Effects of A and B Wolbachia and Host Genotype on Interspecies Cytoplasmic Incompatibility in Nasonia

Seth R. Bordenstein and John H. Werren

Department of Biology, University of Rochester, Rochester, New York 14627

Manuscript received July 29, 1997
Accepted for publication December 8, 1997

ABSTRACT

Wolbachia endosymbionts cause postmating reproductive isolation between the sibling species *Nasonia vitripennis* and *N. girault*i. Most Nasonia are doubly infected with a representative from each of the two major Wolbachia groups (A and B). This study investigates the role of single (A or B) and double (A and B) Wolbachia infections in interspecies cytoplasmic incompatibility (CI) and host genomic influences on the incompatibility phenotype. Results show that the single A Wolbachia harbored in *N. vitripennis* (*w*Av) is bidirectionally incompatible with the single A Wolbachia harbored in *N. girault*i (*w*Ag). Results also indirectly show that the *N. vitripennis w*Bv is bidirectionally incompatible with the *N. girault*i *w*Bg. The findings support current phylogenetic evidence that suggests these single infections have independent origins and were acquired via horizontal transfer. The *w*Av Wolbachia expresses partial CI in the *N. vitripennis* nuclear background. However, following genomic replacement by introgression, *w*Av expresses complete CI in the *N. girault*i background and remains bidirectionally incompatible with *w*Ag. Results show that double infections can reinforce interspecies reproductive isolation through the addition of incompatibility types and indicate that the host genome can influence incompatibility levels. This study has implications for host-symbiont coevolution and the role of Wolbachia in speciation.

WOLBACHIA are maternally inherited bacteria that infect the reproductive tissues of a wide range of insect species, as well as isopods, mites, and nematodes (O'Neill *et al.* 1992; Rousset *et al.* 1992; Johanowicz and Hoy 1995; Sironi *et al.* 1995; Werren *et al.* 1995a). This group of alpha proteobacteria is responsible for various modifications in host reproduction, including parthenogenesis in wasps (Stouthamer *et al.* 1993), feminization in terrestrial isopods (Rousset *et al.* 1992), possible modulation of sperm competition in Tribolium beetles (Wade and Chang 1994), and cytoplasmic incompatibility (CI) in a variety of insect species (Yen and Barr 1971; Hoffmann 1988; Breeuwer and Werren 1990; O'Neill and Karr 1990; Werren 1997a). Each of these phenotypes entails a selective advantage for the bacteria.

CI is phenotypically expressed as embryo mortality in diploid species or as a sex ratio shift biased toward the haploid sex (male) in haplodiploid species. The cytological and biochemical mechanisms of CI are not fully known, but there is good evidence that the expression of incompatibility is due to improper condensation of the paternal chromosomes during mitosis (Ryan and Saul 1968; Breeuwer and Werren 1990; O'Neill and Karr 1990; Reed and Werren 1995). The cytological basis appears to involve disruptions to the kinetics of fertilization (Reed and Werren 1995; Callaini *et al.* 1997). An irregular mass of paternal chromatin is formed, which leads to an unsuccessful formation of the zygote.

There are two cases of CI: unidirectional and bidirectional. In unidirectional incompatibility, sperm from infected males are incompatible with eggs from uninfected females, whereas the reciprocal cross is compatible. Wolbachia are favored to cause CI because selection acts to decrease the number of uninfected individuals in polymorphic populations (Caspari and Watson 1959; Turelli 1994). Bidirectional incompatibility typically occurs when males and females are both infected, but with different strains of Wolbachia. In this case, crosses in both directions are incompatible. Cytoplasmic incompatibility is especially interesting as a possible mechanism for rapid evolution of postmating reproductive isolation between closely related species (Breeuwer and Werren 1990; Turelli and Hoffmann 1991; Coyne 1992; Werren 1997a,b).

CI apparently entails two components: (1) a bacterial "modification" of sperm and (2) a bacterial "rescue" in fertilized eggs (Werren 1997a). Compatible crosses occur when the bacterial strain present in the egg is capable of rescuing the sperm modification. Variation in modification and rescue components among Wolbachia strains presumably is responsible for bidirectional incompatibility. In addition, selection for unidirectional incompatibility could lead to divergence in modification-rescue systems and contribute to the evolution of new incompatibility types within and between host spe-

Corresponding author: Seth R. Bordenstein, Department of Biology, University of Rochester, Rochester, NY 14627.
E-mail: sbst@troi.cc.rochester.edu

cies (Werren 1997b). If bidirectional incompatibility types readily evolve, the likelihood of Wolbachia facilitating a speciation event increases.

Phylogenetic analysis of Wolbachia using sequences from 16S rDNA (O'Neill *et al.* 1992) and the *ftsZ* cell cycle gene (Werren *et al.* 1995b) indicates that there are two major subdivisions of these proteobacteria. These two subdivisions, denoted A and B, are estimated to have diverged 58 to 66 mya, based upon synonymous substitution rates. Some insects naturally harbor a single infection or a double infection with a representative from each subdivision (A and B) (Mercot *et al.* 1995; Rousset and Solignac 1995; Sinkins *et al.* 1995; Werren *et al.* 1995a; Clancy and Hoffmann 1996; Perrot-Minnot *et al.* 1996). In addition, Wolbachia polymorphisms (single) exist both within species and between closely related species (Breeuwer *et al.* 1992; Rousset and Solignac 1995; Clancy and Hoffmann 1996). Characterizing the variation among Wolbachia strains and the number of incompatibility types within and among species can help answer the following questions about the evolution of these heritable microorganisms:

1. What is the role of different Wolbachia types in interspecific cytoplasmic incompatibility? Breeuwer and Werren (1990) investigated cytoplasmic incompatibility between sibling species of Nasonia using cured and doubly infected wild-type strains. They showed that bidirectional incompatibility causing complete reproductive isolation exists between doubly infected *N. vitripennis* (*w*Av,*w*Bv) and *N. girault*i (*w*Ag,*w*Bg). Compatibility between the species is restored upon antibiotic treatment and subsequent curing of the double infections. This bidirectional incompatibility system indicates that the modification and rescue components of these double Wolbachia infections are distinct. However, it may be that only the A or only the B Wolbachia is responsible for causing the bidirectional incompatibility (and isolation) between the species. Alternatively, double infections could express a more complete CI phenotype than single infections. These two scenarios have implications for the potential role of Wolbachia in maintaining isolation between the species. Here we describe experiments that elucidate the role of single and double Wolbachia infections in heterospecific incompatibility between *N. vitripennis* and *N. girault*i.
2. How readily do new incompatibility types evolve within and among closely related species? A phylogenetic analysis of Nasonia Wolbachia, based upon a region of the *ftsZ* cell cycle gene, suggests the A and B Wolbachia variants in *N. vitripennis* have different origins from those infections harbored in *N. girault*i (Werren *et al.* 1995b). This raises the question of how much divergence has occurred in (1) modification and (2) rescue components of the two A Wolbachia variants, denoted *w*Av and *w*Ag, and the two B Wolbachia variants, denoted *w*Bv and *w*Bg. To investigate this, we tested whether the *w*Av- (or *w*Bv)-induced sperm modification of the paternal chromosomes can be rescued by the *w*Ag- (or *w*Bg)-infected egg and vice versa. Here we use genetic crosses to investigate the properties of the modification-rescue components of different A and B Wolbachia types harbored in *N. vitripennis* and *N. girault*i.
3. Can the host's genome influence the expression of CI? In addition to insects, Wolbachia have been found in mites (Johanowicz and Hoy 1995), isopods (Rousset *et al.* 1992), and a close relative in a nematode (Sironi *et al.* 1995). Such findings indicate that Wolbachia can tolerate a variety of cellular environments in diverse hosts and raise the question of whether the host genome influences the Wolbachia symbiont. However, there is only limited evidence of host genomic effects on CI. In inter- and intraspecific studies of CI, host genomic effects need to be considered as a variable influencing CI expression. Experiments are conducted to test the influence of the host species genome on CI in Nasonia.

In this study, we investigate the role of different A and B Wolbachia in interspecies cytoplasmic incompatibility, the variation in Wolbachia strains, the number of different incompatibility types harbored between two sibling species, and the effects of the host genome on the expression of CI between two haplodiploid species, *N. vitripennis* and *N. girault*i.

