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A Genetic Test of the Role of the Maternal Pronucleus in Wolbachia-Induced Cytoplasmic Incompatibility in *Drosophila melanogaster*

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ABSTRACT

Cytoplasmic incompatibility (CI) is a reproductive sterility found in arthropods that is caused by the endoparasitic bacteria Wolbachia. In CI, host progeny fail to develop during early embryogenesis if Wolbachia-infected males fertilize uninfected females. It is widely accepted that this lethality is caused by some unknown Wolbachia-induced modification of the paternal nuclear material in the host testes. However, the direct means by which this modification leads to early embryonic death are currently unresolved. Results from previous studies suggested that CI lethality occurs as a result of asynchrony in cell cycle timing between the paternal and maternal pronuclei. This hypothesis can be tested experimentally by the prediction that the Wolbachia-modified paternal pronucleus should support androgenetic development (*i.e.*, from the paternal pronucleus only). Using specific mutations in *Drosophila melanogaster* that produce androgenetic progeny, we demonstrate that the Wolbachia-induced modification inhibits this type of development. This result suggests that CI occurs independently of the maternal pronucleus and argues against pronuclear asynchrony as the primary cause of CI lethality. We propose that CI occurs instead as the result of either a developmentally incompetent paternal pronucleus or asynchrony between the paternal pronucleus and the cell cycle of the egg cytoplasm.

WOLBACHIA is one of the most successful endoparasitic bacteria known, infecting 20–75% of all insect species (Werren *et al.* 1995; Jeyaprakash and Hoy 2000; Werren and Windsor 2000). Wolbachia, like mitochondria, are maternally transmitted and their widespread success is largely attributable to their ability to selectively favor infected females. The most common means used to achieve this is the induction of a reproductive sterility known as cytoplasmic incompatibility (CI) (reviewed in Stouthamer *et al.* 1999; Tram *et al.* 2003). In CI, host progeny die as early embryos if Wolbachia-infected males fertilize uninfected females, but this effect is suppressed if females carry the same Wolbachia strain. Thus, infected females have a distinct reproductive advantage over uninfected females. It is widely accepted that embryonic lethality due to CI is caused by an unknown Wolbachia-induced modification of the paternal nuclear material in the host testes (Bressac and Rousset 1993; Presgraves 2000; Clark *et al.* 2003). However, the molecular basis of this modification and how it leads to embryonic lethality remain unresolved.

Developmental defects resulting from CI are visible during the first zygotic mitosis (Figure 1). The first mitosis in insects is unique in that the paternal and maternal sets share a common spindle but remain physically separated by nuclear envelope remnants. As a result, the chromosomes segregate along two distinct microtubule bundles during anaphase and do not mix until the end of telophase (Callaini and Riparbelli 1996). In CI embryos, all events from fertilization to pronuclear juxtaposition appear normal (Reed and Werren 1995; Lassy and Karr 1996; Callaini *et al.* 1997; Tram and Sullivan 2002). However, Cdk1 activation (Tram and Sullivan 2002), nuclear envelope breakdown (NEB) (Tram and Sullivan 2002), and chromatin condensation of the paternal pronuclear material (Reed and Werren 1995; Lassy and Karr 1996; Callaini *et al.* 1997; Tram and Sullivan 2002) are delayed relative to the maternal pronuclear material. Maternal chromosomes separate normally during anaphase while the paternal set remains at the metaphase plate. By telophase, chromatin bridges form between separating daughter nuclei (Reed and Werren 1995; Lassy and Karr 1996). The daughter nuclei exhibit severe mitotic defects or fail to undergo mitosis altogether, resulting in aneuploidy and early lethality (Reed and Werren 1995; Lassy and Karr 1996). In Wolbachia-infected eggs, both pronuclei develop synchronously and normal embryonic development is restored (Reed and Werren 1995; Lassy and Karr 1996; Callaini *et al.* 1997; Tram and Sullivan 2002). Thus, the maternal presence of Wolbachia "rescues" or suppresses these developmental defects.

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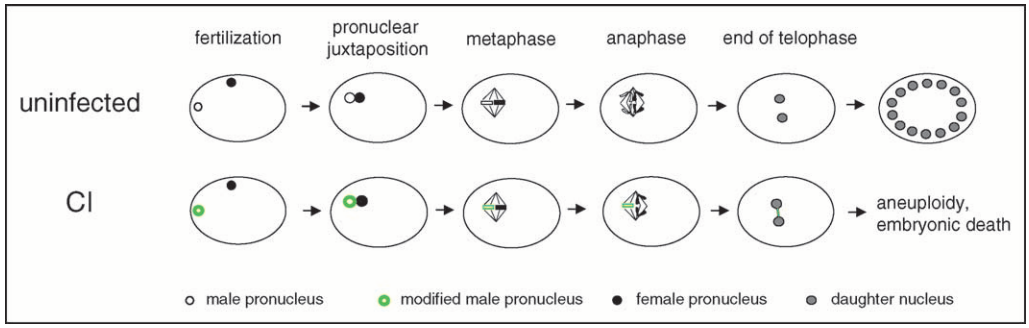

