Host Genotype Determines Cytoplasmic Incompatibility Type in the Haplodiploid Genus Nasonia

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ABSTRACT

In haplodiploid species, Wolbachia-induced cytoplasmic incompatibility (CI) can be expressed in one of two ways: as a "conversion" of diploid fertilized eggs into haploid males or as embryonic mortality. Here we describe CI-type variation within the parasitic wasp genus Nasonia and genetically analyze the basis of this variation. We reach four main conclusions: (i) CI is expressed primarily as conversion in *N. vitripennis*, but as embryonic mortality in the sibling species *N. girault*i and *N. longicornis*; (ii) the difference in CI type between *N. girault*i (mortality) and *N. vitripennis* (conversion) is determined by host nuclear genotype rather than by Wolbachia differences; (iii) *N. vitripennis* "conversion genes" are recessive in hybrid females; and (iv) a difference in CI level between the sibling species *N. girault*i and *N. longicornis* is due to the different Wolbachia infections in the species rather than to the host genotype. These results show that host nuclear genes can influence the type of CI present in a species. On the basis of these findings, we propose a model for how different CI types evolve in haplodiploids due to selection on nuclear genes modifying CI.

WOLBACHIA are a group of cytoplasmically inherited bacteria with an unparalleled host range among bacterial endosymbionts, infecting at least 20% of all insect species as well as other invertebrates, including mites, isopods, and filarial nematodes (Werren *et al.* 1995a; reviewed in Werren 1997 and Stouthamer *et al.* 1999). These bacteria are obligately intracellular and typically infect the reproductive tissues of their host. In arthropods, two major subdivisions of Wolbachia (A and B) diverged ~60 million years ago (MYA; Werren *et al.* 1995b; Zhou *et al.* 1998). These two groups are responsible for various alterations in arthropod reproduction, including parthenogenesis in wasps (Stouthamer *et al.* 1993) and mites (Weeks and Breeuwer 2001), feminization in isopods (Martin *et al.* 1973), male killing in insects (Hurst and Jiggins 2000), and cytoplasmic incompatibility (CI), which has been described in a number of arthropod species (Yen and Barr 1971; Wade and Stevens 1985; Hoffmann and Turelli 1988; Breeuwer and Werren 1990).

CI is the most common effect of Wolbachia. It is a sperm-egg incompatibility that results when an infected male mates with an uninfected female; reciprocal and self-crosses, however, are compatible. Cytologically, although sperm from an infected male fertilizes the uninfected egg, entry of the male pronucleus into mitosis is delayed (Tram and Sullivan 2002) and the paternal chromosomes consequently undergo improper condensation during early mitotic divisions of the egg (O'Neill and Karr 1990; Reed and Werren 1995). This alteration in paternal chromosome behavior ultimately leads to embryonic death in diploids (Laven 1959; Hoffmann and Turelli 1988) and to either increased male production or embryonic death in haplodiploids (Ryan and Saul 1968; Breeuwer and Werren 1990; Breeuwer 1997; Vavre *et al.* 2000). The outcome of incompatibility is a fitness decrease for uninfected females when they mate to infected males. Because infected females (the transmitting sex) are compatible with either infected or uninfected males, they do not suffer this fitness reduction, and CI can therefore rapidly spread Wolbachia through host populations (Turelli and Hoffmann 1991; Turelli 1994).

While CI is clearly advantageous to the spread of Wolbachia, it can have a severe cost to the host. Incompatibility renders infected males and uninfected females at a selective disadvantage, since their gametes are effectively wasted in incompatible crosses. Thus, conflict and coevolution between the Wolbachia and host genomes may spawn genetic interactions between the two parties, ultimately leading to the evolution of host genotypic influences on Wolbachia and its associated phenotypes. Currently a handful of studies have shown host-Wolbachia interactions (Boyle *et al.* 1993; Bordenstein and Werren 1998; Karr 2000; Fujii *et al.* 2001; McGraw *et al.* 2001; Poinsot and Mercot 2001). In these studies, Wolbachia are typically "moved" from the resident-spe-

This article is dedicated to the memory of the late George Saul, who pioneered studies of cytoplasmic incompatibility in Nasonia.

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cies background into a naïve or foreign genetic background by either micro-injection or backcrossing methods. Wolbachia-induced phenotypes are then characterized in this new genetic background. The most common effect described in these studies is a change in CI level when the Wolbachia are in a foreign genetic background (Boyle *et al.* 1993; Bordenstein and Werren 1998; McGraw *et al.* 2001; Poinsot and Mercot 2001). Other Wolbachia-host genotypic interactions include rescue of a Drosophila lethal mutation (Karr 2000) and a phenotypic switch from feminization to male killing when a Wolbachia strain is experimentally transferred from one Lepidopteran species to another (Fujii *et al.* 2001). All these effects reflect "epistatic" interactions between the Wolbachia and the host genome in infected individuals. Characterization and genetic dissection of host nuclear genotypic influences will help us to better understand the genetic machinery underlying Wolbachia-induced reproductive alterations and the potential coevolutionary interactions between Wolbachia and hosts.

The mechanics of CI have been described to an extent in both diploids and haplodiploids. There appear to be two components of CI. The first occurs as a bacterial modification of the sperm and the second as a bacterial rescue in fertilized eggs (Werren 1997). For example, compatibility in a cross between two individuals infected with the same Wolbachia type occurs because Wolbachia in the fertilized egg "rescues" the modification of the paternal chromosomes. However, in crosses between infected males and uninfected females (unidirectional CI) or females harboring a different Wolbachia strain from that in the male (bidirectional CI), the paternal chromosome modification is not rescued, resulting in CI.