MATERIALS AND METHODS

A detailed description of the biology of Nasonia is given by Whiting (1967). In the laboratory, Nasonia are maintained with constant light and temperature (25°) and are raised on fresh fly pupae, *Sarcophaga bullata* (referred to as "hosts"). Under these conditions, generation time is approximately 14 days for *N. vitripennis* and 15 days for *N. girault*i.

Nomenclature: Wolbachia type is denoted in brackets by an italicized lower case *w* and a capital A or B, depending upon the infection status. Zero symbolizes an uninfected host. A corresponding lower case v or g categorizes the Wolbachia strain according to host species from which it is derived. To denote host genotype, V and G are used for *N. vitripennis* and *N. girault*i, respectively. For example, [*w*Av,*w*Bv]V symbolizes the *N. vitripennis* A and B Wolbachia variants in an *N. vitripennis* nuclear background.

Introgression lines consist of an *N. girault*i genotype introgressed into an *N. vitripennis* cytotype. The above terminology applies. For example, [*w*Av]G denotes the *N. vitripennis* A Wolbachia in the *N. girault*i nuclear background.

Strains: A number of strains were used for progeny testing and laboratory experiments, including four strains of *N. vitripennis*, four strains of *N. girault*i, and four introgression lines (Table 1). Note that inferences are based upon these strains and that levels of CI among other lines may differ from those observed.

The following *N. vitripennis* strains were used for progeny testing. All lines were naturally generated from a segregation experiment and contain the same nuclear background (see Perrot-Minnot *et al.* 1996). R5-11 is a bi-infected wild-type

TABLE 1

Strains used, nomenclature, and origin of Wolbachia variant

Strain	Nomenclature	Wolbachia origin
N. vitripennis		
R511	[*w*Av,*w*Bv]V	wild type
12.1	[*w*Av]V	R511
4.9	[*w*Bv]V	R511
8.3	[0v]V	R511
N. giraulti		
RV2	[*w*Ag,*w*Bg]G	wild type
NGOH 206D	[*w*Ag]G	unknown
RV2T	[0g]G	cured from RV2
RV2R	[0g]G	cured from RV2
Introgression lines		
INT R511-G	[*w*Av,*w*Bv]G	R511
INT 12.1-G	[*w*Av]G	12.1
INT 4.9-G	[*w*Bv]G	4.9
INT 8.3-G	[0v]G	8.3

For nomenclature, Wolbachia type is denoted in brackets by a *w* and capital A or B depending on infection status. Zero denotes an uninfected host. The corresponding lower case v or g categorizes A Wolbachia strains according to host species from which it is derived. Host genotype is symbolized as V or G for *N. vitripennis* and *N. giraulti*, respectively.

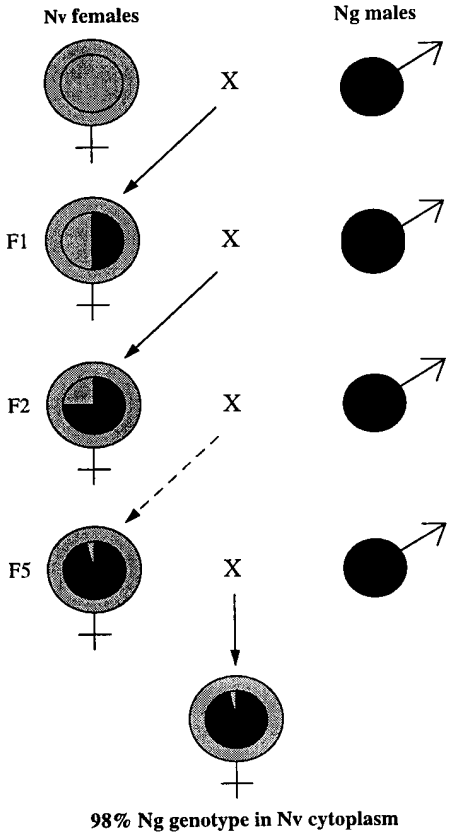

Figure 1.—Protocol for introgression. Outer circles denote cytotype and inner circles denote genotype. Recall that Wolbachia are maternally inherited through the cytotype. *N. vitripennis* females were backcrossed to uninfected *N. giraulti* males for six generations to create the introgression lines. After six backcross generations, the lines were maintained by sibmating without further backcrossing. Successful introgressions were confirmed via PCR.

strain, which is designated [*w*Av,*w*Bv]V; 8.3 is a naturally cured line and is designated [0v]V; 12.1 harbors a single A infection and is designated [*w*Av]V; 4.9 harbors a single B infection and is designated [*w*Bv]V.

Four *N. giraulti* strains were used for progeny testing. RV2 harbors a wild-type double infection and is designated [*w*Ag,*w*Bg]G; RV2T and RV2R are antibiotically cured strains derived from RV2, which are designated [0g]G; NGOH206D is an Ohio field strain that harbors a single A infection and is designated [*w*Ag]G. It is uncertain whether this infection occurs naturally in the field or arose independently under lab maintenance. We presume that this line does not have the identical nuclear genome as the other *N. giraulti* RV2-derived lines.

Four introgression lines were generated by repeated backcrossing of the uninfected RV2R *N. giraulti* genotype into *N. vitripennis* lines R511, 12.1, 4.9, and 8.3 and are designated [*w*Av,*w*Bv]G, [*w*Av]G, [*w*Bv]G, and [0v]G, respectively. These lines were used to control for host genetic background and to test host genomic influences on CI.

Introgression design: The *N. giraulti* nuclear genome from an uninfected lab strain RV2R was introgressed into four *N. vitripennis* cytoplasms by repeated backcrossing (see Figure 1). Lines were started with crosses between uninfected *N. giraulti* males and females from four *N. vitripennis* strains ([0g]G males × [*w*Av,*w*Bv]V, [*w*Av]V, [*w*Bv], and [0v]V females). Resulting hybrid females were backcrossed to the cured males of the paternal species, [0g]G, for six generations. After six backcross generations, the lines were maintained by sibmating without further backcrossing. Successful introgressions were confirmed using (1) a PCR assay with A and B Wolbachia specific primers previously described in Werren *et al.* (1995b) and (2) phenotypic markers characteristic of the two species for host genotype status.

Interspecific compatibility tests: All crosses were set up with one virgin male and one virgin female in a 12 × 75-mm vial. To ensure that all wasps were virgins, they were collected as pupae. To prevent bacterial cross-contamination between the strains (an unlikely occurrence), all surfaces and utensils were washed with 95% ethanol before and after pupae collection of a new strain. Once all wasps emerged, they were set up in their respective crosses in single pair matings and observed for 45 min. Only those crosses with observed copulations were used in the experiments. After 24 hr, the males were discarded from the vial, and each female was hosted with two hosts for egg laying. F_1 progeny were scored for sex, because compatibility is measured according to percent females (hybrids) in haplodiploid organisms (*i.e.*, males are derived from unfertilized eggs or CI-induced paternal genome loss). Family sizes were also recorded.

Compatibility tests in a controlled *N. giraulti* host nuclear background: After introgression of the *N. giraulti* genome into four different *N. vitripennis* cytotypes (*w*Av,*w*Bv; *w*Av; *w*Bv; 0v), crosses were repeated with these introgression lines to test compatibility relationships following genome replacement. All crosses were group mated in sets of two males and five females per vial. Copulations were not observed in these tests because homospecific matings occurred readily (confirmed by preliminary observations). After 24 hr, the males were discarded and the females were hosted singly with two hosts for egg laying. F_1 progeny were scored as described above.