Figure 1.—CI results in mitotic defects observed during the first zygotic mitosis. In normal crosses (both parents uninfected), following fertilization, the male and female pronuclei juxtapose and undergo DNA synthesis. During the first mitotic cycle, chromatin of both pronuclei condenses into chromosomes (prophase, not shown) and aligns on the metaphase plate. In insects, paternal and maternal sets remain separate during the first division but share a common spindle (termed "gonomeric"). Chromosomes separate during anaphase, and by late telophase two daughter nuclei are present, each containing equal amounts of paternal and maternal nuclear material. Multiple rounds of synchronized nuclear divisions give rise to a multinucleated syncytial blastoderm. In CI crosses (male is infected and female is not), the paternal pronucleus carries a Wolbachia-induced modification. Pronuclear juxtaposition occurs normally. However, paternal chromatin condensation and nuclear envelope breakdown are delayed relative to normal maternal pronuclear progression. As maternal chromosomes separate normally during anaphase, improperly condensed paternal chromatin remains at the metaphase plate. By late telophase, paternal chromatin fails to separate normally, resulting in chromosome bridges between daughter nuclei. Subsequent nuclear divisions are abnormal and embryos fail to develop.

Several models have been proposed to explain the basis of CI (Poinsot *et al.* 2003). The "mistiming" model (Callaini *et al.* 1997; Tram and Sullivan 2002; Tram *et al.* 2003) proposes that embryonic lethality is caused directly by the developmental mistiming between the paternal and maternal pronuclei. To induce mistiming, Wolbachia may target components of the cell cycle machinery that specifically delay Cdk1 activation and mitotic progression of the male chromosomes (Tram and Sullivan 2002). Rescue, in this case, would involve the bacteria producing an equivalent delay in Cdk1 activation of the female pronucleus to restore developmental synchrony. The "lock and key" model (Breeuwer and Werren 1990; Hurst 1991; Kose and Karr 1995; Werren 1997; Poinsot and Mercot 1999) proposes that Wolbachia produces a "lock" (*e.g.*, a bacterial protein) that binds some component of the paternal pronucleus, preventing its normal behavior during the first mitosis. To restore pronuclear development, maternally derived Wolbachia would remove the lock in the egg. In the "titration-restitution" model (Kose and Karr 1995; Werren 1997), Wolbachia removes an essential protein from paternal nuclear material in the testes, and maternally derived Wolbachia restores this protein to reestablish normal development. In the latter two models, the primary cause of embryonic death is a developmentally incompetent paternal pronucleus and cell cycle mistiming between the paternal and maternal pronuclei is a secondary effect. Therefore, these models can be generally categorized into two classes on the basis of the mode of embryonic lethality: CI involves either (1) both paternal and maternal pronuclei in the case of the mistiming model or (2) exclusively the paternal pronucleus in the case of the lock and key and titration-restitution models.

We sought to distinguish between these possibilities by genetically testing the role of the female pronucleus in CI. To do this, we used Drosophila mutations that hinder formation of the maternal pronucleus and enhance embryonic development solely from the paternal pronucleus (*i.e.*, androgenetic development). We reasoned that if cell cycle asynchrony between paternal and maternal pronuclei is the cause of developmental failure, the Wolbachia-induced modification should not inhibit the paternal pronucleus from undergoing androgenetic development. We found, however, that the modified paternal pronucleus fails to support this type of development, suggesting that CI does not involve the maternal pronucleus.

MATERIALS AND METHODS

Drosophila lines and crosses: Experiments in this study involved a series of interspecies matings between *Drosophila melanogaster* females and *D. simulans* males. This allowed us to take advantage of maternal mutations in the former species that induce the formation of androgenetic progeny while simultaneously achieving high CI levels characteristic of the latter species. To assay interspecies CI levels, females from uninfected and *w*Mel-infected Oregon-R-derived *D. melanogaster* lines (Ferree *et al.* 2005) were mated with males from a *D. simulans white*^{1} line infected with the *w*Ri *Wolbachia* strain. The *D. simulans white*^{1} line was infected by introgression of *w*Ri bacteria from an infected wild-type *D. simulans* line (Turelli and Hoffmann 1995). Tetracycline treatment (2.5 mg/ml in yeast paste) of a subset of these flies was used to create an uninfected line having the same genetic background as the infected line. PCR of the Wolbachia 16S rRNA gene was used to confirm the infection status of these lines (O'Neill *et al.* 1992).

Two additional *D. melanogaster* lines, α*Tub67C*^{3} *ncd*^{D}/TM3 *Sb* and *ncd*^{D}/*ncd*^{D}, were used to generate α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} females that produce androgenetic progeny (Komma and Endow 1995). Through PCR of the Wolbachia 16s rRNA gene, the *ncd*^{D}/*ncd*^{D} line was found to be infected with the *w*Mel Wolbachia strain, while the α*Tub67C*^{3} *ncd*^{D}/TM3 *Sb* was uninfected. Because Wolbachia are transmitted from mother to offpring in the host, α*Tub67C*^{3} *ncd*^{D}/TM3 *Sb* virgin females

were crossed with ncd^D/ncd^D males to generate Wolbachia-uninfected $\alpha Tub67C^3$ ncd^D/ncd^D females. These females were crossed with Wolbachia-infected and uninfected *D. simulans* $white^1$ males (above) to assess the effects of CI on androgenetic development.