Unlike diploids, in which CI results in embryonic death, haplodiploids show two types of CI expression. For example, in the parasitic wasp *Nasonia vitripennis*, CI causes complete loss of the paternal chromosomes in fertilized eggs (Ryan and Saul 1968; Breeuwer and Werren 1990; Reed and Werren 1995), resulting in haploidization of the egg and its conversion from female development (diploid state) to male development (haploid state). All-male families with little or no embryonic death therefore characterize CI in this species (Saul 1961; Perrot-Minnot *et al.* 1996). These findings were taken to be reflective of how CI would be expressed in other haplodiploid species; this forecast did not turn out to be the case. Recent studies by Breeuwer (1997) and Perrot-Minnot *et al.* (2002) in the spider mite Tetranychus and by Vavre *et al.* (2000) in the hymenopteran Drosophila parasitoid, *Leptopolina heterotoma*, describe a different CI type. In these species, incompatibility primarily results in F_1 mortality of fertilized eggs, similar to incompatibility in diplodiploid species. Therefore, at least two types of CI can occur in haplodiploids: female conversion to males or mortality. The discovery of two CI types in haplodiploid organisms has raised new questions about the evolutionary trajectory of CI-Wolbachia in haplodiploids (Vavre *et al.* 2000). Perhaps the most important question is how the two different CI types (conversion and mortality) evolve.

In this article, our aim is to describe and genetically analyze the levels and types of CI within Nasonia, a genus of three closely related species that has become a model system for research on CI and Wolbachia-associated speciation. *N. giraulti* and *N. longicornis* are sister species that diverged ~0.2 MYA, and their common ancestor diverged from *N. vitripennis* ~0.8 MYA (Campbell *et al.* 1993). All three species are doubly infected with their own species-specific A and B Wolbachia, and studies show unidirectional incompatibility within the species (Saul 1961; Perrot-Minnot *et al.* 1996) and bidirectional incompatibility between the species (Breeuwer and Werren 1990; Bordenstein and Werren 1998; Bordenstein *et al.* 2001). Sequence analysis indicates that the A Wolbachia of all three species are not closely related (Werren *et al.* 1995b; T. Van Opijnen, E. Baudry, J. D. Bartos and J. H. Werren, unpublished data), suggesting independent acquisition of these Wolbachia by horizontal transmission. Similarly, the B Wolbachia of *N. vitripennis* and *N. longicornis* are not closely related, but *N. longicornis* and *N. giraulti* Wolbachia do show a close relationship based on *ftzZ* (Werren *et al.* 1995b) and *wsp* gene sequences (Werren and Bartos 2001).

We show here that CI type differs between *N. vitripennis* (conversion) and *N. giraulti* and *N. longicornis* (mortality). This difference in CI type between the species is determined by the insect nuclear genotype, whereas differences in CI level between *N. giraulti* and *N. longicornis* are determined by the Wolbachia strain.

MATERIALS AND METHODS

Nomenclature: Nasonia strains of all three species are naturally doubly infected with Wolbachia from the two major arthropod subdivisions, A and B (Bordenstein *et al.* 2001). Double infections are indicated in brackets by a *w* for Wolbachia, A and B for the infection group, and lowercase v, g, or l, which corresponds to the Wolbachia strain derived from the host species. A "0" indicates an uninfected host. Host genotype is indicated by a capital V, G, or L (*N. vitripennis*, *N. giraulti*, and *N. longicornis*, respectively) outside the brackets. For example, [*w*Ag,*w*Bg]G symbolizes the *N. giraulti* A and B Wolbachia variant in a *N. giraulti* host.

Strains: A total of six insect strains, which included two (infected and uninfected) from each of the three species *N. giraulti*, *N. longicornis*, and *N. vitripennis*, were used to test cytoplasmic incompatibility. Each strain was maintained in constant light at 25° and was raised on fresh fly pupae (*Sarcophaga bullata*).

Two *N. giraulti* strains were used: [*w*Ag,*w*Bg]G, a double-infected lab strain (named RV2), and [0g]G, an uninfected strain derived from RV2 by antibiotic treatments (established in 1996). Similarly for *N. longicornis*, [*w*Al,*w*Bl]L is a double-infected lab strain (IV7) and [0l]L is an uninfected strain derived from IV7 (in 1996) by antibiotic treatment. *N. vitri-*

pennis, denoted as [*w*Av,*w*Bv]V, is a double-infected strain (R511) and the uninfected [0v]V strain was derived from wild-type strain R511 by spontaneous loss following diapause (Perrot-Minnot *et al.* 1996).

Crossing design: All crosses were set up as single pair matings between virgin females and virgin males. Males and females were collected as pupae. Individual female and male adults were paired and observed for 10–15 min. Only those pairings where copulation occurred were used. After 24 hr, the males were discarded and each female was provided with four hosts and a drop of honey for feeding. After 48 hr, the females were transferred to new vials and given a single host for 6 hr. During this period, females were limited in their access to the head of the host by use of a foam plug that encapsulated the rest of the host. In this way, eggs of ovipositing females could be easily found (on the head of the host) and counted. For crosses where just adult counts were done, no foam plug was used and the female had access to any part of the host for stinging. After 6 hr, the female was discarded from each vial and the samples were used to score eggs and adults. Eggs were scored immediately and adults were scored at death for sex and total family size.

In a different experiment, the timing of mortality was investigated in *N. giraulti* crosses. Following the same treatment as above, sets of hosts were examined for the number of eggs, unhatched eggs (48 hr later), and young, yellow pupae (at days 9 and 10). Adult numbers were scored following host emergence and death. Unhatched eggs at 48 hr indicate embryonic mortality, because hatching typically occurs by 36 hr at 25°.

For the characterization of CI type in F_1 hybrid females, we changed a few aspects of the crossing design. Because F_2 hybrid males suffer from severe hybrid lethality (Breeuwer and Werren 1995), we altered the egg-laying periods to increase the numbers of surviving (and therefore emerging) offspring. Instead of allowing females to feed and to lay eggs on four hosts for 48 hr and then lay eggs on a single host for a 6-hr period, females were set on two hosts for 24 hr and then immediately transferred to a second set of two fresh hosts for an additional 24 hr. Females were then taken off the second set of hosts and discarded. Data analysis and summary data are based upon the total offspring produced in both ovipositioning periods.

