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Paternal chromosome segregation during the first mitotic division determines *Wolbachia*-induced cytoplasmic incompatibility phenotype

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Summary

The most common *Wolbachia*-induced phenotype in insects is cytoplasmic incompatibility (CI), which occurs when sperm from infected males fertilize eggs from uninfected females. CI produces distinct phenotypes in three closely related haplo-diploid species of the genus *Nasonia*: mortality in *N. longicornis* and *N. giraulti*, and conversion to male development in *N. vitripennis*. We demonstrate that the majority of CI-induced mortality occurs during embryogenesis and that the pattern of paternal chromosome segregation during the first mitosis is a good predictor of CI phenotype. In *N. giraulti* and *N. longicornis*, the paternal chromosomes mis-segregate, producing abnormal nuclei connected by chromatin bridges. Consequently, these embryos arrest development with very few and abnormal nuclei. In contrast, the paternal genome in *N. vitripennis* is either not segregated or mis-segregates to one of the two daughter nuclei. Consequently, these embryos continue development utilizing the maternally derived haploid nuclei, resulting in male offspring. The latter class is the first documented example of asymmetric mitotic segregation of abnormal chromosomes. We conclude that in haplo-diploids, CI-induced embryonic lethality occurs only when abnormal paternal genome segregation affects both products of the first mitotic division. This is the first study to associate differences in types of CI with specific cytological defects.

Key words: Cytoplasmic incompatibility, *Wolbachia*, *Nasonia*, Chromosome segregation

Introduction

Wolbachia is a genus of obligate intracellular bacteria that infects arthropods and filiarial nematodes worldwide (for reviews, see Werren, 1997; Stouthamer et al., 1999; Bandi et al., 2001). In their arthropod hosts, *Wolbachia* cause a variety of reproductive alterations including feminized development of genetic males (Rousset et al., 1992; Bouchon et al., 1998; Kageyama et al., 2002), male killing (Hurst et al., 1999; Fialho and Stevens, 2000; Hurst et al., 2000), parthenogenesis (Stouthamer et al., 1990; Stouthamer et al., 1993; Arakaki et al., 2001; Weeks and Breeuwer, 2001), and cytoplasmic incompatibility (CI) (Yen and Barr, 1971; Wade and Stevens, 1985; Hoffmann et al., 1986; Breeuwer and Werren, 1990; O'Neill and Karr, 1990; Hoffmann and Turelli, 1997). All of these manipulations increase the number or fitness of infected females in the population, which is advantageous for *Wolbachia* because they are maternally transmitted via the egg cytoplasm.

Cytoplasmic incompatibility is the most common effect of *Wolbachia* infection and involves a sperm-egg incompatibility expressed in fertilized eggs. It results when males infected with *Wolbachia* mate with uninfected females (or with females infected with a different strain of *Wolbachia*). In diploid insects and most haplo-diploids, CI results in severely reduced egg hatch (Hoffmann et al., 1986; Breeuwer, 1997; Vavre et al., 2000; Vavre et al., 2002; Bordenstein et al., 2003). Three closely related species of the haplo-diploid wasp *Nasonia* differ in the type of CI that they express. The sibling species *N. giraulti* and *N. longicornis* show predominantly embryonic mortality whereas *N. vitripennis* shows predominantly conversion of CI embryos to male development (Bordenstein et al., 2003). Genetic analysis of *Nasonia* revealed that differences in CI type among these species are due to nuclear genetic effects rather than differences in the *Wolbachia* (Bordenstein et al., 2003).

Though the molecular mechanism of CI is not understood in any insect, genetic and cytological analysis of CI in *Drosophila simulans* and *N. vitripennis* show that the paternal genome is excluded from participating in embryonic development (Ryan and Saul, 1968; Breeuwer and Werren, 1990; Reed and Werren, 1995; Callaini et al., 1996; Lassy and Karr, 1996; Callaini et al., 1997; Tram and Sullivan, 2002) (for a review, see Tram et al., 2003). In CI embryos, the paternal chromosomes are undercondensed, delay entering metaphase, and are segregated improperly at anaphase (Ryan and Saul, 1968; Callaini et al., 1996; Lassy and Karr, 1996; Callaini et al., 1997). In *D. simulans* this leads to embryonic mortality. In *N. vitripennis*, in which unfertilized eggs normally develop into males, exclusion of the paternal genome leads to male development. Because cytological analysis of CI in haplo-diploids has only

been performed in *N. vitripennis*, in which the vast majority of CI embryos develop as males, it is not known how CI induces embryonic mortality in haplo-diploids.

To gain insight into the cellular basis of the species-specific CI phenotypes in haplo-diploids, we analyzed chromosome behavior in early embryogenesis in the three *Nasonia* species described above. We show a striking correlation between the segregation behavior of the paternal chromosomes during the first mitotic division and the differences in the rate of embryonic mortality in the three *Nasonia* species. In the majority of *N. vitripennis* CI embryos, the paternal genome is excluded from one or both daughter nuclei produced from the first mitotic division. The paternal genome either fails to segregate or segregates to only one of the two daughter nuclei. The remaining haploid nucleus continues to divide and viable haploid males result. In contrast, in *N. giraulti* and *N. longicornis* CI embryos, the paternal genome improperly and unequally segregates to both daughter nuclei. As a result, severe segregation defects are observed in subsequent mitotic divisions. Egg hatch rates and egg-to-adult counts demonstrate that almost all of the CI-induced mortality in these species can be accounted for by the observed segregation patterns of the paternal chromosomes during the first mitotic division. CI-induced embryonic lethality occurs when abnormal paternal chromosome segregation affects both products of the first mitotic division, whereas CI results in male development when the paternal chromosomes do not segregate or are segregated to only one of the two daughter nuclei.

Results

CI crosses in three *Nasonia* species exhibit different egg hatch rates

Although it had been previously reported that *Wolbachia*-induced CI produces over 80% mortality in *N. longicornis* and *N. giraulti* and a slight but significant amount (16%) of mortality in *N. vitripennis* (Bordenstein et al., 2003), cytological-based phenotypic analyses have not been performed. Thus, we wanted to first determine if mortality observed in the *Nasonia* species occurs during embryogenesis by directly comparing the number of larvae that hatch from the number of eggs that were laid (Table 1). In *N. vitripennis*, control crosses between uninfected males and females and crosses between infected males and infected females laid on average 19±5 (n=71) and 22±5 (n=73) eggs, respectively. Of these, 19±5 and 22±5 eggs hatched, giving hatch rates of 98±3% and 99±2% for the control uninfected and infected crosses, respectively.