In all crosses, 3- to 5-day-old virgin *D. melanogaster* females were mated with 1- to 2-day-old *D. simulans* males. These crosses were performed *en masse* at a ratio of one female to three males to increase interspecies mating frequencies. After 2 days in the presence of males, each female was placed alone in a vial, allowed to lay embryos for 24 hr, and transferred into a new vial. For 2 successive days, females were transferred into new vials after each 24-hr period. Embryos were incubated at 23° for 24–30 hr and subsequently scored as hatched or unhatched. Females that produced only unhatched embryos over the course of 3 days were considered unfertilized and were removed from the analysis.

The $white^1$ allele carried on the paternal (*D. simulans*) X chromosome was used as a phenotypic marker for classification of hybrid progeny. Androgenetic individuals, carrying two *D. simulans* X chromosomes due to diploidization of the paternal set, were identified as white-eyed females. Normal hybrids heterozygous for $white^1$ were identified as red-eyed females. Normal red-eyed males failed to develop past the third instar larval-to-pupal transition due to a lethality effect of this interspecies cross (Sturtevant 1920; Sawamura 2000). Therefore, of males, only exceptional white-eyed X/O males, resulting from loss of the *D. melanogaster* X chromosome during meiosis, appeared. Other exceptionals, gynandromorphs, resulting from loss of the *D. melanogaster* X chromosome following the first mitosis, also resulted from this cross; these individuals were identified as having mosaics of white and red eye patches. The numbers of individuals in each genotype class from all mothers in a given cross (control or CI) were summed for statistical analysis.

Statistical analysis: A global contingency test was used to determine if CI differentially affects the development of androgenetic progeny relative to other sibling hybrid progeny. To do this, the observed progeny numbers in each genotype class (normal hybrid females, androgenetic hybrid females, and other exceptionals) were compared across experimental groups (control *vs.* CI) in a three-by-two contingency table (SYSTAT, version 10.2). The criterion for a significant interaction was a *P*-value ≤ 0.05.

For comparison, a similar contingency test was performed using the same data, except that the observed number of androgenetic progeny from CI crosses was replaced with the expected number of androgenetic progeny from CI crosses if cell cycle asynchrony directly causes embryonic lethality. This value was calculated on the basis of the assumption that although the cell cycle timings of paternal and maternal pronuclei are asynchronous in CI embryos, the developmental potential of the paternal pronucleus *per se* is normal. If true, then the Wolbachia-induced modification should not hinder diploidization of the paternal set during early development. Therefore, the proportions of androgenetic progeny resulting from control and CI crosses should be similar. Thus, the expected number of androgenetic progeny for this model ($x_{\text{exp-mistiming}}$) was calculated by the following equation:

$$x_{\text{exp-mistiming}} = (N_{\text{total-CI}})(x_{\text{observed}}/N_{\text{total-control}}).$$

Here, $N_{\text{total-CI}}$ is the total number of embryos laid in CI crosses, x_{observed} is the observed number of androgenetic progeny produced from control crosses, and $N_{\text{total-control}}$ is the total number of embryos laid in control crosses.

Molecular analysis of exceptional genotypes: Analytical restriction digestions were used to confirm that F_1 androgenetic progeny were homozygous for *D. simulans* autosomes. Primer pairs were designed to amplify short segments of coding sequence from the following genes in both species: *grapes* (*grp*) located on the left arm of chromosome 2 (Fogarty *et al.* 1997) and *aurora* (*aur*) located on the right arm of chromosome 3 (Glover *et al.* 1995). Each amplified product contains a unique restriction site that is present in one species but not in the other. Detailed information for all primer pairs and their amplified products is shown in the appendix.

Genomic DNA from individual flies was purified using the QIAamp DNA micro kit (QIAGEN, Valencia, CA). One microliter of DNA from a single individual was used as a template for PCR. Thermocycler conditions consisted of an initial melting period of 92° for 2 min; 35 cycles of 92° for 30 sec, 57° for 30 sec, and 72° for 30 sec; and a final extension period of 72° for 5 min. Following amplification, each product was digested with the appropriate restriction enzyme (appendix) for 2 hr and separated on a 1.25% agarose gel.

RESULTS

We genetically tested if developmental asynchrony between the paternal and maternal pronuclei kills CI embryos by assessing the ability of the Wolbachia-modified paternal pronucleus to support androgenetic development (Figure 2). To do this, we took advantage of *D. melanogaster* females doubly mutant for αTub67C, a maternal tubulin isoform required for proper meiotic spindle function in the egg, and the Kinesin-like motor protein, nonclaret disjunctional (NCD). It was previously shown that females carrying one copy of $\alpha Tub67C^3$ and two copies of ncd^D produce exceptional androgenetic progeny, *i.e.*, whose genotype is derived entirely from the paternal set (Komma and Endow 1995). Although a portion of embryos from $\alpha Tub67C^3$ ncd^D/ncd^D females develop normally, most die due to either defective formation of the maternal pronucleus or failure in its migration to the paternal pronucleus (Komma and Endow 1995). However, a fraction of these latter embryos develop into adults as a result of abnormal, early diploidization of the paternal chromosomes (Komma and Endow 1995). Of these embryos, those fertilized by sperm carrying an X chromosome give rise to androgenetic females, while those carrying a Y chromosome fail to develop because they lack an X chromosome. Therefore, of androgenetic progeny, only females develop from this cross. Taking advantage of this system, we crossed $\alpha Tub67C^3$ ncd^D/ncd^D females with Wolbachia-infected males to determine if the modified paternal pronucleus is able to support androgenetic development. We reasoned that if CI lethality occurs solely by cell cycle asynchrony between pronuclei, then androgenetic development from the paternal pronucleus, *i.e.*, without the maternal pronucleus, should not be inhibited by the Wolbachia-induced modification. If, however, this lethality results from a developmentally incompetent paternal pronucleus, then androgenetic development should be inhibited in CI crosses.