Host genomic effects on CI: In the experiment above, the strength of CI induced by *w*Av appeared to increase upon

TABLE 2

Percent females from crosses with uninfected [0v]V males

	Females		
[0v]V males	[*wAg*,*w*Bg]G	[0g]G	Self
Replicate 2	0.0 (1)	—	86.2 ± 4.2 (13)
Replicate 3	91.4 ± 6.5 (5)	—	87.5 ± 4.5 (18)
Replicate 4	92.0 ± 3.5 (6)	—	87.7 ± 4.0 (9)
Replicate 5	89.8 ± 4.8 (14)	89.7 ± 5.5 (11)	88.3 ± 3.3 (13)
Replicate 6	92.9 ± 4.8 (5)	93.9 ± 2.7 (9)	89.1 ± 2.7 (17)
Replicate 7	91.6 ± 3.2 (5)	94.3 ± 2.1 (4)	88.3 ± 3.4 (12)
Pooled data	88.6 ± 15.8 (36)	92.0 ± 5.6 (24)	87.9 ± 3.7 (82)

Values are means ± SD. Mean percent females (hybrids) is determined by the proportion of females in the F_1 progeny. Sample sizes are given in parentheses and are the number of families scored. All copulations were observed.

genome replacement of the *N. vitripennis* nuclear background with the *N. giraulti* nuclear background (see results). This result suggested that the host genome can influence expression of cytoplasmic incompatibility. However, the two experiments were conducted in different ways and at separate times. To confirm host genetic effects on the *w*Av-induced CI phenotype, crosses were made at the same time with both standard [*w*Av]V and introgression [*w*Av]G lines. All crosses were set up in single pair matings. Only those crosses with observed copulations were used in the experiment. After 24 hr, the males were discarded from the vial, and each female was hosted with two hosts for egg laying. F_1 progeny were scored as described above.

Statistics: Differences in compatibility relationships were examined by nonparametric Mann-Whitney U tests. Mean family sizes were compared by *t*-tests. Some sample sizes include pooled data from multiple replicates that were not significantly different at the 0.05 level.

RESULTS

In Nasonia, compatibility is measured by the percent females among progeny. Incompatibility is expressed as production of all- or nearly all-male families. This occurs because paternal chromosome loss in incompatible crosses results in haploid (male) production in this haplodiploid insect. In contrast, under the experimental design used here, normal compatible sex ratios are female biased (80–95%).

Effects of single and double Wolbachia infections on interspecies CI: Interspecies crosses were made to characterize the incompatibility properties of the Wolbachia variants harbored in *N. vitripennis* and *N. giraulti*. Bi-infected (*w*Av,*w*Bv), mono-infected (*w*Av or *w*Bv), and uninfected (0v) *N. vitripennis* males were crossed to bi-infected (*w*Ag,*w*Bg) *N. giraulti* females. As controls on compatibility types, these same males were crossed to uninfected females of both species and to same strain females.

Results from compatibility tests with uninfected *N. vitripennis* males are shown in Table 2. In all replicates (except replicate 2), interspecific crosses between uninfected *N. vitripennis* males and bi-infected or uninfected *N. giraulti* females are compatible and yield normal female-biased sex ratios. Control self-crosses also yield normal sex ratios (87.9% females, pooled data). Thus, in the absence of Wolbachia in males, successful hybrid

TABLE 3

Percent females from crosses with [*w*Av,*w*Bv]V males

	Females			
[*wAv*,*w*Bv]V males	[*w*Ag,*w*Bg]G	[0g]G	[0v]V	Self
Replicate 1	—	0.0 ± 0.0 (3)	6.8 ± 21.6 (14)	87.4 ± 4.5 (18)
Replicate 2	—	0.0 ± 0.0 (3)	0.0 ± 0.0 (13)	84.0 ± 9.2 (14)
Replicate 3	0.0 ± 0.0 (8)	0.0 ± 0.0 (1)	0.0 ± 0.0 (17)	86.1 ± 6.4 (19)
Replicate 4	0.0 ± 0.0 (13)	0.0 ± 0.0 (2)	0.0 ± 0.0 (8)	88.3 ± 2.5 (11)
Replicate 5	0.0 ± 0.0 (8)	0.0 ± 0.0 (11)	0.0 ± 0.0 (19)	83.8 ± 6.3 (20)
Replicate 6	0.0 ± 0.0 (6)	0.0 ± 0.0 (12)	0.0 ± 0.0 (5)	87.2 ± 6.9 (17)
Replicate 7	0.0 ± 0.0 (4)	—	0.0 ± 0.0 (12)	87.9 ± 4.3 (14)
Pooled data	0.0 ± 0.0 (39)	0.0 ± 0.0 (32)	1.1 ± 8.9 (88)	86.1 ± 6.1 (113)

Values are means ± SD. Mean percent females (hybrids) is determined by the proportion of females in the F_1 progeny. Sample sizes are given in parentheses and are the number of families scored. All copulations were observed.

TABLE 4

Percent females from crosses with [*w*Bv]V males

	Females			
[*w*Bv]V males	[*w*Ag,*w*Bg]G	[0g]G	[0v]V	Self
Replicate 1	—	0.0 ± 0.0 (3)	0.2 ± 0.4 (10)	88.8 ± 3.8 (13)
Replicate 2	0.0 ± 0.0 (3)	0.0 ± 0.0 (3)	0.2 ± 0.8 (12)	85.4 ± 8.3 (18)
Replicate 3	0.0 ± 0.0 (14)	0.0 ± 0.0 (1)	0.1 ± 0.3 (13)	88.8 ± 3.6 (20)
Replicate 4	0.0 ± 0.0 (12)	0.0 ± 0.0 (4)	0.0 ± 0.0 (14)	85.5 ± 2.9 (5)
Replicate 5	0.0 ± 0.0 (10)	0.3 ± 1.4 (17)	2.2 ± 6.3 (14)	85.1 ± 9.0 (19)
Replicate 6	0.0 ± 0.0 (6)	0.0 ± 0.0 (5)	0.0 ± 0.0 (20)	87.3 ± 5.6 (18)
Replicate 7	0.0 ± 0.0 (3)	0.0 ± 0.0 (3)	0.0 ± 0.0 (4)	—
Pooled data	0.0 ± 0.0 (48)	0.0 ± 0.0 (36)	0.4 ± 2.5 (87)	86.9 ± 6.5 (93)

Values are means ± SD. Mean percent females (hybrids) is determined by the proportion of females in the F_1 progeny. Sample sizes are given in parentheses and are the number of families scored. All copulations were observed.

production occurs between *N. vitripennis* males and *N. girault*i females (Breeuwer and Werren 1990; these results).

Table 3 shows results from compatibility tests with doubly infected (*w*Av,*w*Bv) *N. vitripennis* males. Interspecific crosses between these males and bi-infected (*w*Ag,*w*Bg) *N. girault*i females yield no hybrids (0% females). However, crosses with uninfected *N. vitripennis* males yield many hybrid (female) progeny (Table 2). These results confirm previous findings that double Wolbachia infections completely prevent hybrid production between the two wasp species (Breeuwer and Werren 1990). In addition, females are typically not produced when bi-infected *N. vitripennis* males are crossed to uninfected females of either the same or sister species. This result is concordant with unidirectional incompatibility dynamics (*i.e.*, infected males are incompatible with uninfected females). These results show that the double (*w*Av,*w*Bv) infection in *N. vitripennis* induces complete (or nearly complete) levels of CI. Normal female-biased sex ratios occur in the self-crosses.

Table 4 shows the results of crosses with *N. vitripennis* males singly infected with *w*Bv. The *w*Bv infection in *N. vitripennis* also induces complete (or nearly complete) CI (0% females) when interspecifically crossed to bi-infected or uninfected *N. girault*i females. Therefore, both the *w*Bv and *w*Av,*w*Bv infections in *N. vitripennis* males induce strong interspecific cytoplasmic incompatibility. Results show that the sperm modification induced by the *w*Bv infection in *N. vitripennis* cannot be rescued by either of the *w*Ag or *w*Bg infections harbored in the *N. girault*i-infected egg. It is likely that variation in the modification-rescue components of CI of these strains prevents hybrid production between the species. Self-crosses yield normal sex ratios.

