

ORIGINAL ARTICLE

Superinfection of cytoplasmic incompatibility-inducing *Wolbachia* is not additive in *Orius strigicollis* (Hemiptera: Anthocoridae)M Watanabe¹, K Miura^{1,2}, MS Hunter³ and E Wajnberg⁴¹Graduate School of Biosphere Sciences, Hiroshima University, Hiroshima, Japan; ²National Agricultural Research Center for Western Region, Hiroshima, Japan; ³Department of Entomology, University of Arizona, Tucson, AZ, USA and ⁴INRA, 400 Route des Chappes, Sophia Antipolis Cedex, France

Cytoplasmic incompatibility (CI) allows the intracellular, maternally inherited bacterial symbiont *Wolbachia* to invade arthropod host populations by inducing infertility in crosses between infected males and uninfected females. The general pattern is consistent with a model of sperm modification, rescued only by egg cytoplasm infected with the same strain of symbiont. The predacious flower bug *Orius strigicollis* is superinfected with two strains of *Wolbachia*, *wOus1* and *wOus2*. Typically, superinfections of CI *Wolbachia* are additive in their effects; superinfected males are incompatible with uninfected and singly infected females. In this study, we created an uninfected line, and lines singly infected with *wOus1* and *wOus2* by antibiotic treatment. Then, all possible crosses were conducted among the four lines. The results indicated that while *wOus2* induces high levels of CI, *wOus1*

induces very weak or no CI, but can rescue CI caused by *wOus2* to a limited extent. Levels of incompatibility in crosses with superinfected males did not show the expected pattern. In particular, superinfected males caused extremely weak CI when mated with either singly infected or uninfected females. An analysis of symbiont densities showed that *wOus1* densities were significantly higher than *wOus2* densities in superinfected males, and *wOus2* densities were lower, but not significantly, in superinfected relative to singly infected males. These data lend qualified support for the hypothesis that *wOus1* interferes with the ability of *wOus2* to cause CI by suppressing *wOus2* densities. To our knowledge, this is the first clear case of non-additive CI in a natural superinfection. *Heredity* (2011) **106**, 642–648; doi:10.1038/hdy.2010.100; published online 11 August 2010

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Introduction

Wolbachia are maternally transmitted intracellular bacteria infecting a large proportion of arthropods and nematodes (Bandi *et al.*, 1998; Stouthamer *et al.*, 1999; Werren *et al.*, 2008). A recent meta-analysis of *Wolbachia* surveys estimated that 66% of insect species harbor *Wolbachia* at varying frequencies (Hilgenboecker *et al.*, 2008). *Wolbachia* infections can induce reproductive alterations such as feminization (Rigaud *et al.*, 1997), thelytokous parthenogenesis (Stouthamer, 1997), male killing (Hurst *et al.*, 2000) and cytoplasmic incompatibility (CI; Hoffmann and Turelli, 1997; Charlat *et al.*, 2002) as well as influence the general fitness and life history of hosts in both positive and negative ways (for example Min and Benzer, 1997; Dobson *et al.*, 2004). CI is the most common effect of *Wolbachia* and has been described in crustaceans (Moret *et al.*, 2001), arachnids (Breeuwer, 1997) and in most groups of insects that have been examined (for reviews, see Stouthamer *et al.*, 1999; Werren *et al.*, 2008). The incompatibility occurs in crosses

between males and females with differing *Wolbachia* infection status. It can be either unidirectional, when infected males mate with uninfected females, or bidirectional, in crosses in which both males and females are infected with different CI-inducing *Wolbachia* strains (Hoffmann and Turelli, 1997). Unidirectional CI can also occur between a CI-inducing strain and a strain that does not induce CI, where the incompatible crosses occur between males with the CI-causing strain and females of the non-CI strain, or uninfected females (Duron *et al.*, 2006; Zabalou *et al.*, 2008). Although the strain-specific mechanism of CI is still not well understood, the general pattern is consistent with a model in which sperm is modified in such a way that only the same strain of symbionts in the egg cytoplasm can rescue them and allow normal embryonic development (Werren, 1997). CI symbionts spread because infected females gain in fitness relative to uninfected females that can successfully reproduce only with uninfected males. Superinfections (that is co-infection with two or more *Wolbachia* strains) occur naturally (Sinkins *et al.*, 1995; Zhou *et al.*, 1998; Dobson *et al.*, 2004) and have been generated artificially as well (Rousset *et al.*, 1999; Walker *et al.*, 2009). These superinfections typically have additive effects, such that a superinfected male is unidirectionally incompatible with both singly infected and uninfected