CI lethality levels are naturally low in *D. melanogaster* conspecific matings (Hoffmann 1988; Bourtzis *et al.*

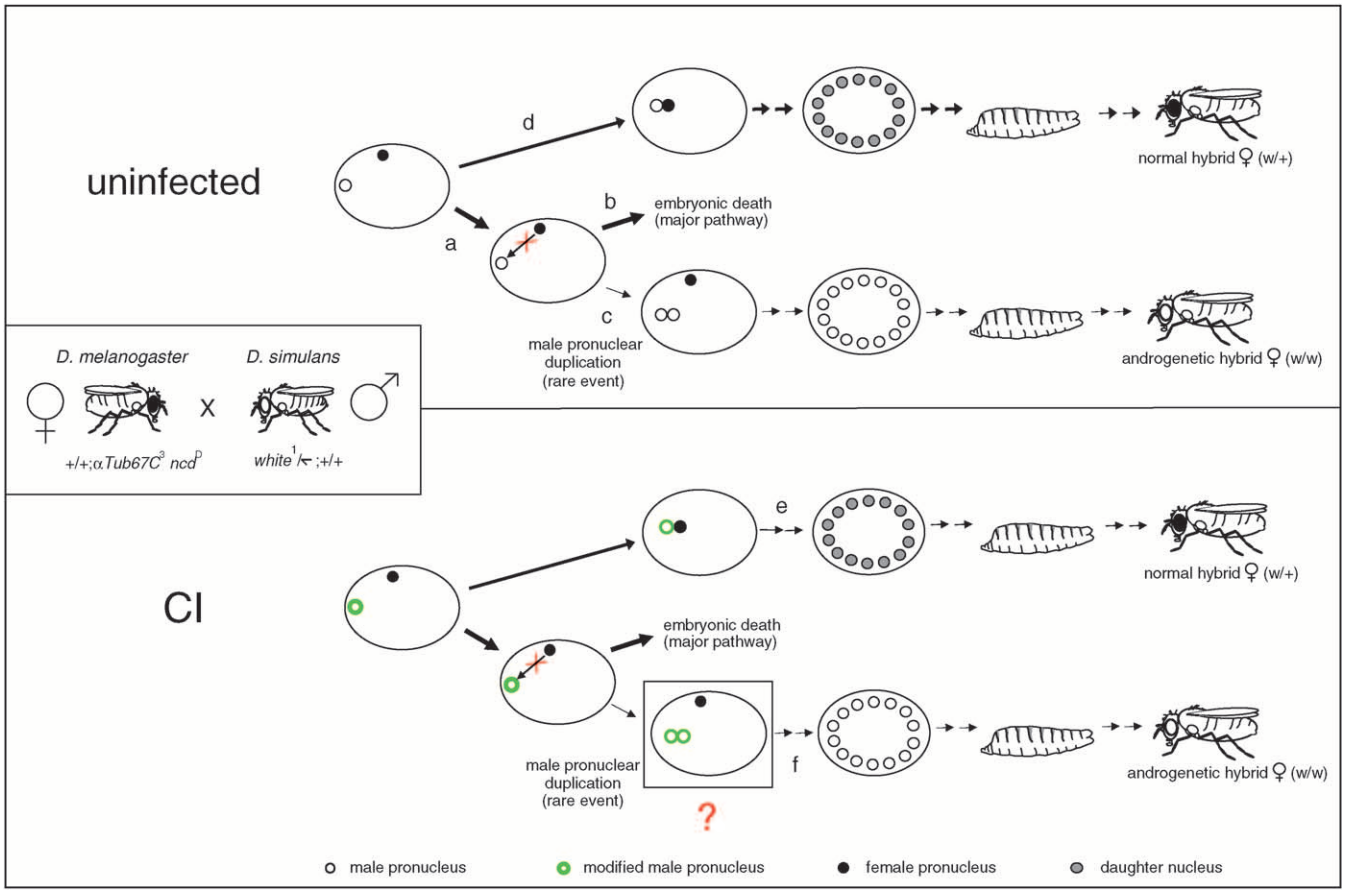

FIGURE 2.—Androgenetic progeny can be used to test whether the female pronucleus is involved in Wolbachia-induced cytoplasmic incompatibility (CI). The majority of embryos from *D. melanogaster* α*Tub67C*[3] *ncd*[D]/*ncd*[D] females die as a result of improper formation of the maternal pronucleus or its failure to migrate toward the paternal pronucleus (a and b). However, in a fraction of these embryos, the paternal set diploidizes (c), allowing these embryos to develop through adulthood. Because their nuclear material is derived solely from the father, these androgenetic progeny can be identified phenotypically by recessive markers carried on any of the four paternal chromosomes. In our experiments, we used the *white*[1] allele on the paternal X chromosome. The maternal mutations are incompletely penetrant, resulting in some progeny that arise via normal development (d). When uninfected α*Tub67C*[3] *ncd*[D]/*ncd*[D] females are crossed with Wolbachia-infected males, a large percentage of these normal progeny fail to develop past the first mitosis due to CI lethality (e). The question addressed with these crosses is whether the Wolbachia-modified paternal pronucleus is able to undergo diploidization (f), as assessed by the presence of androgenetic progeny. For both control and CI crosses, *D. melanogaster* α*Tub67C*[3] *ncd*[D]/*ncd*[D] females were mated with *D. simulans* males to achieve high lethality in the CI cross. As a result, all progeny are hybrid females; hybrid males die as third instar larvae.

