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Overcoming cytoplasmic incompatibility in *Drosophila*

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The endocellular microbe *Wolbachia pipientis* infects a wide variety of invertebrate species, in which its presence is closely linked to a form of reproductive failure termed cytoplasmic incompatibility (CI). CI renders infected males unable to father offspring when mated to uninfected females. Because CI can dramatically affect fitness in natural populations, mechanisms that abate CI can have equally large impacts on fitness. We have discovered that repeated copulation by *Wolbachia*-infected male *Drosophila simulans* significantly diminishes CI. Repeated copulation does not prevent *Wolbachia* from populating developing spermatids, but may reduce the time during spermatogenesis when *Wolbachia* can express CI. This restoration of fertility in premated infected males could have important implications for *Wolbachia* transmission and persistence in nature and for its exploitation as an agent of biological pest control.

Keywords: cytoplasmic incompatibility; *Wolbachia*; sperm; fertilization; *Drosophila*

1. INTRODUCTION

Processes that influence reproductive success and reproductive compatibility are essential to an individual's fitness and a population's maintenance and growth. Likewise, mechanisms responsible for reproductive isolation within a species affect reproductive success and can therefore negatively affect fitness. One such form of reproductive isolation is cytoplasmic incompatibility (CI). In all cases studied, CI is intimately associated with the presence of an endosymbiotic microorganism *Wolbachia* (Binnington & Hoffmann 1989; Hertig 1936; Hsiao & Hsiao 1985; Laven 1959; Yen & Barr 1973). CI renders infected males unable to father offspring when mated to uninfected females. Infected males mate normally and transfer sperm, which fertilize the egg, but the egg fails to complete karyogamy and dies (Callaini *et al*. 1996; Jost 1970; Lassy & Karr 1996). Reciprocal crosses between uninfected males and infected females are viable but, because *Wolbachia* are transmitted vertically through the female, all resulting progeny are infected. Thus, due to the non-reciprocal nature of the crossing types, *Wolbachia* will spread through an uninfected population (Fine 1978; Hoffmann *et al*. 1986; Turelli & Hoffman 1995). Insofar as multiple mutually incompatible mating types can arise within a single species, an infected species can subdivide into reproductively isolated daughter species and gene flow can diminish (Fine 1978; Laven 1959). Thus, infected strains maintain a reproductive advantage over uninfected strains (Hurst & McVean 1996; Laven 1967; Turelli & Hoffman 1995), and suggest that *Wolbachia*-infected strains will spread at the expense of uninfected populations. Indeed, such a predicted spread of infected *Drosophila simulans* has occurred in California during the past five years (Turelli & Hoffman 1995; Turelli & Hoffmann 1991). Not surprisingly, CI has received considerable attention from evolutionary biologists and geneticists, and has been touted as a potential means of biological pest control (Karr 1994; Laven 1959).

CI is not a rare phenomenon affecting few species, but instead occurs in a wide variety of insects, including flies, wasps, and mosquitoes, spanning at least six orders of insects that last had a common ancestor over 200 million years ago (Breeuwer *et al*. 1992; O'Neill *et al*. 1992; Rousset *et al*. 1992*a*; Werren *et al*. 1995). *Wolbachia*-infected strains of the isopod, *Armadillium vulgare* have been shown to cause feminization of males (Rousset *et al*. 1992*b*). More importantly, molecular phylogenetic studies of *Wolbachia* in these divergent host species reveals that they are essentially monophyletic, consisting of two strains, A and B (Werren *et al*. 1995). In agreement with the phylogenetic data, *Wolbachia* can be successfully transferred between species, and such transinfected strains can express CI (Boyle *et al*. 1993; Braig *et al*. 1994; Clancy & Hoffmann 1996; Giordano *et al*. 1995). The fact that a symbiotic bacterium can so profoundly influence the reproductive success of its host in such widely divergent species strongly suggests that CI affects highly conserved, fundamental processes central to the reproductive success of its host.

In laboratory *Drosophila* strains, CI results in high levels of egg lethality (greater than 75%) in incompatible crosses, i.e. between infected male and uninfected female *D. simulans*. Within-strain crosses between infected or uninfected *D. simulans* and inter-strain crosses between uninfected males and infected females are compatible, resulting in lower levels (less than 10%) of egg lethality. In nature, CI expression in *D. simulans* is generally lower and more variable than the laboratory, ranging from 20–70% egg mortality, and is thought to correlate with frequency of infection and/or bacterial density (Turelli & Hoffman 1995). This discrepancy between laboratory and wild CI levels is often ascribed to imperfect maternal

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transmission, temperature, male age or naturally occurring antibiotics (Hoffmann *et al*. 1986; Turelli & Hoffman 1995).