In the *N. vitripennis* CI cross, 81±10% of eggs hatched, indicating that approximately 19±10% of CI embryos die during embryogenesis. These results are consistent with the previous report that CI induces a low level of mortality in *N. vitripennis* (Bordenstein et al., 2003) and further demonstrate that mortality occurs during embryogenesis.

In *N. giraulti*, the uninfected and infected control crosses yield 98±4% (n=26) and 99±2% (n=23) egg hatch rates, respectively (Table 1). In contrast, only 34±11% (n=47) of CI eggs hatched, indicating that approximately 66±11% of *N. giraulti* CI embryos die during embryogenesis. This is consistent with Bordenstein et al.'s estimate that 75% of *N. giraulti* CI eggs did not hatch and supports their conclusions that the majority of *giraulti* CI embryos die during embryogenesis and that additional death occurs during the larval and/or pupal stages (Bordenstein et al., 2003).

Table 1. Egg hatch rates in *Nasonia*

Cross*	No. of eggs laid	% Egg hatch	$n^{\dagger}$
N. vitripennis			
♂×♀	19±5	98±3	71
♂×⚫	22±5	99±2	73
⚫×♀	21±5	81±10	98
N. giraulti			
♂×♀	15±4	98±4	26
♂×⚫	18±4	99±2	23
⚫×♀	15±4	34±11	47
N. longicornis			
♂×♀	14±4	54±20	62
♂×⚫	14±5	64±22	71
⚫×♀	15±4	25±11	93

*Open symbols represent uninfected individuals and filled symbols represent infected individuals.

†n=total number of females scored.

In *N. longicornis*, 54±20% (n=62) and 64±22% (n=71) of eggs hatched in the uninfected and infected control crosses (Table 1), respectively, indicating that almost half of the control embryos die as a result of factors other than *Wolbachia*-induced CI. In the CI cross, we observed a 25±11% (n=93) egg hatch rate, indicating that there was a 75% embryonic mortality rate in CI crosses. Because of the high mortality rate in the control crosses, *Wolbachia*-induced CI mortality probably accounted for only a fraction of the mortality observed in CI crosses. If we normalize our egg hatch results, setting the uninfected control as 100%, then the egg hatch rate in *N. longicornis* CI was 46%, indicating that *Wolbachia*-induced CI was responsible for 54% mortality during embryogenesis. These results differ from those of Bordenstein et al. (Bordenstein et al., 2003) who reported that 93% of embryos in the uninfected control cross and 19% of embryos in the CI cross survived to adulthood (Bordenstein et al., 2003). To determine why our egg hatch rates were so low, we examined overall mortality (percentage of eggs that develop to adulthood). We found that 57% and 69% of eggs from uninfected (n=28) and infected (n=24) crosses developed to adulthood. These data are in agreement with our results for the egg-hatch assay. The differences between Bordenstein et al. (Bordenstein et al., 2003) and our results suggest that between the time of Bordenstein et al.'s analysis and our analysis, the uninfected and/or infected *N. longicornis* stocks may have acquired deleterious mutations, possibly as a consequence of bottlenecks during diapause storage of these strains. In the *N. longicornis* CI cross (n=53), 10% of the eggs laid developed to adulthood. This is lower than our egg hatch results, indicating that additional lethality occurs during the larval and/or pupal stages.

In summary, our egg-hatch assays indicate that CI produces extensive embryonic lethality in *N. giraulti* (66%) and *N. longicornis* (54%) and some embryonic lethality in *N. vitripennis* (19%). Consistent with earlier reports, the predominant CI phenotype in *N. vitripennis* is male development (Ryan and Saul, 1968; Breeuwer and Werren, 1990). We have shown, however, that a significant level of embryonic lethality also occurs in *N. vitripennis*. In *N. longicornis* and *N. giraulti*, the hatch rates are slightly higher

than egg-to-adult survival, indicating that CI may also cause some lethality during the larval and pupal stages. In agreement with earlier reports (Bordenstein et al., 2003), all surviving CI embryos in *N. vitripennis* and *N. giraulti* developed into males (data not shown). Although CI in *N. longicornis* also resulted in male-biased families, 19% of *N. longicornis* CI crosses produced one or two females (data not shown), in agreement with Bordenstein et al. (Bordenstein et al., 2003).

CI produces four distinct cytological classes

To determine if CI type in haplo-diploids can be distinguished cytologically, we analyzed 0 to 1-2-hour collections of CI embryos from *N. vitripennis*, *N. giraulti* and *N. longicornis* and classified them into one of four categories. With these collections, we focused our analysis on embryos that range in age from telophase of cycle 1 through to and including cycle 3. During this period of development, there are only two to five nuclei, which allows us to more easily deduce the identity of the paternal genome. Because *Nasonia* do not have distinguishable sex chromosomes, we could not directly identify the paternal genome based on cytological means (Johnson and Ray, 1972). However, substantial genetic studies have shown that it is the paternal genome that is excluded from the developing embryo (Ryan and Saul, 1968; Breeuwer and Werren, 1990; Beukeboom and Werren, 1993). Thus, we reason that abnormal-looking nuclei observed in CI embryos are of paternal origin.

Four cytological categories emerged from this analysis. CI embryos that contained the predicted number of nuclei (2^n, *n*=nuclear cycle-1) and all of which appear to be morphologically normal were classified in Category 1 (Fig. 1A). Because CI in *N. vitripennis* and *N. giraulti* is complete, meaning all fertilized eggs are affected, we reason that *N. vitripennis* and *N. giraulti* CI embryos that contain only normal nuclei developed from unfertilized eggs. Thus, *N. vitripennis* and *N. giraulti* Category 1 embryos are predicted to develop into males. We found that this is a good predictor of unfertilized eggs because the percentage of CI embryos that were classified in Category 1 was in agreement with the percentage of embryos from an uninfected control cross that develop into males (data not shown). Because males normally develop from unfertilized eggs in *Nasonia*, the number of eggs from a control cross that develop into males corresponds to the number of eggs that were not fertilized. CI in *N. longicornis,* by comparison, is incomplete (Bordenstein et al., 2003); occasionally one or two females emerge, presumably because the paternal genome was properly segregated. Thus, *N. longicornis* CI embryos in Category 1 may be either an unfertilized egg or the rare fertilized egg that escaped the effects of Wolbachia modification. Category 2 embryos contained an odd number of nuclei, one of which is highly condensed and the remaining nuclei are morphologically normal. In these embryos, it appears that the paternal genome did not segregate (Fig. 1B,C). Because two normal-looking nuclei are produced and the paternal genome does not participate in