1994; HOFFMANN *et al.* 1994). To achieve high CI lethality levels in our experiments, we performed interspecies matings between *D. melanogaster* females and *D. simulans* males infected with the *w*Ri *Wolbachia* strain. This interspecific CI cross has been used in one previous study investigating the sperm contribution to CI (PRESGRAVES 2000). Uninfected parents of this cross produce viable but infertile hybrid females, while hybrid males die at the late larval stage (STURTEVANT 1920; SAWAMURA 2000). This male-specific hybrid lethality does not interfere with our analysis since all surviving androgenetic hybrid progeny are females due to duplication of the paternal X chromosome, as well as all three autosomes.

To assess CI lethality levels achievable through this interspecies cross, uninfected Oregon-R-derived *D. melanogaster* females were mated with either uninfected or *w*Ri-infected *D. simulans white*[1] males. Crosses with uninfected *D. simulans white*[1] males resulted in an overall hatch rate of 92.5%, a larval-to-adulthood survivorship of 51.2%, and all adult progeny were hybrid females (Table 1). This suggests that similar numbers of males

TABLE 1

Progeny survivorship of *Drosophila melanogaster* wild-type females crossed with *D. simulans white*[1]/Y;+/+ males

Cross	Fertilized mothers	Embryos laid	Embryos hatched (%)	F_1 adults[a] (%)
Control	$n = 31$	4712	4357 (92.5)	2232 (51.2)
CI	$n = 36$	8711	1286 (14.8)	838 (65.0)

[a] All adult hybrid progeny from these crosses were red-eyed females.

TABLE 2

Progeny survivorship of *D. melanogaster* +;α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} females crossed with *D. simulans white*^{1}/Y;+/+ males

Cross	Fertilized mothers	Embryos laid	Embryos hatched[a] (%)	F_1 adults[b] (%)
Control	$n = 185$	30,671	7,648 (25.0)	1,904 (24.9)
CI	$n = 194$	36,238	2,248 (6.2)	537 (23.9)

[a] Percentages are relative to the number of embryos laid.
[b] Percentages are relative to the number of embryos hatched.

TABLE 3

Genotype frequencies of exceptional hybrid progeny resulting from *D. melanogaster* +;α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} females crossed with *D. simulans white*^{1}/Y;+ males

Cross	$+_{mel}/white^1_{sim}$ females	$white^1_{sim}/white^1_{sim}$ females	Other exceptionals[a]
Control	1781	7	116
CI	505	1 ($x_{\text{exp-mistiming}} = 8.3$)[b]	31

[a] These include hybrid X_{sim}/O males and gynandromorphs, which result from loss of the X_{mel} chromosome either during meiosis or following the first zygotic mitosis, respectively.
[b] Observed and expected values differ to $P < 0.02$.

and females hatched into larvae but the males died before reaching adulthood. However, crosses with wRi-infected *white*^{1} males yielded an overall hatch rate of 14.8% (Table 1), indicating that CI kills hybrid embryos at levels comparably high to those of *D. simulans* conspecific matings (Hoffmann *et al.* 1986). The larval-to-adulthood survivorship for these crosses was 65.0% (Table 1), and like control crosses, all adult progeny were hybrid females. CI, therefore, does not appear to appreciably affect hybrid male larval lethality or hybrid female survivorship to adulthood. Additionally, *D. melanogaster* females infected with the *w*Mel Wolbachia strain when crossed with *w*Ri-infected *D. simulans white*^{1} males restored the embryonic hatch rate to 71.8%, confirming that the observed embryonic lethality is due to Wolbachia and is not an effect of the hybrid cross.

To determine the baseline frequency of androgenetic progeny produced from interspecies crosses, uninfected *D. melanogaster* α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} females were mated with uninfected *D. simulans white*^{1} males. These crosses yielded an embryonic hatch rate of 24.9% (7648/30,671 embryos) resulting from the mutant maternal genotype (Table 2). Of 7648 embryos hatched, 1904 (24.9%) developed into adulthood, demonstrating that the maternal genotype causes a larval–pupal lethality of ~50%, in addition to the ~50% larval lethality due to the hybrid cross (Table 2). Of the adult hybrid progeny, 1781 (93.5%) were normal red-eyed (+/*white*^{1}) females, 7 (0.4%) were white-eyed (*white*^{1}/*white*^{1}) androgenetic females, and 116 (6.1%) were X/O (*white*^{1}/O) males and gynandromorphs resulting from loss of the *D. melanogaster* X chromosome during meiosis and after the first embryonic division, respectively (Table 3).