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Table 1 Predicted results of crosses among hosts of two strains of *Wolbachia* (A and B) when the *Wolbachia* strains are both present in superinfected (A+B) individuals as well as in singly infected (A and B) individuals or are absent (U, uninfected)

Female type	Male type			
	U	A	B	A + B
U	+	–	–	–
A	+	+	–	–
B	+	–	+	–
A + B	+	+	+	+

The pattern shown here pertains when both strains cause CI individually, and there is bidirectional incompatibility between the two strains. In general, incompatibility (–) is predicted when the male host harbors a *Wolbachia* strain not present in the female mate. Successful matings (+) occur when males have the same, or fewer strains of *Wolbachia* than the female. Note that superinfected males are predicted to be incompatible with all but superinfected females. (modified from Dobson *et al.*, 2004).

females (Table 1), whereas superinfected females can rescue infections in both superinfected and singly infected females.

The predacious flower bug *Orius strigicollis* (Poppoius) (Heteroptera: Anthocoridae) is one of the most useful biological control agents of various minute insect pests, such as thrips, and is common in agricultural fields in Japan. The species is also commercially available in Japan as a biological control agent for greenhouses (Yano, 2004). In this study, we show that this population of *O. strigicollis* is superinfected with *Wolbachia*, and the superinfection does not cause strong CI, even though one of the strains it carries causes strong CI when alone; this is not the expected additive effect of a superinfection (see Table 1). We speculate that the superinfection may have spread instead because of its superior ability to rescue CI.

Materials and methods

Insects

O. strigicollis was obtained from Sumitomo Chemical Co. Ltd. The strains were originated from Wakayama, Japan. *O. strigicollis* individuals were maintained in the laboratory (16 h light, 60% humidity, 25 °C) and reared on diet, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) eggs and pure water with cotton.

Wolbachia strains and origin of Orius lines of different infection status

The *O. strigicollis* culture we acquired was fixed for a superinfection of two different *Wolbachia* strains: *wOus1* and *wOus2* (*wOus1* + 2). We note that natural populations of this species in different areas of Japan show more variation with respect to *Wolbachia* infection. In most populations, the greatest number of individuals is superinfected, but some singly infected individuals of both types are also found (Watanabe *et al.*, submitted). The range of infection frequencies for *wOus1* + 2, *wOus1* and *wOus2* in several populations are 23–100, 0–25 and 0–77%, respectively.

In this study, we used primers of the *wsp* region of *Wolbachia* for diagnostic PCR (see below). We previously

examined another suite of *Wolbachia* genes, that is the housekeeping genes for MLST analysis, and also found consistent differences between the two *Wolbachia* strains in these genes (Baldo *et al.*, 2006).

An uninfected line and lines singly infected with either *wOus1* or *wOus2* lines were derived from superinfection with *wOus1* + 2. To establish these lines, superinfected *wOus1* + 2 *Orius* were cured of one or more symbionts by feeding last-instar nymphs honey solution containing 50 mg ml⁻¹ of tetracycline for 48 h in each of three consecutive generations. Nymphs treated with antibiotics were isolated until adults in each generation. In establishment of uninfected line, females mated with males of the same line. In establishment of *wOus1* or *wOus2* line, we used uninfected males to avoid incompatibility. After oviposition, we tested for infection status of females by diagnostic PCR. All crossing experiments were carried out at least five generations after the last antibiotic treatment to avoid any direct effect of the antibiotics.