Table 5 shows results from interspecific compatibility tests with *w*Av-infected *N. vitripennis* males. Crosses with these males to bi-infected (*w*Ag,*w*Bg) *N. girault*i females yield 15.8% females, on average. In six of the seven replicates, partial CI is expressed. The sum of these findings indicates that *w*Av does not induce complete incompatibility by itself. Only in the presence of

TABLE 5

Percent females from crosses with [*w*Av]V males

	Females			
[*w*Av]V males	[*w*Ag,*w*Bg]G	[0g]G	[0v]V	Self
Replicate 1	60.0 ± 0.0 (1)	0.0 ± 0.0 (2)	10.6 ± 12.6 (16)	82.5 ± 4.5 (16)
Replicate 2	0.0 ± 0.0 (1)	0.0 ± 0.0 (3)	8.0 ± 13.8 (14)	83.9 ± 9.0 (16)
Replicate 3	7.1 ± 10.1 (16)	—	5.9 ± 9.0 (18)	81.9 ± 6.9 (18)
Replicate 4	24.3 ± 25.0 (10)	4.2 ± 4.7 (5)	14.4 ± 14.4 (18)	85.6 ± 4.7 (11)
Replicate 5	22.7 ± 16.6 (9)	16.6 ± 23.4 (11)	5.7 ± 7.6 (18)	85.7 ± 4.6 (15)
Replicate 6	8.9 ± 10.6 (5)	9.0 ± 20.9 (11)	5.0 ± 14.0 (16)	84.2 ± 4.7 (16)
Replicate 7	11.0 ± 18.0 (10)	33.7 ± 22.4 (8)	10.8 ± 14.7 (17)	86.1 ± 3.9 (10)
Pooled data	15.8 ± 19.1 (52)	13.9 ± 21.5 (49)	8.9 ± 12.7 (117)	84.2 ± 5.8 (102)

Values are means ± SD. Mean percent females (hybrids) is determined by the proportion of females in the F_1 progeny. Sample sizes are given in parentheses and are the number of families scored. All copulations were observed.

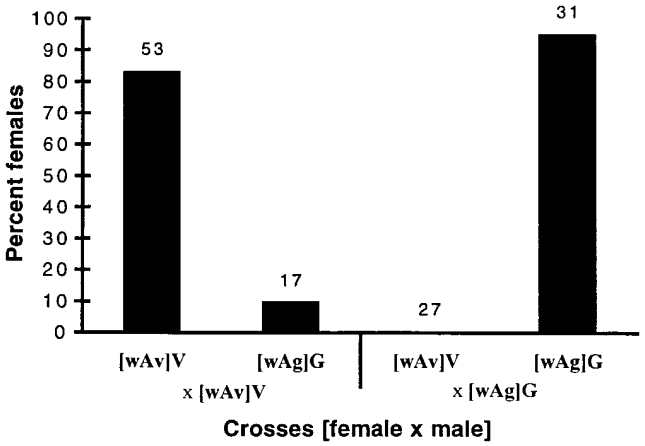

Figure 2.—Bidirectional incompatibility between A Wolbachia types from *N. vitripennis* and *N. giraulti*. Crosses between *w*Av and *w*Ag are incompatible, while self-crosses are compatible. *w*Av and *w*Ag thus constitute two independent incompatibility types in Nasonia. Compatibility is scored according to percent females in F_1 progeny. Sample sizes are the number of families scored.

*w*Bv is complete incompatibility expressed. One reason for this finding could be that the *w*Av-induced sperm modification is partially rescued by the *w*Ag,*w*Bg-infected egg (*i.e.*, *w*Ag-infected eggs can partially rescue the *w*Av-induced sperm modification). If this were the case, then the expectation would be to find significantly higher compatibility levels when *w*Av-infected *N. vitripennis* males are crossed to bi-infected than to uninfected *N. giraulti* females. Results show that this is not the case. No significant differences are found in all replicates of these crosses (Mann-Whitney U, $\alpha = 0.05$). Standard deviation values are high in these cases because of variation in expressivity of CI. When we pool the data from all seven replicates, we again find no significant differences in compatibility levels between these crosses (Mann-Whitney U, $P = 0.335$). Thus, the double infection (*w*Ag,*w*Bg) in the N. *giraulti* egg does not rescue the *w*Av-induced sperm modification in *N. vitripennis*.

Additional crosses were designed to further examine the compatibility relationships of *w*Av and *w*Ag and the variation in their modification and rescue components. In this case, we used an *N. giraulti* line (NGOH206D) that harbors a single A infection (*w*Ag). It is unclear whether this single infection arose independently or through segregation of an ancestral bi-infected line. *w*Av-infected *N. vitripennis* males were crossed to *w*Ag-infected and uninfected *N. giraulti* females. Reciprocally, *w*Ag-infected *N. giraulti* males were crossed to *w*Av-infected and uninfected *N. vitripennis* females. Within-strain crosses were also set up as controls. Figure 2 shows that crosses between the single A variants in their respective host genetic backgrounds yield only 0–10% hybrids, while self-crosses yield normal sex ratios (80–90% females). These data indicate that *w*Av and *w*Ag are bidirectionally incompatible and thus constitute two distinct incompatibility types. Table 6 shows additional results from the same experiment that are consistent with *w*Av acting as a weakly expressing CI variant in *N. vitripennis*. *w*Av in *N. vitripennis* males still induces partial incompatibility (9–10% females) by itself to *w*Ag-infected and to uninfected *N. giraulti* females, while *w*Ag in *N. giraulti* males induces strong interspecies CI (0% females) in crosses to *w*Av-infected and to uninfected *N. vitripennis* females. The findings indicate that *w*Av is a weak CI variant, but induces a sperm modification that cannot be rescued by *w*Ag-infected eggs. Reciprocally, *w*Ag is a strong CI variant but induces a sperm modification that is also not rescued by *w*Av-infected eggs.

Effects of Wolbachia and CI on family size: From the above crosses, data on mean family sizes ± SD were analyzed to address whether Wolbachia influence host fitness in Nasonia and how CI may affect family sizes. The data show two trends (Table 7). First, bi-infected (*w*Ag,*w*Bg) *N. giraulti* females produce more adult offspring than uninfected (0g) *N. giraulti* females in all crosses (Table 7A). This observed fitness difference has implications for how vertically transmitted symbionts coevolve with their hosts. We discuss these implications below.

Second, *N. giraulti* females (bi-infected or uninfected) crossed to *N. vitripennis* males singly infected with *w*Av produce fewer adult offspring than the same females crossed to all other *N. vitripennis* or *N. giraulti* males (Table 7A). Three possibilities can explain this result. First, the effect could be specific to the *w*Av variant. For example, *w*Av induces partial CI, which may cause a reduction in brood size because of an incomplete loss of the paternal chromosomes, resulting in aneuploidy or developmental problems in the offspring (Breeuwer and Werren 1993b). However, reduced adult brood sizes are found only in interspecific crosses rather than in both inter- and intraspecific crosses with *w*Av-infected

TABLE 6

Compatibility relationships between [*w*Av]V and [*w*Ag]G

Crosses (males × females)	n^a	Females (%)[b]
1. [*w*Av]V × [*w*Ag]G	17	10.1 ± 12.7
2. [*w*Av]V × [0g]G	15	8.9 ± 11.6
3. [*w*Av]V × [*w*Av]V	56	82.6 ± 6.5[c]
4. [*w*Ag]G × [*w*Av]V	27	0.0 ± 0.0
5. [*w*Ag]G × [0v]V	44	0.0 ± 0.0
6. [*w*Ag]G × [*w*Ag]G	31	94.9 ± 1.7

Compatibility relationships are measured according to percent females of F_1 progeny. All copulations were observed.

[a] Sample sizes are the number of families scored.

[b] Values are means ± SD.

[c] For Cross 3, one replicate could not be pooled. The mean ± SD for this replicate was 77 ± 11 ($n = 18$).