The *white*^{1} allele is sufficient as a means to identify androgenetic progeny. However, this marker directly indicates only the parental origin of the X chromosome and not the autosomes. To confirm that *white*^{1}/*white*^{1} hybrid females are also homozygous for *D. simulans* autosomes, we performed restriction analysis on amplified sequences from genes located on chromosomes 2 and 3 from both parents and one individual of each hybrid genotype (Figure 3). Amplified products were designed to contain a unique restriction site present for one species but not the other, allowing us to determine from which parent(s) the F_1 hybrid chromosomes are derived (appendix). As expected, the white-eyed hybrid female exhibited restriction digestion signatures identical to those of the *D. simulans* father, whereas the red-eyed hybrid female, *white*^{1}/O male, and gynandromorph showed restriction digestion signatures of both *D. simulans* father and *D. melanogaster* mother combined. These results show that white-eyed hybrid females are, indeed, androgenetic, arising solely from the paternal set.

To determine if CI affects androgenetic development, uninfected α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} females were crossed with *w*Ri-infected *white*^{1} males. These crosses resulted in an overall embryonic hatch rate of 6.2% (2248/36,238 embryos) (Table 2) and a CI-specific lethality level of 76.0%. Of 537 adult hybrid progeny, 505 (94.0%) were normal *white*^{1}/+ females, 1 (0.2%) was a *white*^{1}/*white*^{1} androgenetic female, and 31 (5.8%) were X/O males and gynandromorphs (Table 3). A three-by-two global contingency test was used to statistically compare these genotype numbers to those derived from control crosses described above. This analysis shows that all genotype classes were proportionally decreased by CI ($\chi^2 = 0.503$, $P = 0.778$, d.f. = 2). Therefore, androgenetic progeny are subject to CI lethality levels comparable to those of their siblings. Additionally, a similar global contingency test was performed, except that the observed number of androgenetic progeny for CI crosses was replaced with the expected number of androgenetic progeny predicted to result if the paternal and maternal pronuclei are together required for CI expression ($x_{\text{exp-mistiming}} = 8.3$, see materials and methods for calculations). This value, if obtained, would have differed significantly in proportion to those of the other genotype classes ($\chi^2 = 8.532$, $P = 0.014$, d.f. = 2). Taken together, these results show that CI significantly decreases the number of androgenetic progeny, suggesting that CI lethality does not involve the maternal pronucleus.

DISCUSSION

Three models have been previously proposed to explain the basis of CI (Poinsot *et al.* 2003). The mistiming

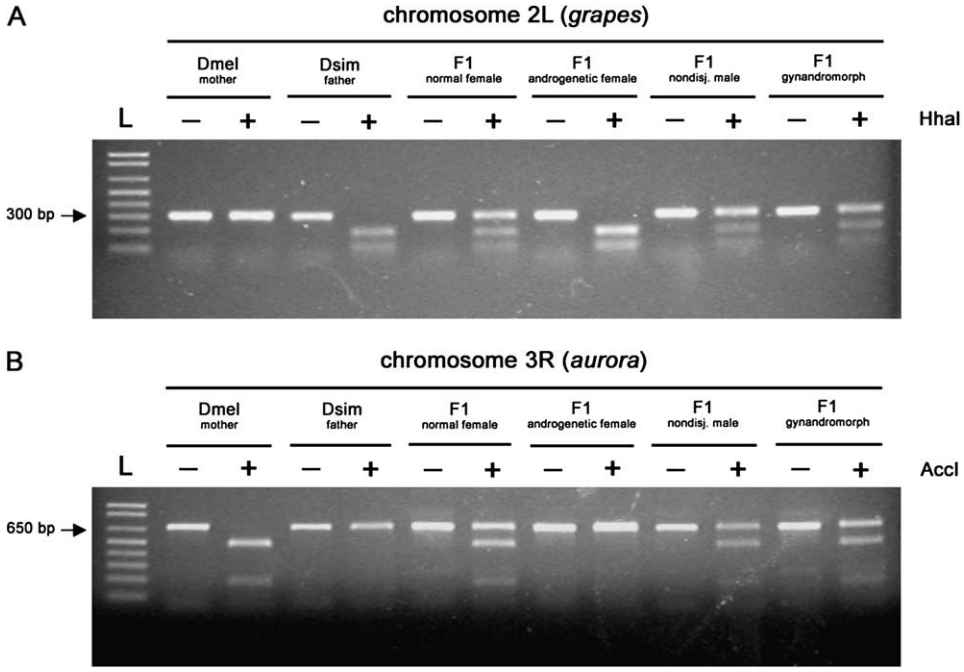

Figure 3.—Molecular genotyping of hybrid progeny using restriction analysis of PCR products amplified from chromosomes 2 and 3. (A) *Hha*I restriction digestion patterns are shown for a 306-bp PCR product amplified from the *grapes* gene located on the left arm of chromosome 2 for the following individuals (from left to right): a *D. melanogaster* α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} female, a *D. simulans white*^{1} male, a normal *white*^{1}/+ hybrid female, an androgenetic *white*^{1}/*white*^{1} hybrid female, a *white*^{1}/O nondisjunctional hybrid male, and a gynandromorphic hybrid that is mosaic for patches of *white*^{1}/O and *white*^{1}/+ cells. Undigested (−) and *Hha*I-digested (+) products are shown for each individual. The *D. simulans* product contains a single *Hha*I restriction site that, when cleaved, produces two fragments of 195 and 111 bp. The *D. melanogaster* product contains no *Hha*I restriction site. (B) *Acc*I restriction digestion patterns are shown for a 675-bp PCR product amplified from the *aurora* gene located on the right arm of chromosome 3 for the same individuals in A. Undigested (−) and *Acc*I-digested (+) products are shown for each individual. In this case, the *D. melanogaster* product contains a single *Acc*I restriction site that, when cleaved, produces two fragments of 522 and 153 bp. The *D. simulans* product does not contain an *Acc*I restriction site. (A and B) In both gels the first lane is a molecular ladder (L) with fragment sizes of 100, 200, 300, 400, 500, 650, 850, and 1000 bp.