Introgression lines: Differences in CI levels or type between the three species were observed. We designed experiments to test if these species-level differences were due to differences in Wolbachia or in the host genetic backgrounds of the species. We generated introgression lines that harbor the Wolbachia of one species in the genetic background of the paternal species. These introgression lines were generated by backcrossing six or more generations of hybrid females to males of the other species. This design theoretically should result in at least a 98% genome replacement and in the retaining of the cytoplasm of the parental female (infected or uninfected). Crosses with these introgression lines were set up according to the methods described above. The actual percentage of genome replacement may be different from the theoretical prediction due to stochasticity and genetic incompatibilities that prevent the movement of some genes into a foreign species background. Severe genetic incompatibilities are known to occur in *N. vitripennis-N. giraulti* F_2 hybrid males (Breeuwer and Werren 1995); however, these incompatibilities tend to be recessive and their presence favors genome replacements during introgression due to selection against recombinant genotypes.

Calculating the percentage of conversion CI and the percentage of mortality CI: We estimated the number of eggs that experience CI in an incompatible cross and the percentage of these eggs that get converted or die. The percentage of conversion CI is the percentage of eggs that experience CI and get converted. It is calculated by dividing the difference between the mean number of males in the incompatible cross and the mean number of males in the compatible cross by the difference between the mean number of females in the compatible cross and mean number of females in the incompatible cross; the percentage mortality CI equals 100% minus the percentage conversion CI.

Statistical analysis: We present descriptive statistics and significance values from Mann-Whitney *U*-tests using MINITAB 11.0 and 12.23. Summary data are shown as means ± standard deviations. Sample sizes, which are the number of families scored in a cross, are denoted as *N*.

RESULTS

We report a set of interconnected experiments that are used to investigate the bases of CI differences between Nasonia species. To facilitate interpretation of results, the key findings of all the experiments are summarized in Table 1 and described in more detail below.

Intraspecific CI: *CI in N. vitripennis:* In *vitripennis*, incompatibility results in the production of all-male families (Perrot-Minnot *et al.* 1996; Table 2). Experiments show a dramatic decrease in the number of adult females produced in the incompatible cross relative to the compatible cross (means: 0.0 *vs.* 22.0, $P < 0.0001$). However, the decrease in adult females is not due to mortality because there is a large increase in the number of adult males (23.8 *vs.* 1.6, $P < 0.0001$), matching the decline in the number of females. This CI-induced male increase is caused by the conversion of diploid fertilized eggs to haploid (male) eggs by paternal genome loss (Ryan *et al.* 1985; Reed and Werren 1995). As a result, the adult family sizes in the incompatible cross were not significantly different from the adult family sizes in the compatible cross (23.8 *vs.* 23.6, $P = 0.93$). However, small amounts of mortality were found in *vitripennis* when we compared the number of eggs laid to the number of surviving adults in incompatible crosses (28.3 *vs.* 23.8, $P = 0.01$), but not compatible ones (26.4 *vs.* 23.6, $P = 0.15$). Thus, while there may be a slight increase in mortality, the primary effect of CI in *vitripennis* is complete paternal genome loss and conversion of fertilized diploid eggs to haploid eggs that develop into males.

CI in N. giraulti: Incompatible crosses within *giraulti* show a different pattern (Table 2). Comparing adult family sizes in the incompatible and compatible cross reveals a significant decrease in family sizes (3.9 *vs.* 22.7, $P < 0.0001$). The compatible cross showed a small, but marginally significant, decrease in the numbers of eggs relative to surviving adults (24.8 eggs *vs.* 22.7 adults, $P = 0.06$), whereas the incompatible cross revealed a severe and strongly significant decrease (23.2 eggs *vs.* 3.9 adults, $P < 0.0001$). Evidence of high levels of mortality was also found in comparisons of numbers of adult males and females in the two crosses. The number of females declined significantly to zero in the incompati-

TABLE 1

Summary of results

Strain	Cross (infected male × uninfected female)	Phenotype	Conclusion
		CI type	
Parental	*N. vitripennis* × self	Conversion	CI type varies between the species
	N. giraulti × self	Mortality	
	N. vitripennis × *N. giraulti*	Conversion	The *N. vitripennis* genotype determines CI type
	N. giraulti × *N. vitripennis*	Conversion	
F_1	*N. giraulti* × F_1 hybrid	Mortality	*N. vitripennis* female conversion gene(s) are recessive
Introgression	[*N. vitripennis* Wolbachia] *N. giraulti* × *N. giraulti*	Mortality	The *N. vitripennis* Wolbachia are not sufficient to induce conversion CI in *N. giraulti*
		CI level	
Parental	*N. giraulti* × self	Strong	CI level varies between the species
	N. longicornis × self	Moderate	
	N. giraulti × *N. longicornis*	Strong	CI level parallels that of the male cytogenotype
	N. longicornis × *N. giraulti*	Moderate	
Introgression	[*N. giraulti* Wolbachia] *N. longicornis* × *N. longicornis*	Strong	CI-level variation is determined by properties of the Wolbachia rather than by those of host genotype
	[*N. longicornis* Wolbachia] *N. giraulti* × *N. giraulti*	Moderate	

ble cross (0.0 *vs.* 21.9, $P < 0.0001$), and in contrast to *vitripennis* incompatibility, there was no similarly sized increase in the number of males (3.9 *vs.* 0.9, $P < 0.0001$). The pattern shows that fertilized eggs (females) are mostly dying in the incompatible cross. Severe mortality is therefore the primary effect of CI within *giraulti*.

We next determined the stage of development at which CI-induced mortality occurs in *giraulti* (Table 3). Several developmental stages (eggs, larvae, pupae, and adults) were examined. Results indicate that severe mortality occurs between the egg and first instar larval stage, during embryonic development (19.6 eggs *vs.* 4.9 hatched larvae, $P < 0.0001$). Embryonic mortality is estimated to be 75%, which parallels the 83% mortality estimated from scoring just egg and adult numbers in previous crosses (Table 1). Other stages of development, pupae and adults, did not show similar levels of mortality. The control cross (uninfected male × uninfected female) yielded normal family sizes and female-biased sex ratios.