DNA extraction and diagnostic PCR

To determine infection status of individuals of *O. strigicollis*, DNA was extracted in 32 µl STE buffer (5 N NaCl, 500 mM EDTA (pH 8.0) and 1 M Tris-HCl (pH 8.0)) and incubated with 2 µl of proteinase K (0.5 mg ml⁻¹) at 56 °C for 2 h. The homogenate was heated at 99.9 °C for 5 min to inactivate the proteinase K, and then used as a template for PCR. PCR amplifications were conducted under the following conditions: 16.5 µl of 2 × AmpliTaq Gold PCR Master Mix (PE Applied Biosystems, Tokyo, Japan), 1.3 µl forward and reverse primer (10 pmol µl⁻¹) and 13.9 µl of sterile water, a total of 33 µl PCR reaction volume. We monitored the infection status of the infected and uninfected laboratory stocks through PCR using the *Wolbachia*-specific *wsp* primers, *wOus1*-f (5' TAA ATA CTT CTG AAA CAA ATG TTG 3') and *wOus1*-r (5' AAA AAT TAA ACG CTA CTC CA 3'), *wOus2*-f (5' GAT GTA GTA TCT GAT GAC AAG 3') and *wOus2*-r (5' GGA CGT TGA TCT CTT TAG TAG 3') (Watanabe *et al.*, submitted). Each *Wolbachia* sequence was deposited in GenBank (*wOus1*: AB094361, *wOus2*: AB094365). PCR amplification was carried out in an ABI thermocycler (PE Applied Biosystems PCR System 9700, PE Applied Biosystems) with the following thermal profiles: 95 °C for 10 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min 30 s and 72 °C for 1 min 30 s, followed by incubation at 72 °C for 1 min 30 s. The PCR included a negative control (sterile water instead of DNA) to detect contamination. The PCR products were resolved on a 1.5% agarose gel, stained with ethidium bromide and visualized under an UV transilluminator. To confirm that DNA was properly extracted, ITS primers were used to amplify nuclear ribosomal DNA as a positive control for the template DNA quality (Hinamoto *et al.*, 2004).

Crossing experiments

Crossing experiments were performed to reveal how the different *Wolbachia* strains influenced CI modification and rescue. Four-day-old virgin females, either uninfected or infected with *wOus1* + 2, *wOus1* or *wOus2* were isolated and individually mated with *wOus1* + 2, *wOus1*, *wOus2* and uninfected males (also 4 days old). Each of the 16 crosses was replicated 7–12 times. All mating was

observed, and subsequently each female was allowed to oviposit onto a plant, *Sedum rubrotinctum* with diet in a vial (1 cm diameter × 5 cm length). The plants that served as oviposition substrates were removed every second day, and replaced with a fresh plant, whereas the eggs laid on the older foliage were counted. Females were allowed to oviposit for 8 days. Egg-hatching rates were scored 1 week after egg collection. Parents of each cross were tested by PCR for confirmation of the expected *Wolbachia* infection status. Egg-hatching rates were compared among the treatments by means of a logistic regression, a generalized linear model specially designed for modeling binomial data using the logistic-link function (McCullagh and Nelder, 1989; Wajnberg and Haccou, 2008). Computations were performed using SAS software (SAS Institute Inc., 1999). The sex ratio was tested by the Fisher's exact test using R ver. 2.7.1 software (R Development Core Team, 2005).

Comparative fecundity of uninfected, singly infected and superinfected lines

To determine whether female *Orius* with different symbiont combinations differ in fecundity, 4-day-old females of all four types were mated with 4-day-old males of the same strain for 48 h in plastic vials (1 cm diameter × 5 cm length). Then, females were placed in another vial of the same type as mentioned above and supplied with a plant for oviposition so that host fecundity could be evaluated. We counted the number of eggs at 2, 4, 6 and 8 days. The total number of eggs in each treatment produced by superinfected, uninfected and singly infected females of both types was analyzed with a repeated measures generalized linear model specially designed for modeling count data using a log-link function (McCullagh and Nelder, 1989; Wajnberg and Haccou, 2008). Computations were performed using SAS software (SAS Institute Inc., 1999).