model (Callaini *et al.* 1997; Tram and Sullivan 2002; Tram *et al.* 2003) presumes that Wolbachia slows the cell cycle timing of the paternal pronucleus relative to the maternal pronucleus. In this case, pronuclear asynchrony *per se* leads to embryonic death. The lock and key and titration-restitution models propose that Wolbachia adds a bacterial factor to or removes an important host factor from the paternal pronucleus, respectively, leading to its developmental failure (Breeuwer and Werren 1990; Hurst 1991; Kose and Karr 1995; Werren 1997; Poinsot and Mercot 1999). Previous cytological studies are consistent with each of these models (Poinsot *et al.* 2003) and, therefore, have not led to any strong conclusions on how CI operates.

A fundamental difference among these models is the role of the maternal pronucleus in CI. In the mistiming model, the maternal pronucleus is directly involved in embryonic lethality, whereas in the lock and key and titration-restitution models, it plays no role. In this study, we used a genetic assay based on androgenetic development to distinguish between these possibilities. This involved crosses between *D. melanogaster* α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} mothers that produce androgenetic progeny and Wolbachia-infected *D. simulans* males. Although the precise mechanism of androgenetic development is not known, previous cytological observations have shown that the majority of embryos derived from α*Tub67C*^{3} *ncd*^{D}/*ncd*^{D} mothers contain defective meiotic spindles (Komma and Endow 1995). As a result, the maternal pronucleus either is severely abnormal or fails to develop altogether in these embryos (Komma and Endow 1995). Another study demonstrated that embryos carrying other mutant alleles of αTub67C produced abnormally short astral microtubules nucleated from the sperm basal body, which are important for migration of the maternal pronucleus to the paternal pronucleus (Mathe *et al.* 1998). Together, these findings suggest that the early steps of androgenetic development involve diploidization of the paternal set and subsequent development without the maternal pronucleus. In effect, this removes any source of pronuclear developmental mistiming, allowing us to ask if the Wolbachia-modified paternal pronucleus retains the ability to develop alone. Our results demonstrate that the modified paternal pronucleus fails to support androgenetic development, suggesting that the female pronucleus is not necessary for CI lethality.

To our knowledge, this is the first study reporting the production of androgenetic hybrids, having all *D. simulans* chromosomes in a *D. melanogaster* cytotype. That these exceptionals are homozygous for all *D. simulans* chromosomes is supported by the presence of two copies of the *white*^{1} allele carried on the paternal X chromosome and by *D. simulans*-like restriction digestion patterns for PCR products amplified from chromosomes 2 and 3. Classical interspecies crosses between *D. melanogaster* females and *D. simulans* males established that hybrid progeny that do not contain at least one *D. simulans* X chromosome (*e.g.*, $X_{mel}Y_{sim}$ males and exceptional $X_{mel}X_{mel}Y_{sim}$ females) are inviable (Sturtevant

1920, 1929). More recent work suggests that this effect may be due to an interaction between the *Hybrid male rescue* (*Hmr*) locus on the *D. melanogaster* X chromosome and the *Lethal hybrid rescue* locus (*Lhr*) on *D. simulans* chromosome 2 (Barbash *et al.* 2000). Because androgenetic females contain all *D. simulans* chromosomes, no such genetic conflicts should exist. Indeed, this appears to be the case, as suggested by the emergence of these individuals in our crosses. Furthermore, androgenetic hybrid females are fully fertile when mated with *D. simulans* males (our unpublished results). The observed frequency of androgenetic progeny reported here is lower than that found for conspecific crosses (Komma and Endow 1995). This effect may be attributable either to a low expressivity of the $\alpha Tub67C^{3}$ or ncd^{D} alleles in hybrid embryos or to a general loss of strength of these alleles over time.

Our finding that CI reduces the number of androgenetic progeny is inconsistent with the mistiming model, which presumes that cell cycle asynchrony between paternal and maternal pronuclei is the direct cause of embryonic lethality. Instead, it is possible that Wolbachia renders the paternal pronucleus developmentally incompetent, an idea compatible with either the lock and key or the titration-restitution model. Bacterial manipulation of some component of the paternal pronuclear material may present a physical constraint to normal progression of the paternal set through the first mitosis. This idea is supported by previous cytological studies in CI embryos from *D. simulans* and the haplodiploid wasp *Nasonia vitripennis*, in which paternal chromatin appears undercondensed and is subsequently lost from the zygotic nucleus (Breeuwer and Werren 1990; Lassy and Karr 1996; Callaini *et al.* 1997).