TABLE 7

(A) Adult family sizes from interspecific and intraspecific crosses

	Ng females		Nv females	
Males	[*w*Ag,*w*Bg]G	[0g]G	[0v]V	Self
[*w*Av,*w*Bv]V	81 ± 13 (5)	65 ± 15 (6)	110 ± 11 (7)	101 ± 9 (7)
[*w*Av]V	57 ± 11 (7)	46 ± 10 (7)	102 ± 10 (7)	97 ± 6 (7)
[*w*Bv]V	78 ± 11 (6)	69 ± 16 (7)	104 ± 13 (7)	94 ± 10 (6)
[0v]V	80 ± 16 (6)	74 ± 9 (3)	109 ± 13 (6)	109 ± 13 (6)
[*w*Ag,*w*Bg]G	95 ± 19 (6)	—	—	—
[0g]G	—	79 ± 15 (7)	—	—

(B) Adult family sizes from intraspecific crosses

	Ng females			
Males	[*w*Ag,*w*Bg]G	[0g]G	[0v]G	Self
[*w*Av,*w*Bv]G	84 ± 36 (14)	79 ± 20 (12)	64 ± 26 (26)	96 ± 19 (17)
[*w*Av]G	57 ± 20 (30)	51 ± 20 (18)	58 ± 29 (31)	85 ± 24 (12)
[*w*Bv]G	97 ± 32 (28)	102 ± 15 (24)	94 ± 26 (23)	91 ± 20 (15)
[0v]G	95 ± 24 (36)	74 ± 9 (37)	94 ± 10 (22)	94 ± 10 (22)

Values are means ± SD. For (A), means are based upon the means of each replicate experiment (*e.g.*, the result of each experiment is taken as a single datum). For (B), means are based upon pooled data from two replicate experiments. Sample sizes are shown in parentheses. Nv, *N. vitripennis*; Ng, *N. giraulti*.

males. Thus, the effect is not intrinsic to *w*Av. The second possibility is that all interspecific crosses between *N. vitripennis* males and *N. giraulti* females yield fewer adult offspring than intraspecific crosses. Although this may be the case, it does not explain why even significantly lower family sizes occur in interspecific crosses with *w*Av-infected *N. vitripennis* males than with bi-infected, wBv-infected, and uninfected *N. vitripennis* males (*t*-tests, $P < 0.01$). The third possibility is that an interaction between *w*Av-induced CI and the hybrid genetic background causes increased mortality of F_1 hybrids, perhaps by inducing aneuploidy.

Effect of host species genotype on interspecific CI: The compatibility tests described above were conducted in different host genetic backgrounds (*e.g.*, *N. vitripennis* and *N. giraulti*). The following experiments were designed to examine the effects of host species genotype on compatibility relationships between the A and B Wolbachia variants harbored in the two species. The *N. giraulti* host nuclear genome was introgressed, by repeated backcrossing, into bi-infected, each mono-infected, and the uninfected *N. vitripennis* cytoplasms. Males from these introgression lines were crossed to bi-infected [*w*Ag,*w*Bg]G, uninfected [0g]G, [0v]G, and same strain females.

Table 8 shows results from these compatibility tests. Crosses with bi-infected (*w*Av,*w*Bv), mono-infected (*w*Bv), and uninfected (0v) *N. giraulti* males yield similar compatibility relationships to crosses with the same Wolbachia infection in *N. vitripennis* males (Tables 2–4). This suggests that the host genome does not influence the expression of CI in these variants. For example, *w*Av,*w*Bv and *w*Bv in both the *N. vitripennis* and *N. giraulti* host genetic backgrounds induce strong CI (0% females) to *w*Ag,*w*Bg-infected *N. giraulti* females. These results confirm that *w*Ag,*w*Bg-infected eggs do not rescue either of the *w*Av,*w*Bv- or *w*Bv-induced sperm modifications. There is thus significant variation in the modification-rescue components of these Wolbachia types.

Introgression of *w*Av into an *N. giraulti* background showed a dramatic change in CI levels. Crosses with these males to bi-infected (*w*Ag,*w*Bg) and uninfected (0g and 0v) *N. giraulti* females yield 0% females, whereas crosses to self-females yield normal sex ratios (Table 8). The results indicate that *w*Av in *N. giraulti* induces complete (or nearly complete) CI, whereas earlier results showed that *w*Av in *N. vitripennis* induces partial CI (*e.g.*, 15.8% females, Table 5). Thus, upon genome replacement of the *N. vitripennis* nuclear background with the *N. giraulti* nuclear background, the strength of CI expression of this *w*Av variant increased to 100%. This finding indicates that the host genome influences the expression of CI.

Additional crosses were conducted to examine compatibility relationships of *w*Av and *w*Ag in a controlled *N. giraulti* genetic background. Results from our prior interspecific crosses indicated that *w*Av in *N. vitripennis* and *w*Ag in *N. giraulti* were bidirectionally incompatible, whereas self-crosses were compatible (Figure 2). Thus, both bacterial strains in their respective host backgrounds induced sperm modifications that could not be rescued by the other. Bidirectional CI also occurs between *w*Av and *w*Ag in the controlled *N. giraulti* host genome. [*w*Av]G males × [*w*Ag]G females and the re-

TABLE 8

Compatibility relationships between different Wolbachia types in a controlled *N. giraulti* nuclear background

	Females			
Males	[*w*Ag,*w*Bg]G	[0g]G	[0v]G	Self
[*w*Av,*w*Bv]G	0.0 ± 0.0 (14)	0.0 ± 0.0 (12)	0.0 ± 0.0 (26)	94.3 ± 2.2 (17)
[*w*Av]G	0.0 ± 0.0 (30)	0.0 ± 0.0 (18)	0.0 ± 0.0 (31)	86.8 ± 27.6 (12)
[*w*Bv]G	0.0 ± 0.0 (28)	0.2 ± 0.8 (24)	0.1 ± 0.4 (23)	93.9 ± 2.9 (15)
[0v]G	93.2 ± 1.8 (36)	93.6 ± 4.4 (37)	84.7 ± 26.8 (22)	84.7 ± 26.3 (22)

Values are means ± SD. Mean percent females (hybrids) is determined from compatibility tests with different *N. vitripennis* and *N. giraulti* Wolbachia types in a controlled *N. giraulti* genetic background. Total sample sizes include pooled data from three replicates that were not significantly different. Sample sizes are given in parentheses and are the number of families scored.

ciprocal cross yield 0% hybrids, whereas self-crosses yield normal sex ratios (Figure 3). These data demonstrate that bidirectional CI between the single A variants is due to differences in the bacteria rather than in the host genomes. *w*Av and *w*Ag constitute two distinct incompatibility types among the two sibling Nasonia species. In addition, our data again support a potential host genetic effect on strength of CI expression of *w*Av. Crosses between *w*Av-infected *N. vitripennis* males and *w*Ag-infected and uninfected *N. giraulti* females typically yield 9–10% hybrids (Table 6), while the same crosses with *w*Av in *N. giraulti* males yield 0% hybrids (Table 9). The findings indicate that the strength of CI induced by *w*Av increased in the *N. giraulti* nuclear background. However, the experiments above were set at different times with slightly modified methods (*e.g.*, single pair observed matings in interspecies crosses versus unobserved group matings in crosses using a controlled nuclear background).

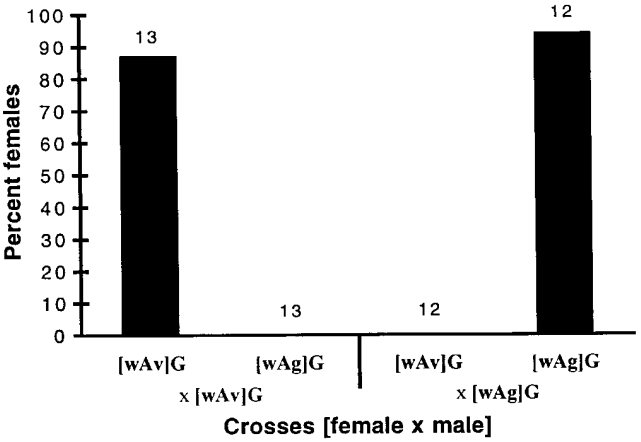

Figure 3.—Bidirectional incompatibility between *w*Av and *w*Ag in a controlled *N. giraulti* genetic background. When placed in the *N. giraulti* background, *w*Av remains bidirectionally incompatible with *w*Ag. However, the strength of CI increases significantly in the new genetic background. This suggests a host genomic effect on the *w*Av CI phenotype. Compatibility is scored according to percent females in F_1 progeny. Sample sizes are the number of families scored.

To confirm host genomic effects on the CI phenotype, we set up and observed crosses at the same time, with both standard [*w*Av]V and introgressed [*w*Av]G lines. Males from both lines were crossed to bi-infected and uninfected *N. giraulti* females. Crosses with *w*Av-infected *N. vitripennis* males yield significantly higher compatibility levels than the same crosses with *w*Av-infected *N. giraulti* males (Figure 4, Mann-Whitney U, $P << 0.001$). The finding supports a host genomic effect on incompatibility levels. This effect could manifest itself through a change in bacterial density or other nuclear genome-Wolbachia interactions.