CI in N. longicornis: In *longicornis* the pattern of CI is similar to that of its sister species, *giraulti* (Table 2). There is a significant decrease in adult family sizes from the compatible and incompatible cross (19.7 *vs.* 4.3,

TABLE 2

Compatible and incompatible intraspecific crosses in Nasonia

Cross (male × female)	No. of eggs	No. of adults	No. of males	No. of females	Sex ratio (% females)
N. vitripennis					
[0v]V × [0v]V	26.4 ± 6.4 (35)	23.6 ± 7.3 (31)	1.6 ± 0.9	22.0 ± 7.0	93.2 ± 7.2
[*w*Av,*w*Bv]V × [0v]V	28.3 ± 5.5 (35)	23.8 ± 6.8 (36)	23.8 ± 6.8	0.0 ± 0.0	0.0 ± 0.0
N. giraulti					
[0g]G × [0g]G	24.8 ± 6.2 (42)	22.7 ± 7.0 (36)	0.9 ± 0.7	21.9 ± 6.8	96.2 ± 3.5
[*w*Ag,*w*Bg]G × [0g]G	23.2 ± 7.0 (42)	3.9 ± 2.5 (38)	3.9 ± 2.5	0.0 ± 0.0	0.0 ± 0.0
N. longicornis					
[0l]L × [0l]L	21.2 ± 5.8 (33)	19.7 ± 4.8 (32)	1.2 ± 0.6	18.5 ± 4.6	94.0 ± 5.2
[*w*Al,*w*Bl]L × [0l]L	22.6 ± 6.3 (33)	4.3 ± 2.4 (35)	3.5 ± 2.1	0.8 ± 1.0	33.2 ± 4.1

TABLE 3

CI-induced mortality occurs during embryonic development in *N. giraulti*

Cross (male × female)	Eggs	Hatched larvae	Pupae	Adults
[0g]G × [0g]G	21.4 ± 5.5 (25)	21.3 ± 0.3 (35)	16.8 ± 5.4 (30)	17.9 ± 5.3 (28)
[*w*Ag,*w*Bg]G × [0g]G	19.6 ± 8.1 (25)	4.9 ± 3.6 (35)	3.0 ± 1.7 (30)	3.3 ± 2.3 (32)

Number of hatched larvae is extrapolated by subtracting the mean number of unhatched eggs (48 hr after hosting) from the mean number of total eggs.

$P < 0.0001$), which is primarily due to a severe decrease in the number of adult females (18.5 *vs.* 0.8, $P < 0.0001$). Egg and adult numbers for the compatible cross do not differ (21.2 eggs *vs.* 19.7 adults, $P = 0.17$), while the same comparison for the incompatible cross revealed a dramatic decrease in numbers from eggs to adults (22.6 eggs *vs.* 4.3 adults; $P < 0.0001$). These findings confirm that, like *giraulti*, mortality is the cause of the decrease in family size in incompatible crosses of *N. longicornis*. However, in contrast to both *giraulti* and *vitripennis*, some females are produced in incompatible crosses (0.8 adult females per family, 4% of the females produced in compatible crosses).

Analysis of CI-level variation between *N. giraulti* and *N. longicornis*: In addition to CI-type differences among *vitripennis*, *giraulti*, and *longicornis*, there are also differences in level of CI between *longicornis* (incomplete CI) and *giraulti* (complete CI). To distinguish whether these CI-level differences are due to the different types of Wolbachia present in the two species or to host genotype-Wolbachia interactions, we characterized CI levels in both interspecific crosses and crosses using introgression lines that contained the cytoplasm (Wolbachia-infected and uninfected) of one species and the nuclear background of the other species. Males and females from these introgression lines were used to determine whether they expressed the CI level of their Wolbachia or nuclear background.

Interspecific crosses: Table 4 shows that compatible crosses between a *longicornis* male and *giraulti* female yield larger family sizes than those yielded by incompatible crosses (20.7 *vs.* 5.3, $P < 0.0001$). Comparisons of the number of eggs to the number of adults within the compatible (22.5 eggs *vs.* 20.7 adults, $P = 0.1481$) and incompatible (21.1 eggs *vs.* 5.3 adults, $P < 0.0001$) crosses also show that mortality is the primary effect of CI in the interspecies cross *longicornis* male × *giraulti* female. As in the *longicornis* intraspecific cross, the number of females declined dramatically, but not completely (3.1 *vs.* 19.5, $P < 0.0001$), indicating that properties of the male cytogenotype determine CI level.

Similar results were found in the reciprocal interspecific cross: *giraulti* male × *longicornis* female. There was a severe reduction in total adult family sizes between the compatible and the incompatible cross (24.3 *vs.* 6.5, $P < 0.0001$). Likewise, there was a significant difference between the numbers of eggs laid and the surviving adults in the incompatible cross (26.0 eggs *vs.* 6.5 adults, $P < 0.0001$). As in interspecific crosses using *giraulti* males, zero females were produced in the interspecific cross, indicating that male cytogenotype is the determinant of the CI level.

Crosses with N. giraulti-N. longicornis introgression lines: To more precisely determine whether the variation in incompatibility level (*e.g.*, number of females produced) arises from differences in the Wolbachia or in the host genetic background, we performed crosses with introgression lines containing the cytoplasm (infected and uninfected) of one species and nuclear background of the other species.