Quantitative PCR

We performed quantitative PCR analysis of host abdomens. These tissues were isolated from individuals from superinfected and singly infected lines. Individual insects were carefully dissected with fine forceps under

a binocular microscope. Isolated tissues were immediately subjected to DNA extraction. Quantitative PCR was carried out with the ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Reaction volumes of 25 µl contained 12.5 µl of SYBR Green PCR Master Mix (PE Applied Biosystems), 10.5 µl of sterile water, 0.5 µl each of 10 µM forward and reverse primer, and 1 µl of target DNA in single wells of a 96-well plate (PE Applied Biosystems). For the selective amplification of a small portion of the *O. strigicollis* COI gene (90 bp) and *Wolbachia* *wsp* (*wOus1*, 85 bp and *wOus2*, 75 bp), the following primers were designed and used: OSCO1-F (5'-CTG CCC CCA TCT ATT ACA TTA CTT ATT-3'), OSCO1-R (5'-TGC TGA AAG AGG AGG ATA TAC TGT TC-3'), *wOus1wsp*-F (5'-ATG TTG AAG GGC TTT ACT CAC AAT T-3'), *wOus1wsp*-R (5'-GCT GTT AAA CTG TCT GCA ACA TTT G-3'), *wOus2wsp*-F (5'-TCA CAA TTG ACT AAA GAT GCA ACT GT-3'), *wOus2wsp*-R (5'-CAA TCC TGA AAA CGC TGT TAC ACT-3'). PCR primers and probes were designed using PRIMER EXPRESS 1.5 (PE Applied Biosystems). Cycle parameters were 50 °C for 2 min and 95 °C for 10 min to activate AmpliTaq Gold for preventing primer dimers, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The number of each *Wolbachia* and COI genes were calculated from the intensity of the fluorescence on the basis of a standard curve obtained from standard samples.

To compare *Wolbachia* density between superinfected and singly infected abdomens, we analyzed the data using a Student's *t*-test (R software ver. 2.7.1; R Development Core Team, 2005).

Results

CI modification

Our results suggested that the two *Wolbachia* strains differed in their ability to cause CI. In the predicted incompatible cross with *wOus1* (*wOus1* males × uninfected females), the mean egg-hatch rate was 81.0%, not significantly different from either of the predicted control crosses (the reciprocal cross), Table 2 ($\chi^2 = 0.03$, $df = 1$, $P = 0.9$) and *wOus1* males × females infected with the same strain ($\chi^2 = 0.06$, $df = 1$, $P = 0.8$). On the other hand,

Table 2 Egg-hatch rate (mean % ± s.d.) of progeny of crosses among lines of *Orius strigicollis* that are superinfected (*wOus1* and 2), singly infected (*wOus1* and 2), and uninfected with *Wolbachia*

Female type	Male type				
	Uninfected	<i>wOus1</i>	<i>wOus2</i>	<i>wOus1+2</i>	
Uninfected	83.7 ± 7.1 ^{abc} (12, 415)	81.0 ± 6.4 ^{bcd} (10, 370)	8.5 ± 8.6 ^g (10, 388)	56.4 ± 18.2 ^e (12, 385)	58.6 ± 31.6 ^A (44, 1558)
<i>wOus1</i>	82.9 ± 9.2 ^{bcd} (12, 449)	82.4 ± 4.2 ^{bcd} (12, 463)	20.5 ± 14.9 ^f (9, 248)	49.6 ± 18.2 ^e (13, 410)	61.1 ± 27.7 ^A (46, 1570)
<i>wOus2</i>	89.4 ± 5.3 ^a (10, 368)	73.5 ± 7.6 ^d (7, 214)	85.7 ± 7.1 ^{ab} (10, 385)	76.4 ± 9.3 ^{cd} (12, 635)	81.6 ± 9.7 ^B (39, 1602)
<i>wOus1+2</i>	85.7 ± 7.6 ^{ab} (10, 514)	81.0 ± 7.7 ^{bcd} (7, 311)	86.6 ± 7.1 ^{ab} (10, 361)	84.7 ± 4.0 ^{ab} (10, 411)	84.8 ± 6.7 ^B (37, 1597)
	85.2 ± 7.7 ^A (44, 1746)	80.0 ± 6.9 ^B (36, 1358)	51.1 ± 37.9 ^C (39, 1382)	65.7 ± 19.8 ^D (47, 1841)	