To render the paternal pronucleus developmentally incompetent, Wolbachia may target components of the sperm chromatin or nuclear envelope (Harris and Braig 2003; Tram *et al.* 2003). Following fertilization, the needle-shaped sperm nucleus is converted into a spherical pronucleus. This process involves the formation of a new pronuclear envelope composed of maternal proteins and the replacement of paternal histones, or protamines, with maternal histones (Tram *et al.* 2003). Despite the fact that the sperm nucleus undergoes proper shape change in CI embryos (Lassy and Karr 1996; Callaini *et al.* 1997; Tram and Sullivan 2002), it is currently not known if formation of the pronuclear envelope and repackaging of the chromosomes occur normally (Tram *et al.* 2003). Both of these events are required for paternal DNA replication (Liu *et al.* 1997; Loppin *et al.* 2001). Interestingly, the chromosome bridges that form between separating daughter nuclei in CI embryos are reminiscent of those that form as a result of genetic or chemical interference of DNA replication and condensation (Uemura *et al.* 1987; Buchenau *et al.* 1993; Christensen and Tye 2003). Bacterial manipulation of paternal chromatin may be read as "damage" by the cell, resulting in the activation of conserved checkpoints operating in the early embryo and subsequent delay of Cdk1 activity on the paternal half of the gonomeric spindle. That Cdk1 activity can be delayed specifically in the paternal half of the spindle is supported by previous experiments in sea urchin embryos in which damage of paternal DNA induces checkpoints that specifically slow the paternal cell cycle independently of the maternal cell cycle (Sluder *et al.* 1995). Any bacterial modification that inhibits DNA replication and (or) condensation would likely be removed or otherwise suppressed in Wolbachia-infected eggs shortly after fertilization to allow these processes to occur normally.

Another possibility is that CI lethality results from asynchrony between the modified paternal pronucleus and the cell cycle of the egg cytoplasm. Previous experiments involving nuclear manipulations in Xenopus established that the cell cycle of the egg cytoplasm is able to progress independently of the nuclear cell cycle. For example, Cdk1 oscillations occurred normally in eggs in which the nucleus was removed (Lohka and Masui 1983; Murray and Kirschner 1989). Furthermore, transplantation of nuclei from nonmitotic cells resulted in DNA synthesis and mitotic cycles characteristic of the recipient eggs (Graham *et al.* 1966), indicating that the cytoplasmic cell cycle influences nuclear divisions. In the case of CI, the egg cell cycle may be initially slowed by checkpoints that are activated by abnormal paternal pronuclear behavior. However, if paternal development is slowed enough, checkpoints would eventually be overridden and the cytoplasmic cell cycle would resume, likely leading to mitotic defects. Rescue, in this case, could involve Wolbachia conditioning the egg cytoplasm to slow its cell cycle timing to accommodate the modified paternal pronucleus. Because the cell cycle of the egg cytoplasm influences the rate of nuclear mitotic progression (Graham *et al.* 1966; Von Beroldingen 1981), an egg whose cell cycle is slowed by the presence of Wolbachia would be compatible with either a modified or an unmodified paternal pronucleus.

The idea that CI results from nuclear/cytoplasmic asynchrony is supported by studies of the "filicidal" ring-Y chromosome in the *D. melanogaster* early embryo. The presence of this chromosome causes the formation of chromosome bridges, atypically small (pycnotic) nuclei, and eventual embryonic death (Stone 1982). Currently it is not understood how the normally dispensable Y chromosome is able to affect the mitotic behavior of the entire nuclear set. However, Stone (1982) suggested that ring-Y-induced embryonic lethality may occur from a loss of nuclear/cytoplasmic cell cycle synchrony. The formation of anaphase bridges may retard nuclear division, forcing daughter nuclei to enter into DNA replication and mitosis under inappropriate states of chromatin condensation. Interestingly, ring-Y mitotic

defects occur in eggs derived from ring-Y-sensitive (RS) lines but not eggs derived from other nonsensitive (non-RS) lines, indicating that these effects are determined in large part by the maternal genotype (Oster 1964). Further, crosses between attached X/ring-Y females and RS-derived males resulted in the absence of mitotic defects, suggesting that RS factors operate only in the egg (Stone 1982). In a similar manner, maternal genotype also contributes to the outcome of CI in Nasonia species. CI in *N. vitripennis* results in complete loss of paternal chromosomes and conversion of embryos into haploid males, while CI in *N. giraulti* results in partial loss of paternal chromosomes and embryonic death (Bordenstein *et al.* 2003). However, crosses between Wolbachia-infected *N. giraulti* males and uninfected *N. vitripennis* females result in conversion only to males and not a mixture of both phenotypes (Bordenstein *et al.* 2003). This maternal effect could be explained by species-specific differences in cytoplasmic cell cycle timing that result in varying degrees of paternal chromatin retention.

Together, these observations make it clear that elucidating the molecular targets of CI will require a thorough examination of molecular and cellular processes that occur between sperm maturation and pronuclear fusion in both wild-type and CI embryos.

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APPENDIX

PCR primers and amplified product information used for autosomal genotyping of hybrid progeny

Primers	Sequence (5′–3′)	Gene (chromosome arm)	Product size (bp)	Unique restriction site (species)	Fragment sizes (bp)
2LGrp5′	ACGCTTCCCAGAACTATTTCC	*grapes* (2L)	306	*Hha*I (*D. sim*)	195,111
2LGrp3′	TATCCTCCAGGGCGTTTTTG				
3RAur5′	CTTTGATATTGGTCGCCTGC	*aurora* (3R)	675	*Acc*I (*D. mel*)	522,153
3RAur3′	GATGCTGCCTTGGAAATGTG				