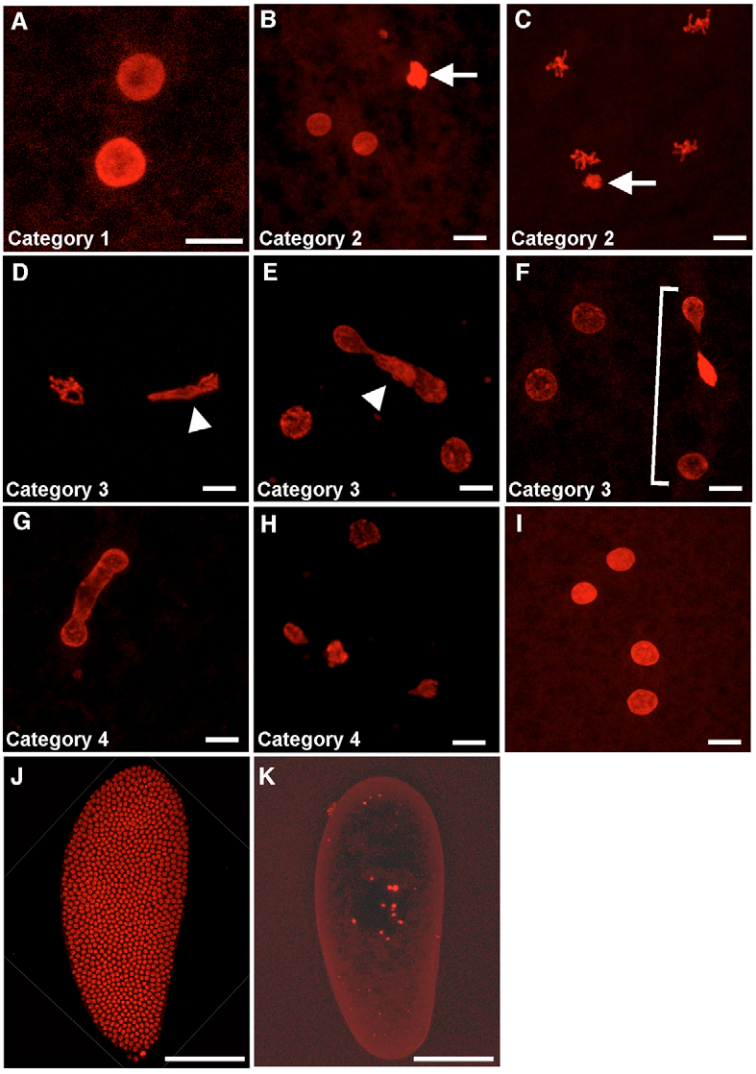

Fig. 1. CI in *N. vitripennis*. (A) Category 1. In 5% of *N. vitripennis* CI embryos, chromosome segregation is normal, indicating that the egg was unfertilized. (B,C) Category 2. In 26% of *N. vitripennis* CI embryos, the paternal genome is not segregated. (B) Nuclear cycle 2, there are two normal nuclei and one highly condensed nucleus (arrow). (C) Nuclear cycle 3, there are four normal nuclei and one highly condensed nucleus (arrow). (D-F) Category 3. In 48% of *N. vitripennis* CI embryos, the paternal genome segregated to one daughter nucleus at the end of nuclear cycle 1. (D) Nuclear cycle 1, one nucleus is normal whereas the other appears to be a composite of two nuclei (arrowhead). (E) Nuclear cycle 2, two normal nuclei are present and the composite nucleus attempts to divide (arrowhead). (F) Nuclear cycle 2, two normal nuclei are present and the composite nucleus divides to give one normal-looking nucleus (bottom of bracket), one misshapen nucleus (top of bracket), and one highly condensed nucleus (middle of bracket). (G,H) Category 4. In 22% of *N. vitripennis* CI embryos, the paternal genome is mis-segregated to both daughter nuclei. (G) Nuclear cycle 2, a chromatin bridge connects the two daughter nuclei. (H) Nuclear cycle 3, all four nuclei are misshapen and of different sizes. (I) Nuclear cycle 3 uninfected control embryo, all nuclei are of uniform size and shape. (J) Nuclear cycle 12 CI embryo; 78% of CI embryos reach at least the syncytial blastoderm stage. (K) CI embryo in which development has arrested; 22% arrested with a few, highly condensed nuclei. Bars, 8 μm (A-I).

development, these embryos are presumably haploid and are predicted to develop into males. Category 3 CI embryos contain both morphologically normal nuclei and nuclei that are misshapen or fused (Fig. 1D,F). The most probable origin of embryos in this category is that the paternal genome segregates to only one of the two daughter nuclei at the end of nuclear cycle 1, producing one nucleus bearing the maternal genome and a daughter nucleus with the maternal genome and part or all of the paternal genome. These embryos are predicted to develop into haploid males because a normal nucleus is produced. CI embryos in which all nuclei are connected by chromatin bridges, are misshapen, or are fused into a single unusually large mass of diffuse DNA were classified in Category 4 (Fig. 1G,H). It is probable that the paternal genome has been improperly segregated to both daughter nuclei in these embryos. Because normal nuclei are not produced, these embryos will probably die in early embryogenesis. Although we were not able to determine whether nuclei are haploid or diploid based on size or intensity of DNA staining, we presume that normal-looking nuclei present in CI embryos are haploid because all *N. vitripennis* and *N. girault*i embryos that survive CI are male, which are haploid.

Cytology of *N. vitripennis* CI embryos

In *N. vitripennis*, 5% (5/102) of the CI embryos displayed the predicted number of nuclei and normal nuclear morphology, indicating the egg was not fertilized, and was classified in Category 1 (Fig. 1A, Table 1). The paternal genome did not segregate in 26% of the embryos and these embryos were classified in Category 2. These embryos contained an odd number of nuclei; cycle 2 embryos contained two nuclei that were uniform in size and shape and one nucleus that was highly condensed (Fig. 1B, arrow), whereas cycle 3 embryos contained four uniformly shaped and sized nuclei and one highly condensed nucleus (Fig. 1C, arrow). The maternal and paternal genomes in many cycle 2 embryos have the unusual configuration shown in Fig. 1B. We do not yet understand the basis of this phenomenon. The majority of *N. vitripennis* CI embryos were classified in Category 3; 48% of *N. vitripennis* CI embryos contained at least one morphologically normal nucleus, indicating that the paternal genome segregated to only one daughter nucleus (Fig. 1D-F). In general, these embryos contained one or two morphologically normal nuclei and one abnormal nucleus that is a fusion of two or three nuclei (Fig. 1D,E, arrowheads). In 22% of CI embryos, the paternal genome was improperly segregated to both daughter nuclei and was classified in Category 4 (Fig. 1G,H). Embryos in cycle 1 telophase and cycle 2 were characterized by chromatin bridges that connect all daughter nuclei (Fig. 1G) whereas cycle 3 embryos were characterized by misshapen nuclei (Fig. 1H). We did not observe any defects in nuclear morphology in 98% (78/80) of control uninfected embryos (Fig. 1I), with the exception that two control embryos contained nuclei that were not uniform in size. Thus, our cytological analysis of CI in *N. vitripennis* predicts that 78% of CI embryos will develop as males and 22% will die. This prediction correlates well with our egg hatch results which indicated that 19±10% of *N. vitripennis* CI embryos die during embryogenesis. In addition, our cytological analysis indicates that 5% of *N. vitripennis* CI embryos are unfertilized. This is consistent with our results which show that the fertilization rate in uninfected *N. vitripennis* is 97% (data not shown).