Numbers within parentheses refer to number of pairs (*n*) and total number of eggs counted for each cross.

The far right column provides means of all crosses by particular female types, and the bottom row provides means of all crosses by particular male types. Mean frequencies marked with the same lower-case letter (in cells) or upper-case letter (in marginal rows or columns) are not significantly different (logistic regression, $P > 0.05$).

the single infection of *wOus2* induced high levels of CI in the predicted CI cross (*wOus2* males × uninfected females), resulting in approximately 10% of the hatching rates of the control crosses. In crosses between uninfected females and singly infected *wOus2* males, only 35 of 388 eggs hatched (Table 2). This egg-hatching rate is significantly lower than for the reciprocal cross ($\chi^2 = 173.30$, $df = 1$, $P < 0.0001$), or for the within-strain cross ($\chi^2 = 168.37$, $df = 1$, $P < 0.0001$).

Surprisingly, given the strong CI induced by *wOus2*, superinfected males caused only weak CI when mated with uninfected females. The egg-hatch rate of this cross was, on average, about two-thirds that of compatible crosses, and quite variable. The egg-hatch rate for this cross was statistically significantly above that for the *wOus2* incompatible cross ($\chi^2 = 86.59$, $df = 1$, $P < 0.0001$), but also significantly below the rate of egg hatch of the control reciprocal cross ($\chi^2 = 44.52$, $df = 1$, $P < 0.0001$), and the within-strain cross ($\chi^2 = 33.91$, $df = 1$, $P < 0.0001$).

CI rescue

The *Wolbachia* strains also appeared to vary in their ability to rescue CI. Although *wOus1* caused either weak or no CI, it showed some ability to rescue sperm modified by *wOus2*; the egg-hatch rates of *wOus1* females mated to *wOus2* males were double that of uninfected females × *wOus2* males ($\chi^2 = 106.14$, $df = 1$, $P < 0.0001$). The rescue function of *wOus1* females when mated to *wOus2* or superinfected males was also variable. The apparent lack of rescue by *wOus2* females was somewhat puzzling. The egg-hatch rates in the cross between *wOus2* females and *wOus1* males were significantly lower than when *wOus2* females mated with males of the same type ($\chi^2 = 33.91$, $df = 1$, $P = 0.0046$). Similarly, hatch rates were lower when *wOus2* females mated with superinfected males relative to within-superinfection matings ($\chi^2 = 4.98$, $df = 1$, $P = 0.0256$). The result is puzzling because, as discussed above, the degree of CI caused by *wOus1* is very weak if it occurs at all; the egg-hatch rate in the predicted CI cross is not significantly different from the control crosses.

As expected, superinfected females rescued sperm from all male types. There were no significant differences among crosses between superinfected females and uninfected, singly infected or superinfected males (Table 2).

Comparative fitness assays

In all crosses, the sex ratio of *O. strigicollis* developed to adults showed no significant differences from 1:1 (Fisher's exact test, $P > 0.05$). There were also no significant differences between the number of eggs produced by singly infected, superinfected or uninfected females on each date (Figure 1).