Because infected males produce mature sperm devoid of *Wolbachia* (as measured by polymerase chain reaction (PCR); T. Karr, unpublished data), and because sperm fertilize eggs in an incompatible cross (Callaini *et al*. 1996; Lassy & Karr 1996), *Wolbachia* presumably alter cellular function(s) during earlier stages of spermatogenesis. We therefore reasoned that natural activities that alter this interaction might abate CI. While studying the effect of various environmental factors on the expression of CI, we discovered that repeated copulation by *D. simulans* males reduces CI expression in subsequent matings.

Although the basis for this effect on male fertility is unknown, it may relate to differences in timing of spermatogenesis during larval and adult periods: recently-eclosed males contain sperm that have been in contact with *Wolbachia* for much of their pre-adult development, whereas the sperm of premated adults have been in contact with *Wolbachia* for a briefer time. We discuss the implications of these results for the population biology of infected *Drosophila* and transmission dynamics of *Wolbachia* in nature.

2. MATERIALS AND METHODS

(a) *Strains used*

The strains used were (i) infected *D. simulans*, originally collected near Riverside, California (DSR), and (ii) an uninfected strain derived from DSR (DSRT) (Hoffmann *et al*. 1990; O'Neill & Karr 1990). The infection status of the DSR and DSRT strains were confirmed by PCR and DAPI staining as previously described (O'Neill *et al*. 1992; O'Neill & Karr 1990).

(b) *CI assays*

CI, reported as the per cent egg mortality, was determined as described (Boyle *et al*. 1993). In brief, CI assays were performed with a manifold that can accommodate 20 single-pair matings. Routinely, at 24 h intervals on 3–4 successive days, food plates were replaced and eggs counted. Unhatched eggs were counted after 24 h, and again 12 h later. CI was calculated as: (unhatched/total eggs) × 100%. Analysis of CI by single female matings yields superior statistics because individual females that are sterile for reasons unrelated to the experiment can be eliminated from the analysis. Single female analysis also provides information on among-female variation in egg laying and egg hatch rates. CI was analysed statistically by standard ANOVA (StatView 4.5, Abacus Concepts).

(c) *Virgin females*

To ensure the virginity of females used in the CI measurements, females were collected at periodic intervals (8–12 h post-eclosion), and stored in food vials prior to mating. Only females that oviposited unfertilized eggs were used in subsequent CI assays. Because maintenance of virgin females on yeasted media can stimulate oviposition of unfertilized eggs, and thus overestimate CI in incompatible crosses, we also performed compatible crosses with similarly treated virgin females as controls.

(d) *Sequential male assay*

Virgin males were collected within 6–8 h of eclosion and aged for one day before mating. Thereafter, each male was mated to a new virgin female on each of four successive days. Each mating pair was placed in a manifold, and assayed for CI. Control CI crosses with males collected and aged as above were performed with singly mated males and females, which were kept together during the same four-day period.

(e) *Premating males*

Individual one-day-old DSR males were placed with five females in food vials for 24 h. Vials were observed for mating activity at 15–30 min intervals for the first 8 h, and only males that had mated at least twice were used in subsequent CI assays. Flies were allowed to continue mating overnight, after which males were isolated from females and used in subsequent CI assays. Following premating, males were allowed to recover for 24–48 h and individual males mated with individual uninfected virgins. CI was determined by egg hatch rates as described above. Uninfected DSRT males were treated identically and CI was measured in the same manner.

(f) *Fluorescence microscopy*

To test for the persistence of *Wolbachia* in sperm, *D. simulans* males were premated as described above, and their testes dissected and fixed in 3.7% formaldehyde. Controls were treated similarly but not premated. *Wolbachia* were visualized with the DNA-specific, fluorochrome, DAPI (Sigma) as described (O'Neill & Karr 1990). DSR larval testes were dissected in Ringers, fixed in 3.7% formaldehyde and stained with DAPI. Images were recorded with a Zeiss Axioplan2 microscope and C-Apo 40X/n.a. 1.2 lens, equipped with epi-fluorescent optics and a Princeton Instruments MicroMax CCD camera.