The cytological phenotypes we have observed in *N. vitripennis* CI embryos resemble those described for *maternal haploid* (*mh*), a maternal effect mutation in *Drosophila melanogaster* (Loppin et al., 2001). In *mh* embryos, the paternal genome is delayed in exiting mitosis relative to the maternal genome and as a consequence forms a chromatid bridge at telophase of nuclear cycle 1 (Loppin et al., 2001), similar to *N. vitripennis* CI embryos (described above) (see Tram and Sullivan, 2002). Although the paternal genome is unequally segregated between the two daughter nuclei in 92% of *mh* embryos, the maternal and paternal chromosomes do not intermingle in the daughter nuclei because they remain enveloped by separate nuclear laminas (Loppin et al., 2001). Because asynchrony between the paternal and maternal genome continues, the paternal genome becomes excluded from the maternal genome in the majority of embryos by nuclear cycle 3, resulting in haploid embryos which develop through the syncytial blastoderm stage. These observations in *mh* embryos suggest that the production of some abnormal nuclei containing paternal chromosome material during the first or second nuclear divisions can be resolved to produce at least one haploid nucleus (Loppin et al., 2001).

We were interested in determining whether a similar mechanism exists in *N. vitripennis*, enabling CI embryos to correct problems resulting from abnormally segregated paternal chromosomes by eliminating the paternal genome in subsequent nuclear divisions. To test this, we examined 4-6-hour-old embryos, which in *N. vitripennis* have reached the cortical nuclear cycles (data not shown). We observe that 78% (n=46) of 4-6-hour-old *N. vitripennis* CI embryos form a normal syncytial blastoderm and are at nuclear cycles 10 through 13 (Fig. 1J), whereas 22% arrest with fewer than 11 abnormal nuclei (Fig. 1K). These observations correlate well with our analysis of 0-2-hour-old CI embryos, which predict that 22% of CI embryos die as a result of improper segregation of the paternal genome to both daughter nuclei (chromatin bridges connecting all nuclei or all nuclei were misshapen). This suggests that in CI embryos, unlike *mh* embryos, when the paternal genome is improperly segregated to both daughters at the end of nuclear cycle 1, the resulting embryo dies. However, when the paternal genome segregates to only one daughter nucleus, embryonic development originates from the single normal nucleus and the aneuploid nucleus does not interfere with development.

Cytology of *N. girault*i CI embryos

In 8% (8/100) of *N. girault*i CI embryos, all nuclei were wild-type in appearance with regard to nuclear number and morphology and were classified in Category 1 (Fig. 2A, Table 2). The paternal genome was not segregated in 7% of all embryos (Category 2, Fig. 2B, arrow) and was segregated to one daughter nucleus in 15% of all CI embryos (Category 3, Fig. 2C, arrowhead). The majority of *N. girault*i CI embryos were classified in Category 4. In 70% of *N. girault*i CI embryos, the paternal genome was improperly segregated to both daughter nuclei (Fig. 2D-F). In 33% (23/70) of these embryos, all nuclei were misshapen (Fig. 2D), 34% had chromatin bridges that connected all nuclei (Fig. 2E), and 33% contained a large single mass of diffuse DNA (Fig. 2F). None

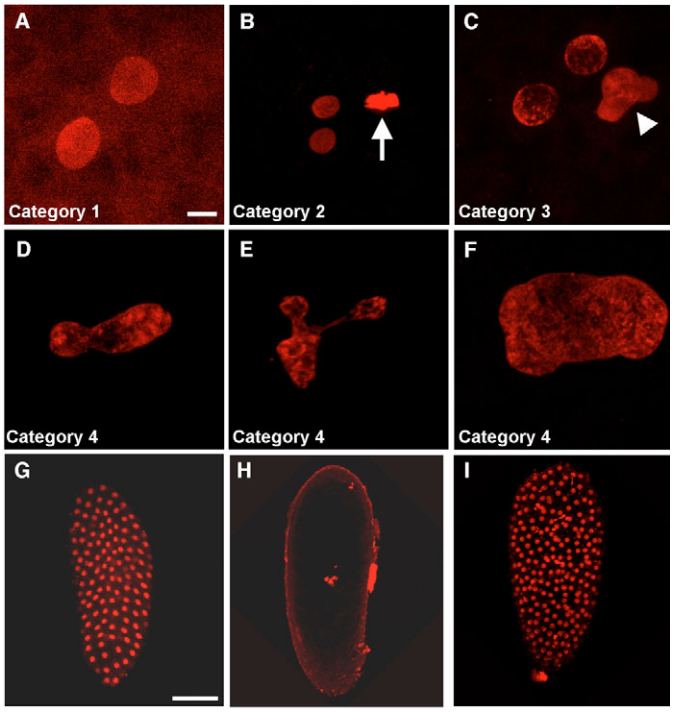

Fig. 2. CI in *N. girault*i. (A) Category 1. Nuclear cycle 2 embryo in which chromosomes were segregated correctly. (B) Category 2. Nuclear cycle 2 embryo in which the paternal genome was not segregated. There are two normal nuclei and one highly condensed nucleus (arrow). (C) Category 3. Nuclear cycle 2 embryo in which the paternal genome had segregated to one daughter nuclear at the end of the previous cycle. There are two normal nuclei and one composite nucleus (arrowhead). (D-F) Category 4. In 83% of *girault*i CI embryos, the paternal genome mis-segregates to both daughter nuclei. (D) Nuclear cycle 2, the two nuclei are connected by a chromatin bridge. (E) Nuclear cycle 3, all four nuclear products are connected by chromatin bridges. (F) Several embryos contained one massive nucleus composed of diffuse DNA. (G) Control uninfected embryo at nuclear cycle 9, note nuclei are evenly spaced from each other. (H) The majority of CI embryos arrest development with a few highly condensed nuclei. (I) A few CI embryos reach the syncytial blastoderm stage but notice nuclei are not evenly spaced from neighbors and chromosome fragments are present. Bars, 8 μm (A-F).

of these nuclear defects were observed in control uninfected embryos (*n*=52, data not shown). Thus, cytology predicts that 70% of *girault*i CI embryos die and 30% develop into males. This is consistent with our egg hatch data which indicated that 66±11% of *girault*i CI embryos die during embryogenesis.