Infection densities of *wOus1* and *wOus2* in superinfected and singly infected strains

The infection densities of *wOus1* *Wolbachia* in the abdomens of superinfected strains were much greater than those of *wOus2* in both males and females (Figure 2; Student's *t*-test, male: $P < 0.01$, female: $P < 0.001$). Similarly, we examined the density of *wOus1* and *wOus2* in abdomens of singly infected individuals and compared them with the density of these *Wolbachia* strains in

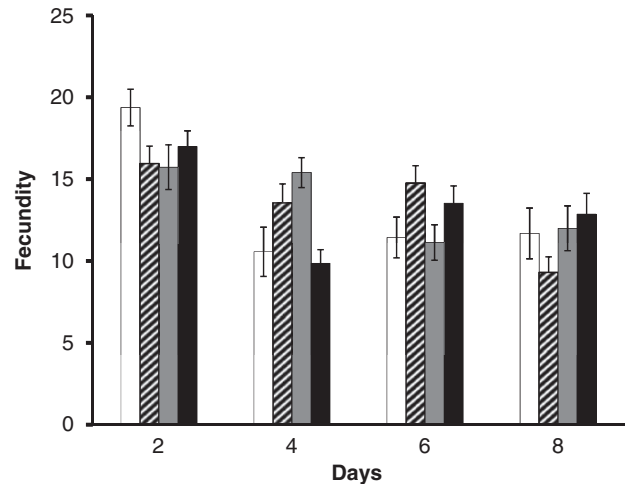


Figure 1 Fecundity of superinfected, singly infected and uninfected females. Data are shown as mean ± s.e. of offspring produced by females at days 2, 4, 6 and 8. There was no significant difference among singly infected (*wOus1* or *wOus2*), superinfected (*wOus1* + 2) and uninfected lines (repeated measures generalized linear model: $\chi^2 = 0.22$, $df = 3$, $P > 0.05$), although there was a significant 'day' effect ($\chi^2 = 29.61$, $df = 3$, $P < 0.001$) and the 'day' effect differs significantly among singly infected, superinfected and uninfected lines (interaction: $\chi^2 = 36.66$, $df = 9$, $P < 0.001$). White bars: uninfected (number of pairs, $n = 16$); striped bars: *wOus1* ($n = 20$); gray bars ($n = 15$): *wOus2*; black bars: *wOus1* + 2 ($n = 15$).

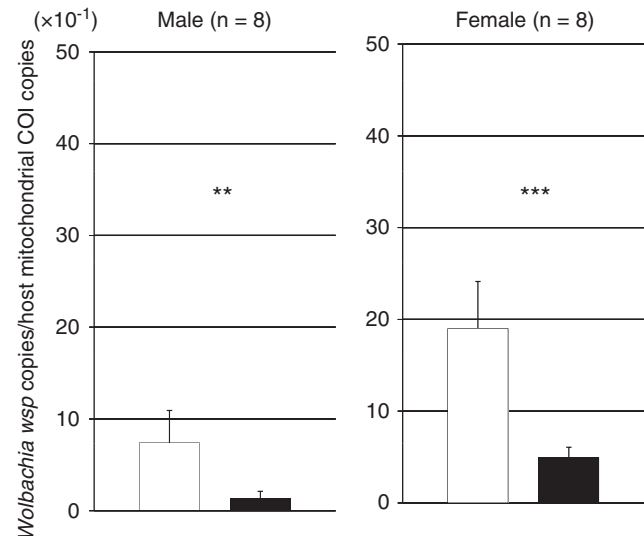


Figure 2 Relative infection densities of *wOus1* and *wOus2* in the abdomens of superinfected individuals. White bars: *wOus1*; black bars: *wOus2*. Different letters indicate statistically significant differences (Student's *t*-test, ** $P < 0.01$, *** $P < 0.001$). Males and females were subjected to the statistical analysis separately.

superinfected individuals. We found that the densities of *wOus1* in superinfected individuals were significantly lower than in singly infected individuals (Figure 3a; Student's *t*-test, male: $P < 0.01$, female: $P < 0.001$). The densities of *wOus2* were also lower in superinfected individuals than in singly infected individuals, but the data were more variable and the difference was not statistically significant (Figure 3b; Student's *t*-test, male: $P = 0.05127$, female: $P < 0.001$). These patterns were observed in both sexes.