3. RESULTS AND DISCUSSION

Because multiple matings reduce male fertility in *D. melanogaster*, a sibling species of *D. simulans* (Fowler 1973; Lefevre & Jonsson 1962), we first performed controls to establish that the repeated mating required for subsequent experiments did not reduce the fertility of compatible crosses. CI was measured in males that had copulated at least twice within one day of eclosion (§ 2). These males were allowed one day to recover and were then mated again to virgin females, and egg hatch rates were counted on each of four successive days. Male premating, followed by a 24 hour recovery in compatible crosses (table 1, crosses *c*,*d*,*e*) yielded estimates of male fertility equivalent to those reported in many studies of unmated *Drosophila* (Boyle *et al*. 1993; Hoffmann *et al*. 1986; Markow *et al*. 1978; O'Neill & Karr 1990). Thus, premating males followed by a 24 hour recovery did not reduce male fertility.

Next, we performed similar experiments to examine the effect of premating of *Wolbachia*-infected strains of *D. simulans* on the expression of CI. The overall egg lethality of uninfected eggs fertilized by premated infected males was 26% during the four days (table 1, cross *b*), whereas eggs fertilized by non-sperm depleted male controls died at much higher rates (75% egg lethality; table 1, cross *a*). This difference is statistically significant (see table 1 legend), and suggests two classes of sperm, each differing in their ability to express CI. One class is represented by first-born sperm in virgin males, which express high levels of CI, and a second class by sperm that arise *de novo* in the adult, expressing CI at lower levels.

We explored further the effect of repeated copulation by monitoring CI in crosses with 1–2-day-old males mated to

Table 1. *Effect of mating history on cytoplasmic incompatibility* (*CI*)

(Egg hatch for each cross was measured under identical conditions in a single-pair mating manifold. Egg counts were reported as per cent CI. Within each cross, neither the number of eggs laid nor the number of unhatched eggs differed during the four days in which CI was measured. Pairwise ANOVA revealed significant differences ($p < 0.001$; Fisher's PLSD) for all cross-comparisons except cross *c* versus cross *d*. DSR, *Wolbachia*-infected strain; DSRT, uninfected strain.)

	cross ♀	cross ♂	treatment	#eggs (4 d)	#unhatched eggs (4 d)	#females	#eggs/female/ day mean (s.e.)	egg mortality (%) mean (s.e.)
a	DSRT	DSR	none	696	517	16	11.6 (3.3)	75.1 (3.1)
b	DSRT	DSR	sperm depleted	736	193	15	12.3 (4.1)	26.2 (2.9)
c	DSR	DSR	sperm depleted	725	90	16	12.3 (1.2)	12.4 (2.0)
d	DSRT	DSRT	sperm depleted	469	43	17	27.6 (2.2)	9.2 (2.8)
e	DSR	DSRT	none	788	91	15	13.1 (1.3)	11.5 (2.2)

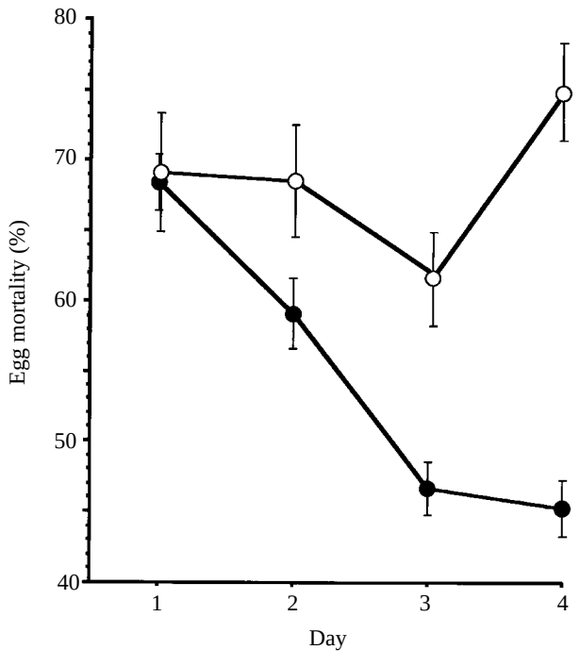

Figure 1. The effect of successive copulation of infected males with uninfected virgin females. CI, reported as per cent egg mortality, was measured from egg hatch rates as described in §2. The effect of prior male sexual activity on CI was measured on four successive days (closed circles), by mating 15 males successively with four virgin females. For comparison, CI from egg hatch rates of 17 single-pair matings measured during the same time span is shown (open circles). Aggregate mean per cent egg mortality are plotted ± standard error.

a virgin female on each of four successive days (figure 1). CI in the first day of mating was approximately 70%, but decreased significantly in subsequent matings. The decrease from the first to the third day of mating is consistent with a dynamic link between CI expression and the rate of sperm production. Moreover, this increase in fertility is opposite to the typical effects of high-frequency mating on male fertility (Markow *et al*. 1978). However, full fertility is not restored (figure 1, 72% egg lethality versus 45% egg lethality), suggesting a concomitant decrease in the fertility of multiply mated males. Therefore, the CI value on days 3 and 4 most likely reflects a combination of both effects.