*N. girault*i CI embryos display two phenotypes that were never observed in *N. vitripennis* CI embryos: chromatin bridges that connect all nuclei present at nuclear cycle 3 and embryos that possess a single massive diffuse nuclear body (Fig. 2E,F). This suggests that when the paternal genome is improperly segregated to both daughter nuclei at end of nuclear cycle 1 in *N. girault*i CI embryos, it also prevents proper segregation of the maternal chromosomes in subsequent nuclear divisions. To determine whether *N. girault*i CI embryos are able to resolve these segregation problems in later nuclear divisions, we examined 4-6-hour-old CI embryos. By this time, control embryos had formed blastoderms and were in nuclear cycles 9-11 (*n*=30) (Fig. 2G). In CI embryos, 70% (38/54) had not formed blastoderms, the majority of which possessed only 1-4 nuclear bodies (Fig. 2H). In the remaining 30%, blastoderms were formed but spacing between nuclei was often irregular and chromosome fragments were present (Fig. 1I). Thus, in *N. girault*i CI embryos, improper segregation of the paternal genome early in embryogenesis prevented further development and embryos often arrest with few nuclei.

Table 2. Paternal genome (PG) behavior in *Nasonia* CI embryos

Category	*n**	1 Normal segregation	2 PG not segregated	3 PG to one daughter	4 PG to both daughters
N. vitripennis	102	5%	26%	48%	22%
N. longicornis	112	13%	2%	21%	65%
*N. girault*i	100	8%	7%	15%	70%

**n*=total number of embryos examined.

Cytology of *N. longicornis* CI embryos

In *N. longicornis*, 13% of the embryos were wild-type in appearance with regard to nuclear number and morphology and were classified in Category 1 (Fig. 3A, Table 2). The paternal genome was not segregated in 2% of all *N. longicornis* CI embryos (Category 2, Fig. 3B, arrow) and was segregated to one daughter nucleus in 21% of all *N. longicornis* CI embryos (Category 3, Fig. 3C,D, arrowheads). The majority of *N. longicornis* CI embryos were classified in Category 4. In 65% (73/112) of CI embryos, the paternal genome missegregated to both daughter nuclei (Fig. 3E-G). In 49% (36/73) of these embryos, all nuclei were misshapen (Fig. 3E), 34% had chromatin bridges connecting all nuclei present in the embryo (Fig. 3F), and the remaining 16% were characterized by large masses of diffuse DNA (Fig. 3G). In control uninfected embryos (*n*=90), 94% of the embryos displayed the predicted number of nuclei of uniform size and shape (Fig. 3H) and 6% contained an odd number of nuclei (Fig. 3I). Our cytological data predicts that 65% of *N. longicornis* CI embryos will die during embryogenesis. This correlates well with our normalized egg hatch result which indicated that CI in *N. longicornis* produces 54% embryonic mortality. At least 22% of the embryos are predicted to develop as males because the paternal genome segregated to one daughter or was not segregated in these embryos. Because the rare female escaper is occasionally observed in *longicornis* CI, the remaining 13% of CI embryos that appeared normal will develop either as male or as female.

Cytologically, *N. girault*i and *N. longicornis* CI embryos are similar to each other and both are distinct from *N. vitripennis*. Whereas the paternal genome segregated to one daughter or was not segregated in the majority of *N. vitripennis* CI embryos, the paternal genome improperly segregated to both daughters in the majority of both *N. girault*i and *N. longicornis* CI embryos (Table 2). Thus, at the end of the first mitotic cycle, the majority of *N. vitripennis* CI embryos contain at least one normal nucleus whereas the majority of CI embryos in *N. longicornis* and *N. girault*i contain two abnormal nuclei often connected by chromosome bridges.

Discussion

The cytological basis of conversion and mortality

Among haplo-diploids, *Wolbachia*-induced CI causes the

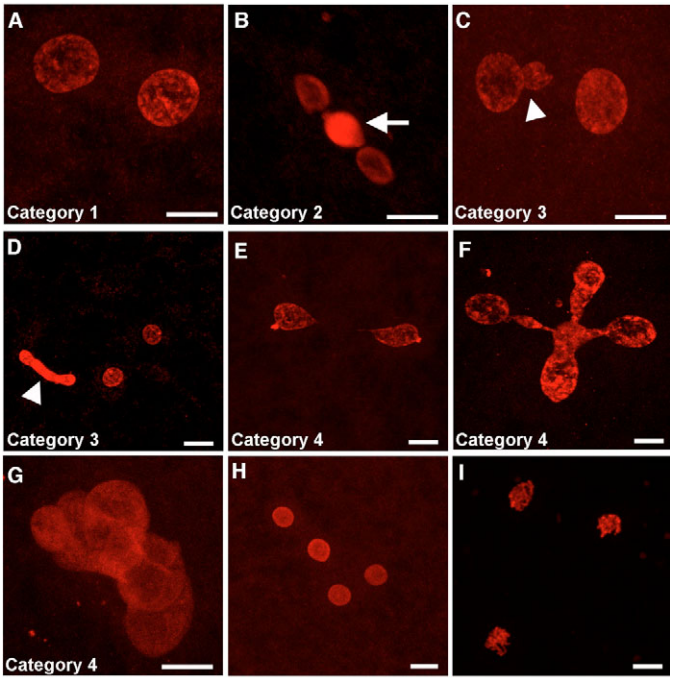

Fig. 3. CI in *N. longicornis*. (A) Category 1. In 13% of *N. longicornis* CI embryos, normal segregation is observed. (B) Category 2. In 2% of *N. longicornis* CI embryos, the paternal genome is not segregated (arrow). (C,D) Category 3. In 21% of *N. longicornis* CI embryos, the paternal genome segregates to one daughter. (C) Nuclear cycle 2, one normal nucleus and one composite nucleus (arrowhead). (D) Nuclear cycle 3, two normal nuclei and one composite nucleus (arrowhead) that attempts mitosis. (E-G) Category 4. In 65% of *N. longicornis* CI embryos, the paternal genome mis-segregates to both daughter nuclei. (E) Nuclear cycle 2, the nuclei are tear-shaped indicating aneuploidy. (F) Nuclear cycle 3, all four nuclei are connected by chromatin bridges. (G) The embryo is composed of a single, diffuse mass of DNA. (H,I) Control uninfected embryos. (H) Nuclear cycle 3, all nuclear are uniform in size and shape. (I) A small number of control embryos contain an odd number of nuclei. Bars, 8 μm.