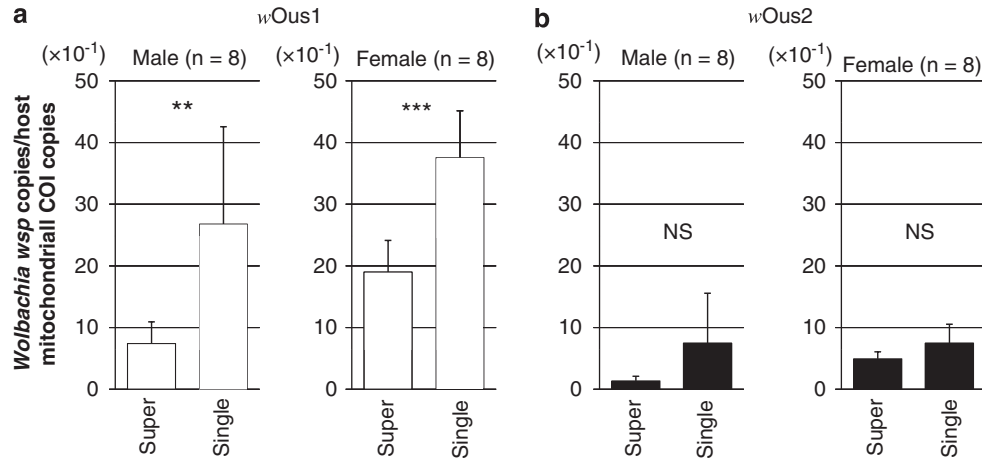


Figure 3 Relative infection densities of the two *Wolbachia* strains in the abdomens of singly vs superinfected individuals. White bars: *wOus1* (a); black bars: *wOus2* (b). Different letters indicate statistically significant differences (Student's *t*-test, NS $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$). Males and females were subjected to the statistical analysis separately.

Discussion

Here, we show that two strains of *Wolbachia*, one of which causes strong CI when alone, induce only weak CI in the superinfected state in the predacious bug *O. strigicollis*. The great majority of studies in which the compatibilities of superinfected, singly infected and uninfected lines have been studied show an additive effect; superinfected males cause CI similar to the strength of the strongest single infection when mated to singly or uninfected females (Sinkins *et al.*, 1995; Perrot-Minnot *et al.*, 1996; Mercot and Poinso, 1998; Rousset *et al.*, 1999; Dobson *et al.*, 2001; Mouton *et al.*, 2005). We are aware of only one other similar example from the nature of a lack of additive CI effects of superinfection, in field populations of spider mites infected with *Cardinium* (which causes CI when alone), *Wolbachia* (which does not) or both (with no CI induced) (Ros and Breeuwer, 2009). In the mite study, it was not feasible to manipulate the infections of mites, however, and, therefore, not possible to determine whether the different phenotypes were due to strain differences in the *Cardinium* in the single and superinfected populations, or interference between the symbionts in the superinfected mites. In another, even more pertinent example, Walker *et al.* (2009) created artificial superinfections of mosquitoes by adding a *Wolbachia* strain that induced CI to a host bearing one that did not. In the superinfected lines, the new strain was stably maintained, but CI was not induced. Further, CI induced by the singly infected males was not rescued by superinfected females, likely because the density of the introduced symbiont was very low in both males and females (Walker *et al.*, 2009). To our knowledge, this study is unique in showing differences in phenotype between single and naturally superinfected lines in which the symbiont strains are identical in both types of infection. The superinfection of CI symbionts in *O. strigicollis* is not additive in its phenotype, and suggests interference between the symbionts such that the CI that is caused by *wOus2* when alone is suppressed when it is in a host co-infected with *wOus1*. Interestingly, although the modification aspect of CI appears to be reduced in the superinfected

males, superinfected females are fully capable of rescuing sperm from the strong CI-inducing strain, *wOus2*, distinct from Ros and Breeuwer (2009) and Walker *et al.* (2009) examples, in which the rescue function of superinfected females was also impaired.