Results are summarized in Table 5. As in its normal *giraulti* genetic background (cross 2), the *giraulti* Wolbachia in the *longicornis* genetic background (cross 4) express nearly complete CI. The average number of females produced in the incompatible cross is 0.0 (0.0% of the normal number of females) using the introgres-

TABLE 4

CI level in interspecific crosses between *N. giraulti* and *N. longicornis*

Cross (male × female)	No. of eggs	No. of adults	No. of males	No. of females	Sex ratio
N. giraulti females					
[0l]L × [0g]G	22.5 ± 6.4 (42)	20.7 ± 7.1 (44)	1.2 ± 0.8	19.5 ± 7.0	93.6 ± 7.5
[*w*Al,*w*Bl]L × [0g]G	21.1 ± 6.5 (42)	5.3 ± 4.7 (39)	2.2 ± 1.5	3.1 ± 4.6	58.5 ± 35.9
N. longicornis females					
[0g]G × [0l]L	25.0 ± 9.2 (31)	24.3 ± 3.2 (29)	1.3 ± 0.6	23.0 ± 2.7	94.5 ± 3.9
[*w*Ag,*w*Bg]G × [0l]L	26.0 ± 8.7 (30)	6.5 ± 2.8 (31)	6.5 ± 2.8	0.0 ± 0.0	0.0 ± 0.0

TABLE 5

Levels and type of CI induced by Wolbachia in a foreign-species genetic background

Cross (male × female)	No. of males	No. of females	Total (*N*)	Sex ratio	% conversion CI	% mortality CI
G cytoplasm in G (1, 2) and L (3, 4) host genome						
1. [0g]G × [0g]G	1.9 ± 1.3	19.7 ± 4.1	21.8 ± 4.44 (15)	90.6 ± 5.3	—	—
2. [*w*Ag,*w*Bg]G × [0g]G	4.1 ± 1.4	0.0 ± 0.0	4.1 ± 1.4 (17)	0.0 ± 0.0	11.2	88.8
3. [0g]L × [0g]L	2.2 ± 0.9	17.3 ± 5.0	19.5 ± 5.3 (37)	88.6 ± 4.8	—	—
4. [*w*Ag,*w*Bg]L × [0g]L	3.8 ± 2.0	0.1 ± 0.5	3.9 ± 2.3 (34)	1.2 ± 5.4	9.3	90.7
L cytoplasm in L (5, 6) and G (7, 8) host genome						
5. [0l]L × [0l]L	1.4 ± 0.9	17.0 ± 4.0	18.4 ± 4.8 (17)	93.1 ± 3.7	—	—
6. [*w*Al,*w*Bl]L × [0l]L	4.1 ± 1.6	1.3 ± 1.1	5.4 ± 2.3 (18)	21.0 ± 16.8	17.2	82.8
7. [0l]G × [0l]G	1.9 ± 0.9	17.9 ± 4.2	19.8 ± 4.9 (36)	90.4 ± 3.1	—	—
8. [*w*Al,*w*Bl]G × [0l]G	3.8 ± 1.3	1.4 ± 0.9	5.2 ± 2.1 (31)	24.8 ± 13.2	11.5	88.5

sion lines and 0.1 (0.6% of the normal number of females) using the nonintrogression lines. These numbers are not significantly different ($P = 0.98$). The *girault*i Wolbachia in either its resident- or a foreign-species genetic background therefore express the same (high) level of CI. Similarly, the *longicornis* Wolbachia in the *girault*i genetic background (cross 8) express the same level of incomplete CI as when it is in its resident *longicornis* genetic background (cross 6). The average number of females produced in these incompatible crosses is 1.4 (7.8% of the normal number of females) using the introgression lines and 1.3 (7.6% of the normal number of females) using the nonintrogression lines ($P = 0.80$). Taken together, the results strongly show that differences in the level of CI are due to properties of the different Wolbachia, not to host nuclear differences between *longicornis* and *girault*i, and that CI type in all crosses is primarily mortality.

Analysis of CI-type variation between *N. vitripennis* and *N. girault*i: CI is expressed primarily as conversion in *vitripennis*, but as embryonic mortality in *girault*i and *longicornis*. We dissected whether Wolbachia and/or the host genome are involved in this difference by characterizing CI type in interspecific crosses between *vitripennis* and *girault*i, as well as in crosses with introgression lines. Results are summarized in Tables 6 and 7 and show a strong effect of the host nuclear genotype.

Interspecific crosses: CI between an infected *girault*i male and an uninfected *vitripennis* female is expressed primarily as conversion (Table 6). Total family sizes between the incompatible and compatible cross are not significantly different (12.7 *vs.* 14.2, $P = 0.45$). In addition, the decrease in the number of females in the incompatible cross (0.0 *vs.* 12.1, $P < 0.0001$) parallels the increase in the number of males (2.1 *vs.* 12.7, $P < 0.0001$). The results indicate that the uninfected *vitripennis* female determines CI type in this direction of the interspecific cross. In a separate experiment, we have established that the *vitripennis* maternal cytoplasm (*e.g.*, mitochondria) has no effect on CI type by characterizing the CI type of females from an introgression line that contains the *girault*i genotype and a *vitripennis* cytoplasm (data not shown).

CI in the reciprocal cross direction, between an infected *vitripennis* male and an uninfected *girault*i female, is also expressed as conversion, but some small level of mortality does occur (Table 6). Comparing adult family sizes in the incompatible and compatible cross reveals a significant reduction in family size (8.1 *vs.* 11.8, $P = 0.0281$). The number of females declines significantly

TABLE 6

CI is expressed primarily as conversion in interspecific crosses between *N. girault*i and *N. vitripennis*

Cross (male × female)	No. of males	No. of females	Total (*N*)	Sex ratio	% conversion CI	% mortality CI
N. vitripennis females						
[0g]G × [0v]V	2.1 ± 1.2	12.1 ± 5.7	14.2 ± 6.0 (23)	83.2 ± 11.5	—	—
[*w*Ag,*w*Bg]G × [0v]V	12.7 ± 4.1	0.0 ± 0.0	12.7 ± 4.1 (23)	0.0 ± 0.0	87.6	12.4
*N. girault*i females						
[0v]V × [0g]G	1.1 ± 0.7	9.9 ± 5.6	11.8 ± 5.0 (10)	81.1 ± 28.9	—	—
[*w*Av,*w*Bv]V × [0g]G	8.1 ± 3.4	0.0 ± 0.0	8.1 ± 3.4 (24)	0.0 ± 0.0	70.7	29.3