Family size effects in the *N. giraulti* genome: Previous results showed that [*w*Av]V males induce reduced adult family sizes in crosses with *N. giraulti* females (Table 7A). As seen in Table 7B, [*w*Av]G males also induce reduced adult family sizes in incompatible crosses with *N. giraulti* females. For example, [*w*Ag,*w*Bg]G, [0g]G, and [0v]G females all produce significantly fewer adult offspring when crossed to [*w*Av]G males, than with [0v]G males or [*w*Bv]G males. Each of these is a com-

TABLE 9

Compatibility relationships between [*w*Av]G and [*w*Ag]G

Crosses (males × females)	n^a	Females (%)[b]
1. [*w*Av]G × [*w*Ag]G	13	0.0 ± 0.0
2. [*w*Av]G × [0g]G	10	0.0 ± 0.0
3. [*w*Av]G × [*w*Av]G	13	87.0 ± 26.3
4. [*w*Ag]G × [*w*Av]G	12	0.0 ± 0.0
5. [*w*Ag]G × [0g]G	18	0.0 ± 0.0
6. [*w*Ag]G × [*w*Ag]G	12	93.7 ± 2.4

All crosses were conducted in a controlled *N. giraulti* host genomic background.

[a] Sample sizes are the number of families scored.

[b] Values are means ± SD. Mean percent females was scored according to the percent F_1 female progeny.

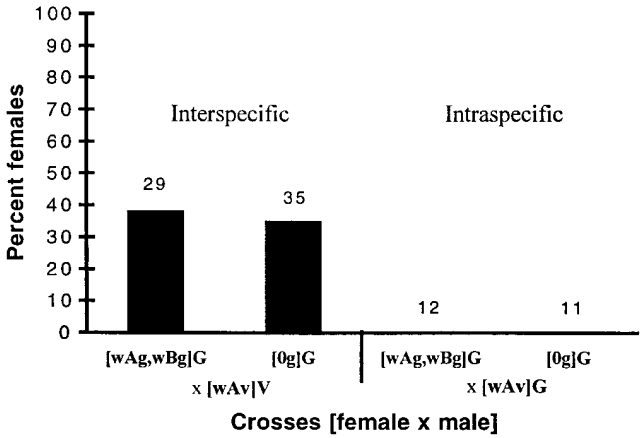

Figure 4.—Increased incompatibility levels of *w*Av in the *N. girault*i nuclear background. *w*Av in *N. vitripennis* typically expresses partial interspecies CI. However, when placed with the *N. girault*i control host genotype, incompatibility levels increased significantly (to 100%). Compatibility is scored according to percent females in F_1 progeny. Sample sizes are the number of families scored.

pletely incompatible cross. However, [*w*Av]G females do not produce smaller family sizes when crossed to self-males (compatible cross) relative to either [*w*Bv]G males (incompatible cross) or [0v]G males (compatible cross). Thus, the effect only happens in incompatible crosses and occurs when sperm from *w*Av-infected males fertilize *N. girault*i eggs. The results indicate that the effect does not require an *N. vitripennis* paternal genome, nor is it dependent upon a hybrid genetic background or partial CI (absent in these crosses). One explanation for the finding is that zygotic lethality occurs due to aneuploidy in these crosses.

Interspecific mating frequencies (IMF): As a result of the experiments above, baseline data on IMF between *N. vitripennis* and *N. girault*i were compiled (Table 10). Data were pooled from inter- and intraspecific crosses with standard lines (*i.e.*, no introgression lines) to make an estimate of IMF. All matings were observed under the same laboratory conditions. IMF values are based upon the percent copulations observed in single pair matings. The average IMF between *N. vitripennis* males × *N. girault*i females was 36% (n = 1297) and *N. girault*i males × *N. vitripennis* females was 53% (n = 208). Self-crosses for *N. vitripennis* and *N. girault*i yielded 98% (n = 852) and 96% (n = 347) mating frequencies. Results indicate significant levels of premating isolation between these two sibling species. Implications for the role of Wolbachia in the evolution of premating isolation are discussed below.

TABLE 10

Interspecies premating isolation

Crosses (male × female)	n^a	Copulations observed (%)[b]
1. Nv × Nv	852	98.4 ± 3.5
2. Nv × Ng	1297	36.2 ± 20.0
3. Ng × Nv	208	53.1 ± 21.7
4. Ng × Ng	347	95.7 ± 7.4

Nv, *N. vitripennis*; Ng, *N. girault*i.

[a] Sample sizes include the total number of observed replicates from all inter- and intraspecific crosses with standard lines.

[b] Values are means ± SD. Mean percent copulations observed is determined by mating observations.

DISCUSSION

Nasonia vitripennis and *N. girault*i naturally harbor double Wolbachia infections that are bidirectionally incompatible. However, it was previously unclear what role the different Wolbachia types play in reproductive isolation between the species. Phylogenetic analysis of Wolbachia based upon the *ftsZ* cell cycle gene shows that the A and B group Wolbachia diverged 58–66 mya (Werren *et al.* 1995b). Both A and B Wolbachia show high levels of horizontal transfer between a number of insect host species (Werren *et al.* 1995b). Given that *N. vitripennis* and *N. girault*i are estimated to have diverged only 250,000–500,000 years ago, it is clear that the A and B bacteria were acquired via horizontal transfer. Current phylogenetic evidence also indicates that the *w*Bv and *w*Bg variants have independent origins (*i.e.*, were acquired by horizontal transfer rather than diverging in the Nasonia species complex). *w*Av and *w*Ag also appear to have independent origins, although the phylogenetic evidence is less strong than for *w*Bv and *w*Bg (Werren *et al.* 1995b). Therefore, it is interesting to know how different the *w*Av and *w*Ag and *w*Bv and *w*Bg modification-rescue systems are. Prior evidence indicated that differences existed in the modification-rescue components of the double Wolbachia infections harbored in the two species. For example, Breeuwer and Werren (1990) showed bidirectional incompatibility causing complete reproductive isolation exists between the double *w*Av,*w*Bv and *w*Ag,*w*Bg Wolbachia infections. However, the differences in the modification-rescue components of the single Wolbachia infections (*e.g.*, *w*Av, *w*Ag, *w*Bv, *w*Bg) and their role in postmating reproductive isolation between the species has remained uncertain. Our results from interspecies compatibility tests with different single / double Wolbachia infections have implications for the origin and evolution of incompatibility types, host-symbiont coevolution, and the role of Wolbachia in speciation.

Results reported here show the following. First, the single A Wolbachia infections harbored in the two species are bidirectionally incompatible. The *w*Ag strain in *N. girault*i expresses a more complete CI phenotype than the *w*Av strain in *N. vitripennis*, and the modification and rescue components of these Wolbachia types are

distinct. These findings support current phylogenetic evidence that suggests that the *w*Av and *w*Ag bacterial strains have independent origins in Nasonia. In addition, our results indicate that these strains constitute two different incompatibility types. Data from CI studies in Drosophila show an analogous result. Three different A Wolbachia strains found in *Drosophila simulans* are all bidirectionally incompatible, and each strain constitutes a separate incompatibility type (O'Neill and Karr 1990; Clancy and Hoffmann 1996). New incompatibility types appear to be evolving rapidly, indicating variation in modification and rescue components among a diversity of Wolbachia strains.

The single *w*Bv infection induces complete (or nearly complete) incompatibility in both species' genomic backgrounds, just as does the double *w*Av,*w*Bv infection. Results show that *w*Bv is at least unidirectionally incompatible with *w*Bg (*i.e.*, *w*Bg-infected egg does not rescue *w*Bv sperm modification) because crosses between *w*Bv males × *w*Ag,*w*Bg females yield 0% hybrids in both nuclear backgrounds. This interpretation presumes no interaction between the *w*Ag and *w*Bg strains in the infected egg that would bias the result. The findings also add support to phylogenetic evidence that suggests the B Wolbachia variants in *N. vitripennis* and *N. giraulti* have independent origins (Werren *et al.* 1996b). It is unclear whether *w*Bg males × *w*Bv females will also yield no hybrids because we have not yet generated a single *w*Bg infection.