fertilized egg to either abort development or convert to male development. Of seven haplo-diploid species examined, six exhibit a significant degree of mortality as a result of CI, suggesting that mortality is the more common CI phenotype among haplo-diploids (Breeuwer, 1997; Vavre et al., 2000; Vavre et al., 2002; Bordenstein et al., 2003). *Nasonia vitripennis* is the only haplo-diploid in which the vast majority of CI embryos are converted to male development (Ryan and Saul, 1968). The prevailing model for *Wolbachia*-induced CI is that *Wolbachia* modify sperm during spermatogenesis and these modifications render the sperm unable to successfully participate in embryonic development. The fate of the paternal genome during the first zygotic mitosis has been hypothesized to be determined by the extent of *Wolbachia* modification present in the sperm (Breeuwer, 1997; Vavre et al., 2001; Vavre et al., 2002; Bordenstein et al., 2003; Tram et al., 2003). Thus, male pronuclei that have been completely modified by *Wolbachia* action will probably produce chromosomes that will not be segregated, resulting in embryos that will develop as haploid males, whereas male pronuclei that have been only moderately modified will probably produce chromosomes that missegregate, resulting in aneuploid nuclei and death. Male pronuclei that have been minimally or not modified at all will produce chromosomes that segregate properly, resulting in diploid embryos that develop into females. Previous cytological analyses of CI in *N. vitripennis* suggest that *Wolbachia* induce male development by preventing the paternal genome from participating in development (Ryan and Saul, 1968; Breeuwer and Werren, 1990; Tram and Sullivan, 2002). Here, we provide the first evidence in support of the model that *Wolbachia*-induced CI phenotypes among haplo-diploids depend on the behavior of the paternal genome during the first mitotic division. We show that embryonic mortality results when the paternal genome is improperly segregated to both daughter nuclei at the end of the first mitosis and that male development results when the paternal genome is not segregated or segregated to one daughter nucleus. In the former case, detrimental aneuploidy is produced in which all nuclei present are affected, whereas in the latter the presence of at least one normal nucleus at the end of the first mitotic division is sufficient to support embryonic development.

Chromatin bridging is a common feature of CI in diverse species

A common feature of CI in all three *Nasonia* species is chromatin bridging (Reed and Werren, 1995) (this study), similar to that observed in *Drosophila simulans* CI embryos (Lassy and Karr, 1996; Callaini et al., 1997). CI-induced chromatin bridges arise from inappropriate segregation of the paternal genome to one or both daughter nuclei at the end of the first mitosis. The most dramatic chromatin bridges are seen in cycle 3 *N. giraulti* and *N. longicornis* CI embryos. In these embryos, chromatin bridges connect all four nuclear products together. Presumably, the paternal genome is inappropriately segregated to both daughter nuclei at the end of nuclear cycle 1, creating a chromatin bridge linking the two daughter nuclei, and when these two daughters undergo mitosis, their daughters also remain attached via chromatin bridges. This phenotype is never observed in *N. vitripennis* cycle 3 CI embryos. Instead, cycle 3 *N. vitripennis* CI embryos in which the paternal genome was inappropriately segregated to both daughter nuclei at the end of cycle 1 are characterized by four misshapen nuclei. These observations indicate that chromatin bridges are more persistent in *N. giraulti* and *N. longicornis* CI embryos than in *N. vitripennis* embryos.

Chromatin bridges can result when chromosome segregation occurs in the presence of incomplete sister chromatid resolution, incomplete DNA replication, or improper chromosome condensation (Raff and Glover, 1988; Buchenau et al., 1993; Bhat et al., 1996; Steffensen et al., 2001). Previous cytological analyses of CI in *N. vitripennis* and *D. simulans* have shown that the paternal chromosomes are undercondensed at metaphase in CI embryos (Ryan and Saul, 1968; Reed and Werren, 1995; Lassy and Karr, 1996; Callaini et al., 1997; Tram and Sullivan, 2002). Undercondensed paternal chromosomes are also observed in *N. giraulti* and *N. longicornis* CI embryos (data not shown). Thus, improper condensation of the paternal chromosomes may contribute to the chromatin bridging observed in CI embryos. It will be important to examine DNA replication in *Nasonia* CI embryos and determine whether incomplete paternal genome replication

also contributes to chromatin bridging. Understanding the primary cause of chromatin bridging in *Nasonia* CI embryos may uncover the molecular basis of *Wolbachia*-induced CI.

Wolbachia may exploit unique properties of the first mitotic spindle in insects

Among the three *Nasonia* species, the Wolbachia-modified paternal genome is not segregated 26%, 7% and 2% of the time in *N. vitripennis*, *N. giraulti* and *N. longicornis*, respectively. Chromosome segregation is ultimately determined by the spindle checkpoint, which ensures that all chromosomes are attached to microtubules before anaphase is initiated (Pinksy and Biggins, 2005). Failed or improper microtubule-kinetochore attachment, chromosome damage and microtubule damage are all known to activate the spindle checkpoint and inhibit sister chromosome segregation (Chan et al., 2005). It is possible that the sensitivity of the spindle checkpoint differs among the three *Nasonia* species, such that the spindle checkpoint is most sensitive in *N. vitripennis* and least sensitive in *N. longicornis.* Thus, *N. vitripennis* is less likely to initiate chromosome segregation in the presence of damaged chromosomes or microtubules or improper microtubule-kinetochore attachment than either *N. giraulti* or *N. longicornis*. As a result, *Wolbachia*-modified paternal genome is segregated less frequently in *N. vitripennis* and more haploid embryos bearing only the maternal genome are produced. A second possibility is that Wolbachia produces the most extensive chromosome damage to the paternal chromosomes in *N. vitripennis* and thus is more likely to activate the spindle checkpoint in *N. vitripennis*. Other research indicates that the difference between these species in mortality versus male conversion CI is a result of the nuclear genotype of the species, rather than differences in Wolbachia (Bordenstein et al., 2003). To elucidate the role that the spindle checkpoint plays in determining CI phenotype in *Nasonia*, it will be important to compare spindle structure and composition in these embryos.