How would a superinfection that causes only weak CI spread in a population with a strong CI-inducing strain? Theory predicts that the spread and maintenance of a strictly maternally inherited symbiont is dependent on infected mothers producing more infected daughters than the number of daughters produced by uninfected females (Bull, 1983). This theory was recently extended to consider the evolution of symbiont densities in superinfected hosts (Engelstadter *et al.*, 2007); superinfected females produce more infected daughters than singly or uninfected females (Engelstadter *et al.*, 2007). We hypothesize that the first infection in *O. strigicollis* was *wOus2*. Theory would predict this symbiont would rapidly spread to fixation, given that CI is strong, and fecundity costs appear to be low or absent (Figure 1; Hoffmann and Turelli, 1997). Although the superinfection of *wOus1* and *wOus2* does not cause strong CI, it does induce weak CI and thus reduces the fitness of *wOus2* females (Table 2). It also shows little or no cost to fecundity (Figure 2), rescues *wOus1* sperm and appears to be marginally better at rescuing *wOus1* sperm than *wOus2* females as well, although this last difference is not statistically significant (Table 2). Given perfect co-transmission, and any slight superiority in its ability to rescue, we might predict that the superinfected females could produce more (superinfected) daughters than the number of daughters produced by *wOus2* or uninfected females, and a superinfection would, therefore, invade.

The finding that superinfected males cause much weaker CI than does one of the strains while acting alone suggests some kind of interference of one strain with the other. Perhaps the most intuitive explanation would involve simple differences in density in the singly and superinfected hosts. At least in some hosts, CI intensity is related to symbiont density (Breeuwer and Werren, 1993; Hoffmann and Turelli, 1997). We predicted that, if density differences underlie the non-additive

phenotype of superinfected hosts, then (1) the densities of *wOus1* would be greater than *wOus2* in doubly infected hosts. This is what we have observed (Figure 2), indicating the potential for competitive exclusion of *wOus2* in tissues occupied by *wOus1*. This could be further tested by localizing the symbionts in tests with singly and doubly infected males. (2) We also predicted densities of *wOus2* to be lower in superinfected males relative to singly infected males. The densities of *wOus2* are indeed lower in doubly infected males, but the difference is not statistically significant, probably because the densities are quite variable (Figure 3b). Interestingly, there is also considerable variability in the egg-hatch rates when superinfected males mate with either uninfected or *wOus1* females (Table 2), indicating that both *wOus2* densities and CI intensity vary among individual superinfected males. In future work, it would be interesting to correlate the degree of CI and density of *wOus2* and *wOus1* in superinfected males to test the possibility that *wOus2* densities and CI intensity co-vary. In summary, our results provide qualified support for the hypothesis that the non-additivity of CI expression in doubly infected males is mediated by a suppression of the density of *wOus2*. More work remains to be performed to resolve this question. However, suppression of the density of one symbiont by the other has been found before in natural infections (Kondo et al., 2005), and density differences may also have been the reason for the lack of CI modification and rescue in the *Wolbachia* superinfection created in mosquitoes by Walker et al. (2009). The higher densities of *wOus1* compared with *wOus2* suggest that *wOus1* might also show a higher rate of maternal transmission in the field; this hypothesis should be investigated.

Lastly, the results presented here may temper our expectations about the potential applications of CI-inducing symbionts as tools in pest suppression when the pest/host population is already infected. Symbionts that cause CI have been considered as a means to directly reduce population size (Brelsfoard et al., 2008), limit longevity of disease vectors (McMeniman et al., 2009) and as a drive system for a beneficial gene (Beard et al., 1993; Bourtzis, 2008). When superinfections are not costly, and the phenotype of the superinfected individuals is the same level of CI as the single infection, there appears to be little cost to 'stacking symbionts' in hosts (Sinkins et al., 1995). However, this example shows that adding symbionts that cause CI when alone may or may not result in the expected phenotype in a superinfected host.

Conflict of interest

The authors declare no conflict of interest.

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