The sperm-modification/egg-rescue components of Wolbachia are apparently evolving rapidly. Results suggest that at least four Wolbachia variants (*w*Av, *w*Ag, *w*Bv, *w*Bg) in Nasonia each represent a different incompatibility type. Not only are single *w*Av and *w*Bv infections bidirectionally incompatible within *N. vitripennis* (Perrot-Minnot *et al.* 1996), but double infections harbored in *N. vitripennis* and *N. giraulti* are also bidirectionally incompatible (Breeuwer and Werren 1990). Results now show that the single A Wolbachia variants harbored in the two species are also bidirectionally incompatible and the B Wolbachia variants are at least unidirectionally incompatible with each other. Similarly, within *D. simulans*, *w*Ha, *w*Ri, and *w*No are bidirectionally incompatible and constitute three different A Wolbachia incompatibility types (Clancy and Hoffmann 1996). More studies in Nasonia can further address the question of how many incompatibility types occur and how much variation exists among modification and rescue systems. *Nasonia longicornis* is the third species of the complex that typically occurs in the western United States. This sibling species also harbors A and B Wolbachia, denoted *w*Al and *w*Bl. It is unclear what the compatibility relationships of these strains are, but they may add to the spectrum of incompatibility types in Nasonia. Phylogenetic evidence suggests that *w*Bl and *w*Bg share a relatively recent ancestor, as do *w*Al and *w*Av (Werren *et al.* 1995b).

Do single and double Wolbachia infections occur in natural populations of Nasonia? Although *N. vitripennis* and *N. giraulti* are typically thought to harbor double infections in the wild, single infections have recently been documented in natural populations of Rochester, NY (S. R. Bordenstein and J. H. Werren, unpublished results). It is unclear what frequency of single and double Wolbachia infections occurs in the wild. Nevertheless, this finding clearly has implications on the population biology of Wolbachia in Nasonia, in addition to the effects of polymorphic infections on interspecies incompatibility. Under incompatibility dynamics for polymorphic infections (single and double infections), it can be predicted that double infections will spread to fixation once they reach a threshold frequency. The basic reason is that double infections create novel incompatibility types because they can rescue the sperm modification induced by single infections, whereas single infections cannot rescue the sperm modification induced by double infections. Thus, double Wolbachia infections will spread within a population in a way analagous to unidirectional incompatibility dynamics.

Double infections have implications for the evolution of Wolbachia-mediated reproductive isolation. For example, multiple infections can reinforce interspecies isolation through the addition of incompatibility types, as is likely to be the case in Nasonia. At least between *N. vitripennis* and *N. giraulti*, double infections harbored in each species likely constitute four separate incompatibility types. The double infections are bidirectionally incompatible and prevent gene flow between the species. If the loss of a single infection (or incompatibility type) from one bi-infected species were to occur, gene flow could still be prevented because the remaining infection would maintain bidirectional incompatibility between the species. However, if we imagine the loss of an infection in a mono-infected species, the resulting uninfected individuals would allow for one-way gene flow between the species (*i.e.*, uninfected males are compatible with infected females, but the reciprocal cross is incompatible). Thus, because of the layering of incompatibility types within a host, double infections can reinforce reproductive isolation induced by CI.

Results reported on family sizes have implications for the evolution of bacterial symbionts in insects and the occurrence of CI phenotypes that kill progeny in haplodiploids. Ecological theory predicts that vertically transmitted symbionts will not persist in host populations if they bear a cost to their hosts. The basic reason is that transmission of the symbiont is dependent upon transmission of the host's gametes. There is thus a longstanding view that such symbiotic agents will evolve mutualisms with their hosts. In almost all crosses with *N. giraulti* females, we observed that bi-infected females produce higher fecundities than uninfected females (Table 7). This result is concordant with the view that vertically transmitted symbionts such as Wolbachia may

confer a benefit in Nasonia (Stolk and Stouthamer 1996). We note that further studies are necessary to distinguish whether the fitness difference is due to a Wolbachia-mediated positive effect or to host nuclear genes. In a limited number of other studies, negative host fitness effects attributed to Wolbachia have been documented in *D. simulans* (Hoffmann and Turelli 1988; Poinsot and Mercot 1997), *Tribolium confusum* (Stevens and Wade 1990), and two Trichogramma wasp species (Stouthamer and Luck 1993).

Data from family sizes also suggest that *w*Av-induced CI may cause lethality in haplodiploids. It is well established that CI in diploid species results in reduced offspring numbers because of zygotic lethality. For example, in Drosophila, an incompatible cross yields an 80–90% loss in progeny because of embryo mortality (O'Neill and Karr 1990). In contrast in the haplodiploid genetic system, CI typically manifests itself as all-male progeny rather than zygotic lethality. While the paternal chromatin are lost in an incompatible cross, the maternal egg develops into a haploid male (Reed and Werren 1995). It is therefore interesting to observe CI phenotypes that kill progeny in haplodiploids. Crosses between *w*Av-infected males of either species to bi-infected and uninfected *N. giraulti* females yielded reduced fecundities in comparison to the same crosses with males that are uninfected or harbor other infected cytotypes. We suggested (in results) that an interaction between *w*Av-induced CI and the *N. giraulti* genetic background may explain the reduced adult family sizes, perhaps by causing aneuploidy. Although the total paternal chromatin is typically lost in incompatible crosses, it is possible that incomplete "imprinting" of paternal chromosomes may result in aneuploidy following CI, leading to lethality. Further studies are necessary to confirm whether CI induced by *w*Av Wolbachia causes aneuploidy in *N. giraulti* embryos.

The role of host genotype in Wolbachia-induced CI has not been widely investigated. An early empirical study showed that genomic replacement via continuous backcrosses between a pair of *Culex pipiens* strains had no influence on incompatibility (Laven 1959). Boyle *et al.* (1993) found by microinjection that Wolbachia from *D. simulans* expressed lower compatibility levels in *D. melanogaster* and attributed this to host genomic effects. Breeuwer and Werren (1993a) introgressed the double infection from *N. vitripennis* into an *N. giraulti* nuclear background and established that bidirectional incompatibility between the species (*i.e.*, between *w*Av, *w*Bv and *w*Ag,*w*Bg) was not due to an interaction with the host species genome. Here we follow up on those studies by showing that bidirectional incompatibility between *w*Av and *w*Ag, and at least unidirectional incompatibility between *w*Bv and *w*Ag,*w*Bg, still occur in a controlled *N. giraulti* genetic background. However, we did find an effect on levels of CI expression. *w*Av typically expressed partial incompatibility in the *N. vitripennis* background (10–30% hybrids) and complete (or nearly complete) incompatibility in the *N. giraulti* background (0% hybrids). We believe that the host genome may be influencing the expression of CI of this particular A Wolbachia strain. Specifically, the host genome could cause an increase in CI via two ways. First, bacterial densities may increase in the new genetic background. Breeuwer and Werren (1993b) showed that there is a positive association between bacterial densities and the strength of CI. Results suggested that complete expression of CI is dependent on a threshold level of bacterial densities. For example, bacterial densities may have increased in the new *N. giraulti* nuclear background and thus caused an increase in incompatibility levels. Second, there may be a direct effect on the expression of CI through a Wolbachia-nuclear genome interaction, possibly because of selection on the host or symbiont. For example, *N. vitripennis* nuclear genes may ameliorate the effects of *w*Av-induced CI by suppressing the sperm modification component. Such selection is expected if infection polymorphisms occur in nature because males that can suppress Wolbachia function will be compatible with more females. It is still possible, however, that stochastic changes in bacterial densities during the introgression scheme (rather than host genomic influences) are responsible for the increased CI expression of *w*Av in *N. giraulti*. Other cases of partial CI have been documented in *D. simulans* and *D. melanogaster*, which also harbor A-type Wolbachia (Hoffmann 1988; Mercot *et al.* 1995).