How does repeated copulation diminish CI? One possibility is that spermatogenesis in premated males proceeds so rapidly that *Wolbachia* cannot populate the developing spermatid and express CI. Accordingly, because bacterial density and CI levels are linearly related (Boyle *et al*. 1993; Breeuwer & Werren 1993), we hypothesized that premated males should contain two- to threefold fewer *Wolbachia*. Premated males, however, contained approximately as many *Wolbachia* as non-depleted males, suggesting that bacterial loss does not account for the reduction in CI (figure 2*a*,*b*).

Another possibility is that in premated males, *Wolbachia* can populate developing spermatids but lack sufficient time to express CI before sperm individualization excludes them. While our data do not address this possibility directly, it is consistent with differences in the timing of sperm development during pre-adult and adult life. Spermatogenesis in *D. melanogaster* (a sibling species of *D. simulans*) begins when spermatogonial cells arise in the rudimentary testes (Lindsley & Tokuyasu 1980). Subsequent stages of spermatogenesis, including spermatocyte cyst formation, growth and meiosis, all occur during the second and third larval stages. Because testis formation is incomplete until the latter stages of pupariation, sperm elongation and individualization occur before and immediately following emergence of the adult. Thus, pre-adult spermatogenesis spans approximately 325 h. In contrast, spermatogenesis in the adult takes approximately 250 h, of which individualization and coiling of mature sperm require 30–40 h (Lindsley & Tokuyasu 1980). Therefore, *Wolbachia* and the sperm of virgin males share a common cytoplasm for about 100 h longer than sperm produced during adulthood. These estimates assume, of course, that *Wolbachia* are present in developing spermatids during larval development. This is confirmed in third instar larval testes (figure 2*c*). Although precise estimates of bacterial numbers await a more rigorous study, the density of bacteria in the larval testes appear similar to those in the adult testes (figure 2*a*,*b*). These results suggest that the penetrance of CI expression may be related to the rate of spermatogenesis.

CI decreases with male age and is correlated with a concomitant reduction in the fraction of infected spermatids (Bressac & Rousset 1993). Male age alone, however, cannot account for the reduction in CI in our experiments because (1) age-dependent reduction in CI is significant