TABLE 7

***N. vitripennis* Wolbachia are not sufficient to induce conversion CI in a *N. giraulti* paternal genetic background**

Cross (male × female)	No. of males	No. of females	Total (*N*)	Sex ratio	% conversion CI	% mortality CI
N. giraulti females						
[0v]G × [0g]G	0.7 ± 0.5	13.0 ± 5.1	13.7 ± 5.2 (9)	94.6 ± 5.8	—	—
[*w*Av,*w*Bv]G × [0g]G	4.6 ± 4.5	0.0 ± 0.0	4.6 ± 4.5 (7)	0.0 ± 0.0	30.0	70.0
N. vitripennis females						
[0v]G × [0v]V	1.8 ± 1.0	13.7 ± 5.7	15.4 ± 6.1 (26)	87.8 ± 7.2	—	—
[*w*Av,*w*Bv]G × [0v]V	17.0 ± 8.2	0.0 ± 0.0	17.0 ± 8.2 (11)	0.0 ± 0.0	110.9	−10.9

to zero in the incompatible cross (0.0 *vs.* 9.9, $P < 0.0001$), but the number of males rises (8.1 *vs.* 1.1, $P < 0.0001$), almost matching the decline in females. Therefore, CI between an infected *vitripennis* male and an uninfected *giraulti* female is also expressed primarily as conversion, although some mortality occurs. The results from this direction of the interspecific cross therefore show that the infected *vitripennis* male also exerts a strong influence on CI type.

Crosses with N. vitripennis-N. giraulti introgression lines: The above interspecific crosses implicate the *vitripennis* maternal genotype and paternal cytotype or genotype in controlling CI type. To precisely test whether the paternal cytoplasm or genotype is the major determinant of CI type, we generated an introgression line that harbors the *vitripennis* cytoplasm (Wolbachia infected and uninfected) in an otherwise *giraulti* genetic background (*i.e.*, [*w*Av,*w*Bv]G and [0v]G). We crossed males of these infected and uninfected introgression lines to uninfected *giraulti* females, who show variation in CI type depending upon the male they are crossed to, and to uninfected *vitripennis* females, who express conversion no matter who they are crossed to.

As shown in Table 7, total adult family sizes in the incompatible cross with *giraulti* females were significantly reduced from that of the compatible cross (4.6 *vs.* 13.7, $P < 0.01$). While the number of females declined in the incompatible cross (0.0 *vs.* 13.0, $P < 0.001$), the number of males increased only slightly (4.6 *vs.* 0.7, $P = 0.06$). Mortality is therefore the primary type of CI expressed when *giraulti* males, infected with *vitripennis* Wolbachia, are crossed to uninfected *giraulti* females. This finding indicates that the *vitripennis* Wolbachia alone are not enough to induce conversion. In the same set of crosses with *vitripennis* females, conversion is the primary CI type (Table 5). Previous interspecific results showed that CI with *vitripennis* females always results in conversion. Conversion therefore will occur if either the infected male or the uninfected female has the *vitripennis* genotype.

Dominance of CI-type genes: We tested the dominance of those female genes that determine CI type by crossing infected (and control uninfected) *giraulti* males to uninfected F_1 hybrid females between *vitripennis* and *giraulti.* We also crossed the same males to pure uninfected *vitripennis* and *giraulti* females as additional control crosses. Data are based upon the sum of the offspring from two successive ovipositioning periods, in which a single female was on two hosts for 24-hr periods.

Results indicate that the conversion-CI genes from *vitripennis* are recessive (Table 8). First, the control pure species crosses reconfirm previous findings that CI in *giraulti* is expressed primarily as mortality and that CI between infected *giraulti* males and uninfected *vitripennis* females is expressed primarily as conversion. Second, for crosses using F_1 hybrid females with a *giraulti* cytoplasm, the mean number of females in the incompatible cross decreased, as expected, from that of the compatible cross (0.1 and 73.9, $P < 0.0001$), while the mean number of males increased only slightly in the same crosses (2.8 and 1.1, $P < 0.005$). Mortality is therefore the primary CI type in these crosses. The observed number of males does not take into account the fact that many F_2 hybrid males die due to hybrid breakdown, while hybrid females suffer little from F_2 breakdown due to recessivity of hybrid genetic incompatibilities (Breeuwer and Werren 1995). We estimate 90% F_2 hybrid male mortality on the basis of F_2 egg and adult counts (data not shown). Therefore, the actual number of males that would have survived if there were no hybrid lethality is ~27.6 in the incompatible cross and 11.3 in the compatible cross. While these "corrected" values show a much larger increase in the number of males in the incompatible cross than observed, they still do not match the severe decline in the number of females and indicate that the primary CI type of F_1 uninfected females is still embryonic mortality. Third and finally, for crosses using F_1 hybrid females with a *vitripennis* cytoplasm, the mean number of females in the incompatible cross decreased, as expected, from that of the compatible cross (0.0 and 56.6, $P < 0.0001$), while the mean number of males increased only slightly in the same crosses (8.6 and 2.4, $P < 0.005$). After correcting the data for 65.9% F_2 hybrid male mortality measured by F_2 egg and adult counts (data not shown), the actual number of males is estimated to be 25.3 and 7.0 for the

TABLE 8

CI-induced mortality is dominant in F_1 hybrid females

Cross (male × female)	No. of males	No. of females	Total (*N*)	Sex ratio	% conversion CI (corrected)	% mortality CI (corrected)
F_1 hybrid females						
[0g]G × [0g]F_1	1.1 ± 1.3	73.9 ± 9.8	75.0 ± 9.7 (23)	98.5 ± 1.8	—	—
[*w*Ag,*w*Bg]G × [0g]F_1	2.8 ± 2.0	0.1 ± 0.5	2.9 ± 2.1 (21)	3.6 ± 10.8	2.3 (33.3)	97.7 (66.7)
[0g]G × [0v]F_1	2.4 ± 2.0	56.6 ± 13.9	59.0 ± 12.9 (8)	95.5 ± 4.6	—	—
[*w*Ag,*w*Bg]G × [0v]F_1	8.6 ± 5.0	0.0 ± 0.0	8.6 ± 5.5 (13)	0.0 ± 0.0	11.0 (32.3)	89.0 (68.7)
Nonhybrid females						
[0g]G × [0g]G	4.9 ± 1.1	108.1 ± 14.3	112.9 ± 14.4 (18)	95.6 ± 1.1	—	—
[*w*Ag,*w*Bg]G × [0g]G	20.0 ± 5.1	0.2 ± 0.8	20.2 ± 5.2 (15)	0.9 ± 3.4	14.0 (16.5)	86.0 (83.5)
[0g]G × [0v]V	8.8 ± 3.9	40.3 ± 16.7	49.1 ± 15.5 (10)	80.5 ± 11.7	—	—
[*w*Ag,*w*Bg]G × [0v]V	54.9 ± 19.8	0.0 ± 0.0	54.9 ± 19.8 (15)	0.0 ± 0.0	114.4	−14.4