Our results on interspecific mating frequencies are interesting and have potential implications for the role of Wolbachia in speciation. We documented that premating isolation occurs in these lines. Although individuals in self-crosses mated readily, *N. vitripennis* males copulated with *N. giraulti* females in 36% of the observed replicates. In the reciprocal cross, copulations occurred in 53% of the observed replicates. It is unclear whether Wolbachia-induced CI has facilitated the evolution of this premating isolation. One could imagine that postmating isolation caused by CI can drive the evolution of premating isolation via natural selection (*e.g.*, reinforcement). This area of research has been unexplored both theoretically and empirically.

The Nasonia species complex remains an excellent system for studying whether Wolbachia can facilitate a speciation event. Resolving whether Wolbachia-induced CI can prevent gene flow between diverging populations and promote the evolution of isolating mechanisms in natural populations is a relevant question. CI could be a primary cause of reproductive isolation or a contributing factor between diverging populations (Werren 1997b). This study shows that the variation in modification-rescue systems, the number of incompatibility types, and single and double infections can all, in principle, contribute to Wolbachia-mediated reproductive isolation. The occurrence of double infections, by the addi-

tion of incompatibility types, is likely to be especially important in strengthening interspecies reproductive isolation induced by CI. This appears to be the case for the Nasonia species complex. Further investigations of this and other systems will help to clarify to what extent Wolbachia facilitate the evolution of reproductive isolation and therefore promote speciation.

We thank Mark Drapeau, John Jaenike, Corbin Jones, Bryant McAllister, Howard Ochman, and Allen Orr for stimulating discussions and comments. We thank Celina Arboleda, Vincent Calhoun, and Renee Goodwin for technical assistance. This research was supported by a National Science Foundation grant DEB 9707665 to J.H.W.

LITERATURE CITED

Boyle, L., S. L. O'Neill, H. M. Robertson and T. L. Karr, 1993 Inter- and intraspecific horizontal transfer of Wolbachia in Drosophila. Science **260:** 1796–1799.

Breeuwer, J. A. J., and J. H. Werren, 1990 Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature **346:** 558–560.

Breeuwer, J. A. J., and J. H. Werren, 1993a Effect of genotype on cytoplasmic incompatibility between two species of Nasonia. Heredity **70:** 428–436.

Breeuwer, J. A. J., and J. H. Werren, 1993b Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis.* Genetics **135:** 565–574.

Breeuwer, J. A. J., R. Stouthamer, D. A. Burns, D. A. Pelletier, W. G. Weisburg *et al.,* 1992 Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid wasp genus Nasonia (Hymenoptera: Pteromalidae) based on 16s ribosomal DNA sequences. Insect Mol. Biol. **1:** 25–36.

Callaini, G., R. Dallai and M. G. Riparbelli, 1997 Wolbachia-induced delay of paternal chromatin condensation does not prevent maternal chromosomes from entering anaphase in incompatible crosses of *Drosophila simulans.* J. Cell Sci. **110:** 271–280.

Caspari, E., and G. S. Watson, 1959 On the evolutionary importance of cytoplasmic sterility in mosquitoes. Evolution **13:** 568–570.

Clancy, D. J., and A. A. Hoffmann, 1996 Cytoplasmic incompatibility in *Drosophila simulans:* evolving complexity. Trends in Ecol. and Evol. **11:** 145–146.

Coyne, J. A., 1992 Genetics and speciation. Nature **355:** 511–515.

Hoffmann, A. A., 1988 Partial cytoplasmic incompatibility between two Australian populations of *Drosophila melanogaster.* Entomol. Exp. Appl. **48:** 61–67.

Hoffmann, A. A., and M. Turelli, 1988 Unidirectional incompatibility in *Drosophila simulans*: inheritance, geographic variation, and fitness effects. Genetics **119:** 435–444.

Johanowicz, D. L., and M. A. Hoy, 1995 Molecular evidence for A-Wolbachia endocytobiont in the predatory mite *Metaseiulus occidentalis.* J. Cell. Biochem. **21A:** 198.

Kose, H., and T. L. Karr, 1995 Organization of *Wolbachia pipientis* in the Drosophila fertilized egg and embryo revealed by an anti-Wolbachia monoclonal antibody. Mech. Dev. **51:** 275–288.

Laven, H., 1959 Speciation by cytoplasmic isolation in the *Culex pipiens* complex. Cold Spring Harbor Symp. Quant. Biol. **24:** 166–173.

Mercot, H., B. Llorente, M. Jacques, A. Atlan and C. Montchamp-Moreau, 1995 Variability within the seychelles cytoplasmic incompatibility system in *Drosophila simulans.* Genetics **141:** 1015–1023.

O'Neill, S. L., and T. L. Karr, 1990 Bidirectional incompatibility between conspecific populations of *Drosophila simulans.* Nature **348:** 178–180.

O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr and H. M. Robertson, 1992 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA **89:** 2699–2702.

Perrot-Minnot, M. J., L. R. Guo and J. H. Werren, 1996 Single and double Wolbachia infections. Genetics **143:** 961–972.

Poinsot, D., and H. Mercot, 1997 Wolbachia infection in *Drosophila simulans:* does the female bear a physiological cost? Evolution **51:** 180–186.

Reed, K. M., and J. H. Werren, 1995 Induction of paternal genome loss by the paternal-sex-ratio chromosome and cytoplasmic incompatibility bacteria (Wolbachia): a comparative study of early embryonic events. Mol. Reprod. Devel. **40:** 408–418.

Rousset, F., and M. Solignac, 1995 Evolution of single and double Wolbachia symbioses during speciation in the *Drosophila simulans* complex. Proc. Natl. Acad. Sci. USA **92:** 6389–6393.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault and M. Solignac, 1992 Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. Ser B **250:** 91–98.

Ryan, S. L., and G. B. Saul, 1968 Post-fertilization effect of incompatibility factors in Mormoniella. Mol. Gen. Genet. **103:** 29–36.

Sinkins, S. P., H. R. Braig and S. L. O'Neill, 1995 Wolbachia superinfections and expression of cytoplasmic incompatibility. Proc. R. Soc. Lond. Ser B **261:** 325–330.

Sironi, M., C. Bandi, L. Sacchi, B. D. Sacco, G. Damiani *et al.,* 1995 Molecular evidence for a close relative of the arthropod endosymbiont Wolbachia in a filarial worm. Mol. Biochem. Parasit. **74:** 223–227.

Stevens, L., and M. J. Wade, 1990 Cytoplasmically inherited reproductive incompatibility in Tribolium flour beetles: the rate of spread and effect on population size. Genetics **124:** 367–372.

Stolk, C., and R. Stouthamer, 1996 Influence of a cytoplasmic incompatibility-inducing Wolbachia on the fitness of the parasitoid wasp *Nasonia vitripennis.* Proceedings of Exp. and Appl. Ent., N.E.V. Amsterdam **7:** 33–37.

Stouthamer, R., and R. F. Luck, 1993 Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretosum.* Entomol. Exp. Appl. **67:** 183–192.

Stouthamer, R., J. A. L. Breeuwer, R. F. Luck and J. H. Werren, 1993 Molecular identification of microorganisms associated with parthenogenesis. Nature **361:** 66–68.

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

Turelli, M., and A. A. Hoffmann, 1991 Rapid spread of an inherited incompatibility factor in California Drosophila. Nature **353:** 440–442.

Wade, M. J., and N. W. Chang, 1994 Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite Wolbachia. Nature **373:** 72–74.

Werren, J. H., 1997a Biology of Wolbachia. Ann. Rev. Entomol. **42:** 587–609.

Werren, J. H., 1997b Wolbachia and speciation, in *Endless Forms: Species and Speciation*, edited by D. Howard and S. Berlocher. Oxford University Press.

Werren, J. H., L. R. Guo and D. W. Windsor, 1995a Distribution of Wolbachia in neotropical arthropods. Proc. R. Soc. Lond. Ser B **262:** 197–204.

Werren, J. H., L. R. Guo and W. Zhang, 1995b Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. Proc. R. Soc. Lond. Ser B **261:** 55–71.

Whiting, A. R., 1967 The biology of the parasitic wasp *Mormoniella vitripennis.* Q. Rev. Bio. **42:** 333–406.

Yen, J. H., and A. R. Barr, 1971 New hypothesis of the cause for cytoplasmic incompatibility in *Culex pipiens.* Nature **232:** 657–658.

Communicating editor: J. Hey