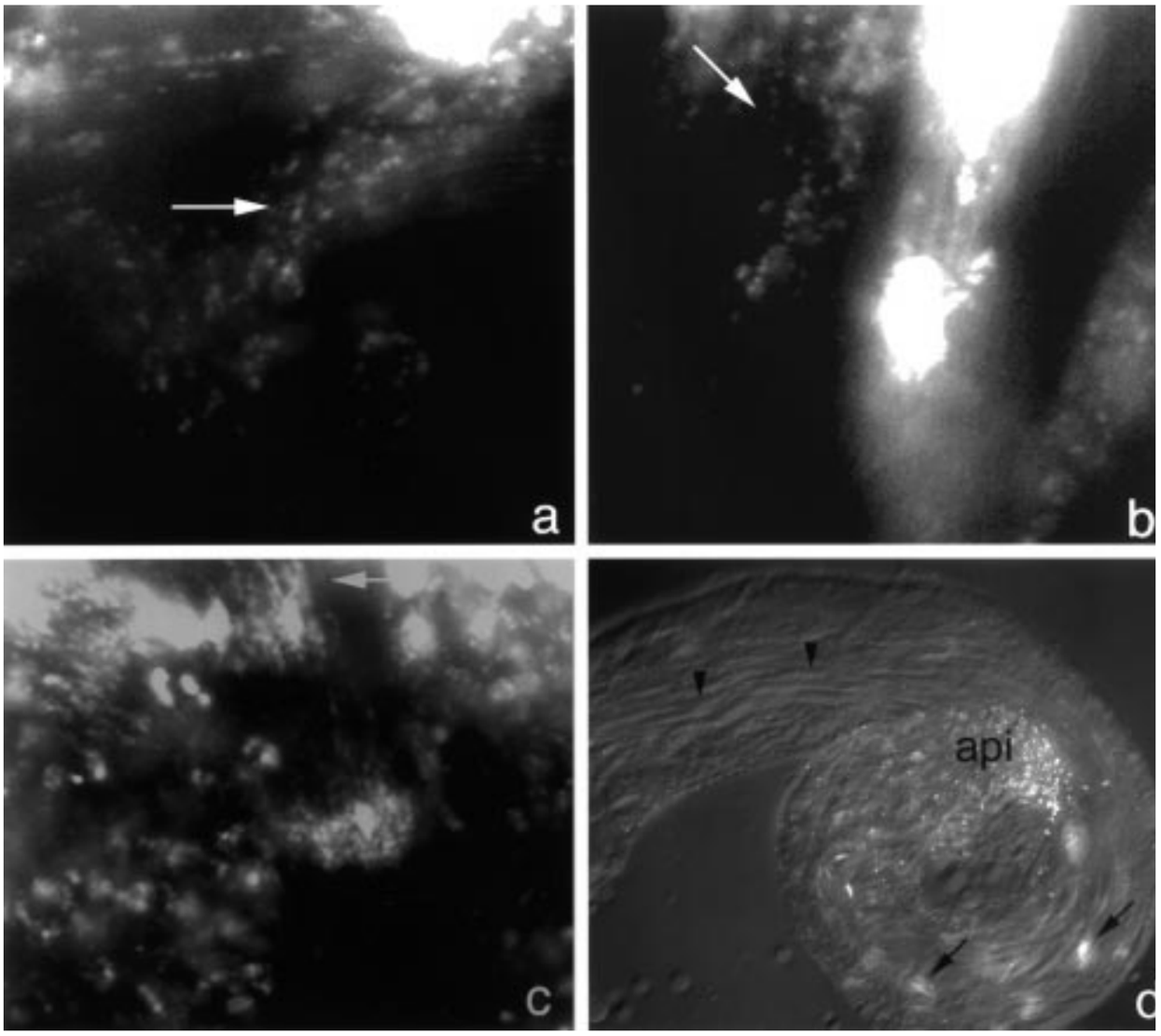

Figure 2. Microscopic examination of *Wolbachia* in testes before and after premating. Testes were fixed and stained with DAPI, a DNA-specific fluorochrome as described in §2. (a) Spermatid bundle in an adult three-day-old virgin male testis showing numerous small punctate DNA-positive bodies (arrow); (b) DNA-positive bodies (arrow) observed in a testis from a male that had mated twice and been allowed to recover for one day prior to fixation; (c) third instar larval testis showing the presence of numerous DNA-positive dots (arrow) in the region of the developing spermatids; (d) low magnification overlay of transmitted light Nomarski DIC and fluorescent images of a single testis. The apical end of the testis (api) contains mitotically active spermatogonial cells that give rise to spermatid bundles containing DNA-positive sperm nuclei (arrows) and elongating sperm tails (arrowheads). The high magnification images shown in a,b were taken from regions immediately distal to the elongating spermatid nuclei (arrows, d), which afford a view of *Wolbachia* that is relatively clear of the much more highly fluorescent diploid nuclei of the testis. Panel magnification: a–c, $\times 650$, d, $\times 200$.

only at ages older than those of males used in the present experiment (Hoffmann *et al*. 1986; Turelli & Hoffman 1995); (2) control experiments with infected males of identical age showed no reduction in CI (table 1); and (3) testes of premated males in the present study were devoid of the uninfected sperm cysts observed in older males (Bressac & Rousset 1993) (figure 2*a*,*b*).

The transmission of *Wolbachia* and the spread of CI in laboratory and natural populations of *D. simulans* have been the subjects of elegant mathematical modelling (Turelli 1994; Turelli & Hoffman 1995; Turelli *et al*. 1992). This work suggests that environmental factors such as male age or naturally existing antibiotics influence the spread of *Wolbachia*. Our results suggest that copulation frequency is another factor influencing the transmission and spread of *Wolbachia*. Clearly, repeated copulation enables male *D. simulans* to ameliorate CI and, because *Wolbachia* are not present in the sperm, matings of premated males and uninfected females will contribute uninfected individuals to the population.

Because CI can effectively reduce the reproductive capacity of uninfected females, CI may form an important element in future biological pest control strategies (Karr 1994; Laven 1967). The effectiveness of this approach will

depend on our knowledge of environmental and genetic factors influencing the expression of CI in natural populations. Our results indicate that multiple matings may be one such factor influencing CI-based biocontrol programmes, as repeated copulation may overcome the intended blockade of reproduction.

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