The unique geometry of the first zygotic spindle provides an explanation for the distinct CI phenotypes observed in *Nasonia* (Fig. 4). Referred to as the gonomeric spindle, distinct bundles of microtubules associate with the maternal and paternal chromosome complements (Huettner, 1924; Sonnenblick, 1950). At metaphase, the maternal and paternal chromosome complements align on separate regions of the plate, physically separated by remnants of the nuclear envelope (Callaini and Riparbelli, 1996). This unique bifurcated spindle structure raises the possibility that the spindle checkpoint is independently regulated in the two halves of the spindle (Callaini et al., 1997; Tram et al., 2003). During the first mitotic division in CI embryos derived from *D. simulans* females, the maternal chromosome complement enters anaphase while the paternal chromosome complement remains arrested on the metaphase plate (Callaini et al., 1997). Thus, in these embryos, it seems that the spindle checkpoint has been activated only in the paternal half of the spindle. Support for this interpretation comes from observations of fused mammalian cells which result in two spindles sharing a common cytoplasm. In these cells, the spindle checkpoint acts locally on its own spindle and the signal does not diffuse to the neighboring spindle (Rieder et al., 1997). In the first division in insect embryos, the situation in very similar with the exception spindles share a common pole.

We propose that when CI produces severe defects in the processing of the paternal chromosomes, the spindle checkpoint is activated in the paternal half of the spindle resulting in the production of the haploid nuclei containing only the maternal chromosome complement (Fig. 4). If the CI-induced defects are less severe, the spindle checkpoint would not be activated and improperly processed paternal chromosomes segregate, producing inviable aneuploid daughter nuclei. It should be noted that in diplo-diploid species inviable progeny are produced irrespective of whether the

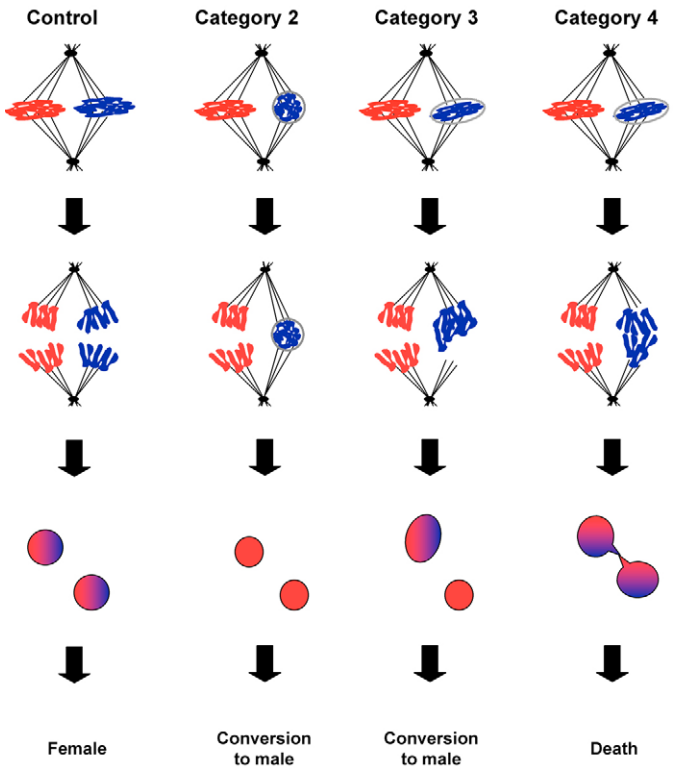

Fig. 4. Model of how *Wolbachia* exploits the unique structure of the gonomeric spindle to produce both embryonic mortality and conversion to haploid male development in *Nasonia*. In control embryos, the maternal (red) and paternal (blue) genomes align in separate regions of the metaphase plate. Both sets of chromosomes are segregated equally to produce two diploid nuclei, where the maternal and paternal genomes mingle for the first time. In CI embryos, the paternal genome is somehow modified by *Wolbachia* and this causes its segregation at anaphase to be aberrant. One explanation for the range in segregation behavior of the paternal genome is that the level of *Wolbachia* modification varies from high to low. When *Wolbachia* modification is high, the paternal genome is not segregated and two haploid nuclei of maternal origin (two red nuclei) are produced (category 2). These embryos develop into males. When *Wolbachia* modification is moderate to low, the paternal genome is segregated abnormally. The paternal genome is either wholly segregated to one side of the spindle (category 3), producing one haploid nucleus of maternal origin (red nucleus) and one abnormal nucleus of maternal and paternal origin (red-blue nucleus); or the paternal genome is unequally segregated to both daughter nuclei (category 4), producing two nuclei that contain the maternal genome and unequal quantities of the paternal genome. Often the resulting nuclei are connected by chromatin bridges. Category 3 embryos continue development into a haploid male whereas category 4 embryos arrest development in early embryogenesis.

spindle checkpoint is activated because haploidy is not compatible with survival. However, in haplo-diploids these different effects can result in different phenotypic outcomes – embryonic lethality versus male haploid conversion.

Insights into the primary cause of spindle checkpoint activation come from cytological observation CI embryos. The earliest documented defects in CI embryos is a delay in nuclear envelope breakdown and Cdk1 activation in the male pronucleus (Tram and Sullivan, 2002). In addition, paternal chromosome condensation during prophase is severely disrupted (Callaini et al., 1997; Tram and Sullivan, 2002). There are two known mechanisms in which these events could lead to the activation of the spindle checkpoint. The most obvious is that disruptions in chromosome processing could lead to failed kinetochore microtubule attachment (Mikhailov et al., 2002). More recently, it has also been demonstrated that non-kinetochore DNA damage can induce a metaphase arrest (Royou et al., 2005). The mechanism by which this occurs is unresolved but there is evidence that this arrest is mediated through the spindle checkpoint as well as the DNA damage checkpoint (Smits et al., 2000; Mikhailov et al., 2002; Skouflas et al., 2004; Royou et al., 2005). Therefore, CI-induced disruption of the integrity or replication of the paternal chromosomes would probably lead to the observed metaphase arrest (Callaini et al., 1997).

