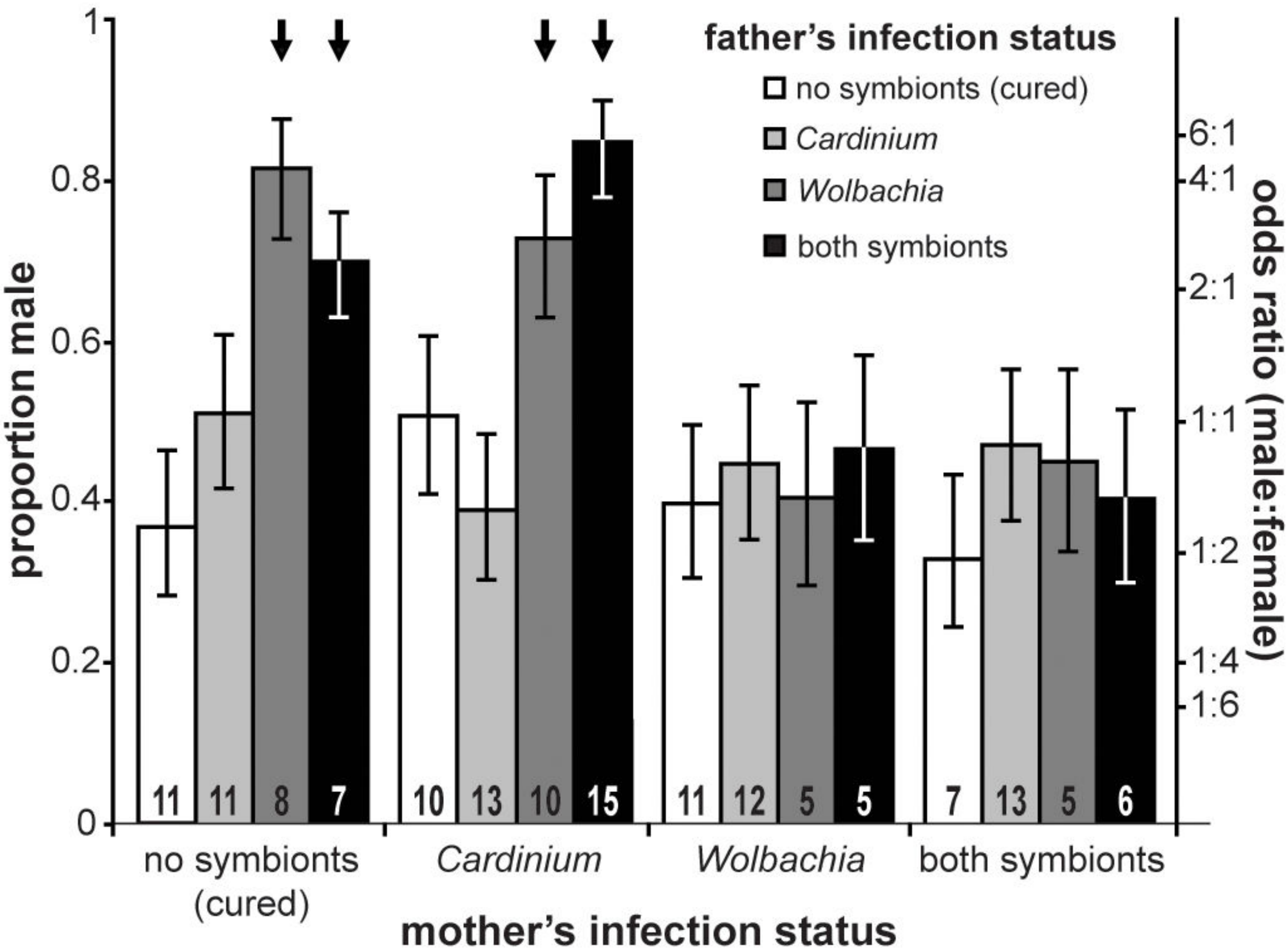

Figure 1.

Back-transformed logistic regression estimates ± SE of proportion male progeny and corresponding odds ratios produced by all 16 crosses. Numbers at the base of each column represent the sample size of mothers for that cross. Crosses where CI was detected are indicated by bold arrows above a column.

Table 1

Potential cytoplasmic incompatibility interactions between *Cardinium* and *Wolbachia* in *Encarsia inaron*.

Potential interaction	**Test cross**	**Control cross**
A) *Cardinium* “rescues” *Wolbachia*- induced CI	Wol. ♂ × Card. ♀	Wol. ♂ × Cured ♀
B) *Cardinium* in the male modifies the strength of *Wolbachia*-induced CI	Both ♂ × Cured ♀	Wol. ♂ × Cured ♀
C) *Cardinium* in the male modifies the strength of *Wolbachia*-induced CI when *Cardinium* is also in the female	Both ♂ × Card. ♀	Wol. ♂ × Card. ♀
D) *Cardinium* in both male and female modifies the strength of *Wolbachia*- induced CI	Both ♂ × Card. ♀	Wol. ♂ × Cured ♀
E) *Cardinium* in a *Wolbachia*-bearing female affects the ability to rescue CI	Wol. ♂ × Both ♀	Wol. ♂ × Wol. ♀
F) *Cardinium* in a *Wolbachia*-bearing male affects CI rescue in a *Wolbachia*-bearing female.	Both ♂ × Wol. ♀	Wol. ♂ × Wol. ♀