Corrected percentages of conversion-CI and mortality-CI values are based on estimates of the number of surviving F_2 hybrid males if there was no F_2 hybrid male inviability. See RESULTS for details.

incompatible and compatible crosses, respectively. This increase in the number of males far from matches the steep decline in the number of females. Mortality is the primary CI type in these crosses as well. Taken together, the results (corrected and uncorrected for F_2 hybrid mortality) show that the genes underlying CI conversion are recessive in F_1 hybrid females.

DISCUSSION

CI appears to be the most common reproductive alteration induced by Wolbachia and has been well studied in both diploid (YEN and BARR 1971; WADE and STEVENS 1985; HOFFMANN and TURELLI 1988) and haplodiploid species (BREEUWER and WERREN 1990; PERROT-MINNOT *et al.* 1996; BREEUWER 1997; VAVRE *et al.* 2000; PERROT-MINNOT *et al.* 2002). Beyond the fitness advantage that it indirectly imparts to Wolbachia, CI has been implicated as a speciation mechanism (LAVEN 1959; BREEUWER and WERREN 1990; WERREN 1998; SHOEMAKER *et al.* 1999; BORDENSTEIN *et al.* 2001) as well as a potential tool for pest biocontrol (SINKINS *et al.* 1997).

In this study, we have reached four main conlusions regarding the evolution of CI in the haplodiploid insect genus Nasonia. First, CI type noticeably varies between species, with *N. vitripennis* showing conversion and the sister species *N. giraulti* and *N. longicornis* showing mortality. Second, CI type is determined by properties of the insect nuclear genotype rather than by those of the Wolbachia infection or other elements in the cytoplasm. Third, the conversion-CI genes are recessive, as evidenced by dominance tests with hybrid females between the two species. Incompatible crosses with hybrid females predominantly showed mortality CI in both species' cytoplasm. And fourth, CI-level differences between *N. giraulti* and *N. longicornis* were found to be due to properties of the Wolbachia rather than to those of the host genome.

There is now growing interest in the role of host genes in the evolution and expression of Wolbachia-induced phenotypes and CI is no exception. There are several reasons for this interest. First, while the mechanism and biochemistry of CI remain unknown, it is likely that Wolbachia induce CI through direct interactions with host gene products, some of which may be involved in processing the male pronucleus during early mitotic events of the embryo (TRAM and SULLIVAN 2002). Second, CI poses a clear fitness cost to the arthropod host, and this cost generates a selective pressure for the host genome to ameliorate the negative effects of CI on the host (TURELLI 1994). Finally, genetic interactions between the Wolbachia and the host could spur genetic changes that indirectly lead to reproductive isolation between populations or between young species (WERREN 1998; BORDENSTEIN 2003). Several studies have shown a change in the level of CI when Wolbachia are moved into a novel or foreign genetic background (BOYLE *et al.* 1993; BORDENSTEIN and WERREN 1998; MCGRAW *et al.* 2001; POINSOT and MERCOT 2001). These effects on CI level occur in infected individuals and likely reflect an "epistatic" interaction between the Wolbachia and the host genome. Other studies have found an effect of the host on the Wolbachia phenotype. Specifically, FUJII *et al.* (2001) found that a feminizing Wolbachia transferred from its standard lepidopteran host into a new lepidopteran host caused male killing. Here, we have found another dramatic host genotypic effect on CI: altering the type of CI from conversion to mortality. In addition, rather than changing the phenotype of Wolbachia in infected hosts, we have found that the genotype of *uninfected* females can affect CI type when their eggs are fertilized by incompatible sperm. An in-

fected *N. giraulti* male crossed to an uninfected *N. vitripennis* female results in the *N. vitripennis* CI type—conversion. Therefore, the expression of conversion or mortality is dependent on the host genotype, even in uninfected individuals.

What cytological mechanisms underlie this variation in CI type? One explanation for the different types of CI is the level of paternal genome loss and frequency of aneuploidy. If modification of the paternal (*i.e.*, sperm) chromosomes is complete, resulting in complete paternal genome loss, then haploidization of the fertilized egg and male production will result. In contrast, if modification is incomplete, only partial destruction of the paternal chromosomes would occur and aneuploidy and embryonic death would result (Breeuwer and Werren 1993; Breeuwer 1997). This suggests that conversion *vs.* mortality may be due to host genetic effects on processing of the sperm pronucleus following fertilization of the egg. These processing steps include removal of the sperm nuclear envelope, decondensation of the sperm chromatin, replacement of sperm chromosomal proteins with maternally supplied histone, assembly of a nuclear envelope, and chromosome replication and condensation (Karr 1996; Poccia and Collas 1996). Subtle differences in the level or activity of proteins involved in these processing steps may underlie the observed CI differences. Candidate proteins include condensin complex proteins, topoisomerase II, and histones H1 and H3 (Karr 1996; Poccia and Collas 1996). Detailed cytological analyses are needed to determine the basis of conversion *vs.* mortality CI and the modifications induced by host genotype on CI type.

Breeuwer (1997) speculated that the CI-induced mortality observed in mites may also be a consequence of aneuploidy, the potential for which is enhanced by their holokinetic chromosome structure. Holokinetic chromosome fragments, generated from partial modification of the paternal chromosomes in incompatible crosses, may readily attach to microtubules and migrate to incipient nuclei during early mitotic divisions of the egg. This process would lead to aneuploidy and thus to CI-induced mortality. For this reason, Breeuwer (1997) suggested that CI-induced mortality may be more common in systems with a holokinetic chromosome structure.