In *N. vitripennis*, asymmetric segregation of damaged paternal chromosomes results in conversion

Our cytological data fit well with previous genetic studies suggesting that CI-induced conversion progeny in *N. vitripennis* result from exclusion of the paternal chromosomes from the first mitosis (Ryan and Saul, 1968). However, we discovered that conversion progeny can also arise from asymmetric segregation of the modified paternal chromosomes to only one of the two daughter nuclei during the first mitotic division. Thus in *N. vitripennis* even when mis-segregation of the paternal chromosomes occur, haploid nuclei bearing only the maternal chromosome complement arise. Whether specific mechanisms are involved in segregating the modified chromosomes to only one of the two daughter nuclei is unclear. Several drive phenomenon have been described that result in asymmetric segregation of specific chromosomes (Taylor, 2003). The paternal sex ratio (PSR) chromosome is an example of a supernumerary chromosome that acts as a selfish DNA element in *N. vitripennis* (reviewed by Werren and Stouthamer, 2003). PSR is transmitted via sperm and it causes the loss of the entire paternal genome at the first mitotic division. The PSR chromosome, however, is segregated along with the maternal chromosomes into the two daughter nuclei. The mechanism by which PSR induces loss of the paternal genome is not known but differs from that of *Wolbachia*-induced CI. In the presence of *Wolbachia*, the PSR chromosome is eliminated along with the paternal genome as occurs during CI (Reed and Werren, 1995; Werren and Stouthamer, 2003).

Genetic basis and evolution of CI-type

Of the seven haplo-diploids examined for CI-type, six result in mortality and only one, *N. vitripennis* results in conversion (Breeuwer, 1997; Vavre et al., 2000; Vavre et al., 2002; Bordenstein et al., 2003). A genetic analysis of the basis of CI-type differences in *Nasonia* revealed that conversion versus mortality CI is caused by genetic differences between the species, rather than by differences in *Wolbachia* between each species (Bordenstein et al., 2003). For example, interspecies crosses show the *N. vitripennis* phenotype regardless of whether the male or female is *N. vitripennis*. F1 hybrid females between the species show a conversion phenotype. Finally, genetic introgression of *N. vitripennis* Wolbachia into *N. giraulti* nuclear genetic background show the mortality CI phenotype typical of *N. giraulti*. These results indicate CI type among the *Nasonia* species is determined by nuclear genetic factors, and imply CI type arises from differences in how the egg processes the sperm for mitosis and/or differences in regulation of the mitotic checkpoints (S phase and/or spindle assembly).

Bordenstein et al. (Bordenstein et al., 2003) speculate that natural selection could have favored evolution of the conversion phenotype in *N. vitripennis*. The reasoning is that ongoing CI within the species would favor genotypes that 'convert' dead embryos into males. Given that *N. vitripennis* is more outbred than either *N. giraulti* or *N. longicornis*, conversion will be selectively favored more strongly in *N. vitripennis*.

This is the first study to associate differences in types of CI with specific cytological defects. Because the differences in CI between these species has been shown to be due to the genetic background of the host rather than differences in the *Wolbachia* and the three species are interfertile (Bordenstein et al., 2003), it may be possible to genetically identify host factors that interact with *Wolbachia* to influence processing of the sperm. The soon to be completed genome sequencing of the three species (J.H.W. et al., unpublished) should facilitate identification of candidate genes involved in these early developmental processes.

Materials and Methods

Stock maintenance

All stocks were maintained at 25±1°C under constant light and were reared on *Sarcophaga bullata* pupae. To examine CI, we used one infected stock and one uninfected stock, which was derived from the infected stock via antibiotic treatment (Breeuwer and Werren, 1990; Bordenstein et al., 2003). The infected stocks we used were LabII (*N. vitripennis*), IV7 (*N. longicornis*), and RV2 (*N. giraulti*) and the uninfected stocks were designated Asymc (*N. vitripennis*), IV7R3-1b (*N. longicornis*), and RV2R (*N. giraulti*). Individuals of each of the *Nasonia* species are normally doubly infected with two different Wolbachia, one each from the A group and B group Wolbachia. The *Wolbachia* present in each of the species are not closely related to each other, with the exception that the B group Wolbachia in *N. longicornis* and *N. giraulti* appear to have derived from a common ancestor (van Opijnen et al., 2005). The A Wolbachia in each species were apparently acquired by horizontal transfer from different insects subsequent to divergence of the *Nasonia* species, as were the B Wolbachia in *N. vitripennis* versus *N. giraulti* and *N. longicornis*. The *Nasonia* strains used in this study are each doubly infected with their species-specific A and B *Wolbachia*.

Egg-hatch assay

To determine whether CI embryos die during embryogenesis, we measured egg hatch. Virgin females and males were collected as pupae and kept in groups of 10-12 per vial. Upon emergence, adults were fed 10% sucrose for 2 days and males were transferred into the females' vials and the insects were allowed to mate overnight. Females were then transferred to individual egg collection chambers, which consisted of two P1000 pipet tips fitted together. The female was placed into the bottom tip and a Sarcophaga pupa was placed into the top tip, with the top of the pupa pointing down. This limits the female to only the head of the pupa for oviposition. Females were allowed to oviposit for 2 hours. Then, the Sarcophaga pupae were collected, dissected, and *Nasonia* eggs were gathered. The eggs were aligned on 2% agar plates containing food coloring, overlaid with Halocarbon oil, and incubated in a humidified Petri dish for 36 hours. Eggs that have hatched look like deflated footballs. We scored the number of hatched eggs and calculated percent egg hatch=number of hatched eggs/total number of eggs laid. A subset of Sarcophaga pupae was not dissected to allow the *Nasonia* embryos to develop until the pupal stage. Approximately 10 days after oviposition, the Sarcophaga pupae were dissected and the total number of *Nasonia* pupae inside were counted and sexed.

Embryo fixation and immunofluorescence analysis

Embryos were collected as described for the egg-hatch assay. Females were allowed to oviposit for 1 to 2 hours. Eggs were either processed immediately for fixing or aged 4 hours before fixing as described in Tram and Sullivan (Tram and Sullivan, 2002). Embryos were incubated in heptane for 3 minutes and then in heptane saturated with 37% formaldehyde for 45 minutes to 1 hour at room temperature. After fixation, the chorion and vitelline membrane were manually removed, using a 25-gauge needle. For immunofluorescence analysis, fixed embryos were treated with RNase A (10 mg/ml; Sigma) for 6-8 hours at 37°C, washed extensively with PBTA (1×phosphate-buffer saline, 0.1% bovine serum albumin, 0.05% Triton X-100, 0.02% sodium azide), washed several times with PBS and mounted in 90% glycerol, PBS containing 1 mg/ml N-N-1-4-phenylenediamine and 1 μg/ml propidium iodide. Microscopy was performed on either a Leica DM IRB inverted microscope equipped with the TCS SP2 confocal system or Zeiss LSM510 multiphoton confocal inverted microscope.

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