Our data and that of Vavre *et al.* (2000) suggest that CI-induced mortality may be the norm for all haplodiploids, regardless of chromosome structure. CI-induced mortality has now been found in four wasp species, including *N. longicornis*, *N. giraulti*, *L. heterotoma* (Vavre *et al.* 2000), and *Trichopria drosophilae* (J. H. Werren, V. C. Calhoun and J. van Alphen, unpublished data), as well as in two mite species (Breeuwer 1997). *N. vitripennis* is the only species in which conversion CI is the predominant CI type, although natural variation within species was recently documented in the mite *Tetranychus urticae* (Perrot-Minnot *et al.* 2002). The overall pattern suggests that mortality is the norm for CI in haplodiploids, whereas male conversion is the exception.

Changes in CI type may have arisen as a consequence of selection in the nuclear genome in response to Wolbachia infection. The basic idea is as follows: In more outbreeding species, nuclear genes that convert incompatible fertilized eggs to viable haploid eggs (males) will be selectively favored over nuclear genes that allow the same incompatible fertilized eggs to die. Consider an outbreeding haplodiploid population in which a mortality-CI Wolbachia strain occurs at polymorphic equilibrium in the population, such that CI recurs between infected and uninfected individuals. The death of fertilized eggs in incompatible crosses would exert strong selection for nuclear genotypes that convert the dead embryos to viable males, presumably by altering early developmental processes such that incompatibility results in complete paternal genome loss. In more inbred species, the conversion gene(s) would be less favored because of the paucity of mating opportunities for males from the resulting all-male families. Therefore, if a cost is associated with conversion, conversion would be even less likely to increase in more inbred species. Evidence suggests that *N. vitripennis* has a more outbreeding population structure than *N. giraulti* and *N. longicornis* have (Drapeau and Werren 1999). Thus, we propose that conversion in *N. vitripennis* evolved as a mechanism to decrease the negative fitness consequences of CI. However, a formal population genetic analysis of this process is needed.

Vavre *et al.* (2000) have outlined a different model for the evolution of conversion and mortality CI types. In one version of their model, they propose an initial invasion of Wolbachia, in which intracellular Wolbachia densities and incompatibility levels are high, resulting in complete paternal genome loss and conversion. However, selection at the host level against the fitness costs posed by high Wolbachia densities leads to a reduction in the number of Wolbachia. The by-product of this density reduction is an incomplete modification of the paternal chromosomes, aneuploidy, and CI-induced mortality. The final phase of this evolutionary trajectory may be the complete loss of Wolbachia. Vavre *et al.* (2000) therefore consider CI-induced mortality in haplodiploids to be a transitory stage in the evolutionary trajectory of CI (*i.e.*, conversion → mortality → loss of Wolbachia). The model also predicts a tight relationship between bacterial densities and CI type (mortality means lower bacterial densities).

Our data refute this model on several accounts. First, bacterial densities do not associate with CI type in Nasonia (Breeuwer 1992). Egg bacterial densities in *N. giraulti* are the highest of the three species, yet this species induces mortality CI. Second, we find no evidence of the kinds of Wolbachia-host coadaptations predicted by Vavre *et al.*'s model (2000). In their model,

nuclear repressors of Wolbachia densities are expected to evolve and lead to reduced CI levels and mortality CI. However, our genetic results show that the difference in CI level between *N. girault*i and *N. longicornis* is not dependent on the host genome, but rather on Wolbachia type. Furthermore, in the *N. vitripennis-N. girault*i combination, there is an effect of host genotype on CI level, but it is in the opposite direction expected: The *N. vitripennis* (conversion) genotype reduces CI level (Bordenstein and Werren 1998). Finally, whereas their model predicts that CI type arises as a by-product of genomic interactions between host and Wolbachia (resulting in a reduction in bacterial density), we clearly show that the genotype of uninfected females determines CI type, independent of Wolbachia type in the male. This is clearly not predicted by their model.

Cytoplasmic incompatibility has now been studied in a total of seven haplodiploid species and only one of these species, *N. vitripennis*, naturally expresses CI-induced conversion as its primary CI type. While a larger sample of species is needed to confirm this pattern, we should begin to ask, why is mortality the more common CI form in haplodiploids? On the basis of our findings, we suggest that mortality is the default CI type for haplodiploids and that the conditions required for the evolution of conversion CI are moderately restrictive. The selective pressure is transient, occurring only when CI is expressed in the population. Thus, if a CI-Wolbachia spreads to fixation and all individuals are compatible with each other, the selective pressure for conversion CI is gone. However, incomplete transmission of Wolbachia could result in chronic levels of incompatibility that would select for conversion. Transmission levels of Wolbachia in the Nasonia species are high, although bacterial transmission can be reduced during the overwintering stage (Perrot-Minnot *et al.* 1996). Another potential source of chronic incompatibility is interspecific bidirectional CI (Breeuwer and Werren 1990; Breeuwer 1992; Bordenstein *et al.* 2001). The species ranges of *N. girault*i (eastern North America) and *N. longicornis* (western North America) are embedded within the broader range of *N. vitripennis* (Darling and Werren 1990). Contact and mating between the species would result in bidirectional CI and selection for conversion. Therefore, interspecies mating either currently or in the past could have selected for the conversion phenotype in *N. vitripennis*.

The evolution of host genotypic influences on CI type suggests that selection for nuclear genes reduces the cost of Wolbachia on host fitness. We have postulated that the genes involved are likely to act early in development to alter sperm pronuclear processing. More generally, Wolbachia-host coevolution is likely to affect genes involved in early development and both male and female gametogenesis. Wolbachia infection may therefore accelerate rates of evolution of these genes and therefore indirectly lead to genetically based reproductive isolation.

In summary, we have found that CI type varies within the Nasonia genus and that the host nuclear genotype is the major genetic determinant of CI type in Nasonia. We have shown that even the genotype of uninfected individuals can influence CI.

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