

Male Development Time Influences the Strength of Wolbachia-Induced Cytoplasmic Incompatibility Expression in *Drosophila melanogaster*

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ABSTRACT

Cytoplasmic incompatibility (CI) is the most widespread reproductive modification induced in insects by the maternally inherited intracellular bacteria, Wolbachia. Expression of CI in *Drosophila melanogaster* is quite variable. Published papers typically show that CI expression is weak and often varies between different *Drosophila* lines and different labs reporting the results. The basis for this variability is not well understood but is often considered to be due to unspecified host genotype interactions with Wolbachia. Here, we show that male development time can greatly influence CI expression in *D. melanogaster*. In a given family, males that develop fastest express very strong CI. The “younger brothers” of these males (males that take longer to undergo larval development) quickly lose their ability to express the CI phenotype as a function of development time. This effect is independent of male age effects and is enhanced when flies are reared under crowded conditions. No correlation is seen between this effect and Wolbachia densities in testes, suggesting that a more subtle interaction between host and symbiont is responsible. The observed younger brother effect may explain much of the reported variability in CI expression in this species. When male development time is controlled, it is possible to obtain consistently high levels of CI expression, which will benefit future studies that wish to use *D. melanogaster* as a model host to unravel CI mechanisms.

WOLBACHIA are intracellular, maternally inherited, α -Proteobacteria known to infect a broad range of invertebrates, including crustaceans, mites, filarial nematodes, spiders, and at least 25% of all insect species (WERREN 1997; JAYAPRAKASH and HOY 2000; FLOATE *et al.* 2006). In arthropods, Wolbachia commonly act as reproductive parasites and manipulate their host's reproduction in a variety of ways, including male killing, feminization of genetic males, parthenogenesis induction, or more commonly via cytoplasmic incompatibility (CI) (WERREN and O'NEILL 1997). It is considered that all these phenotypes provide a reproductive advantage to infected females, thereby allowing Wolbachia to persist and spread into host populations (HOFFMANN and TURELLI 1997).

CI is the best-described reproductive modification caused by Wolbachia. CI results in failure to produce progeny in crosses between an infected male and a female that lacks the same strain of Wolbachia found in the male. The reciprocal cross between uninfected males and infected females is fertile, as are crosses between males and females harboring the same Wolbachia strain. CI provides a reproductive advantage to infected females since they can mate successfully with either infected or uninfected males, while uninfected

females are incompatible with infected males (WERREN and O'NEILL 1997). Therefore, as a consequence of maternal inheritance of Wolbachia-infected individuals will increase in frequency in a host population. Though the molecular mechanisms of CI have not yet been identified, several lines of evidence suggest that the proper functioning of sperm is modified by Wolbachia infection (WERREN 1997; POINSOT *et al.* 2003). Cytological studies demonstrate a delay in nuclear envelope breakdown and a disruption of paternal chromosome condensation in CI embryos during the first mitotic division, leading to their subsequent death (CALLAINI *et al.* 1997; TRAM and SULLIVAN 2002; TRAM *et al.* 2003).

Werren proposed a nomenclature for describing Wolbachia strains based on the modification status of male sperm and the ability of female embryos to rescue this modification. In this system, four phenotypic categories can be expected: *mod+ / resc+*, *mod+ / resc-*, *mod- / resc+*, and *mod- / resc-* (WERREN 1997). In wild *Drosophila simulans* population, five distinct Wolbachia strains have been reported to date (MERCOT and CHARLAT 2004). *wRi*, *wHa*, and *wNo* have been described as *mod+ / resc+* strains. These three Wolbachia strains induce distinctive CI phenotypes in *D. simulans*. The *wRi* strain induces high CI, while *wHa* and *wNo* show partial CI (MERCOT and CHARLAT 2004). In addition, two *mod-* strains have been described from *D. simulans*; *wMa* displays a *mod- / resc+* phenotype (MERCOT and POINSOT 1998a; CHARLAT *et al.* 2003) and *wAu* is

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considered a *mod*-/*resc*- strain, which induces no CI and does not appear to rescue the modification of all *mod*+ strains tested so far (JAMES and BALLARD 2000; REYNOLDS and HOFFMANN 2002; CHARLAT *et al.* 2003, 2004).

In *D. melanogaster*, Wolbachia infection also induces CI (HOFFMANN 1988), but its expression appears much more variable. Early studies have shown CI expression ranging from 0 to 77% incompatible embryos (HOLDEN *et al.* 1993; HOFFMANN *et al.* 1994; SOLIGNAC *et al.* 1994). Genetic characterization of Wolbachia strains, based on gene sequences such as 16S rDNA (HOLDEN *et al.* 1993), *ftsZ* (WERREN *et al.* 1995), *dnaA* (BOURTZIS *et al.* 1994), and *wsp* (ZHOU *et al.* 1998), have all concluded that *D. melanogaster* is predominantly infected by a single *mod*+/*resc*+ strain of Wolbachia known as *wMel*. However, expression of CI by this strain appears to be very variable, with different studies reporting results ranging from very strong to very weak CI (BOURTZIS *et al.* 1996; POINSOT *et al.* 1998; MCGRAW *et al.* 2002; REYNOLDS and HOFFMANN 2002). The basis of this variability is not well understood but is often considered to be due to host genetic background differences. Another possible explanation is that the *wMel* strain actually consists of different cryptic variants. A recent report has characterized five different Wolbachia genetic variants within stocks of *D. melanogaster* (RIEGLER *et al.* 2005), although phenotypic variation associated with these strains is unclear.

Several environmental and physiological factors have been identified that influence the expression of CI in *D. simulans*. For example, infected males exposed to nutritional stress have a decreased ability to induce CI (SINKINS *et al.* 1995; CLANCY and HOFFMANN 1998). Similarly, males that have multiply mated also show reduced expression of CI (KARR *et al.* 1998) as do old males (HOFFMANN *et al.* 1990; TURELLI and HOFFMANN 1995). The influence of these factors on expression of CI in *D. melanogaster* is unclear.

Only one study has shown very strong CI in *wMel*-infected *D. melanogaster* (REYNOLDS and HOFFMANN 2002). In this study, Reynolds and Hoffmann clearly showed the importance of a male age effect in *D. melanogaster*. They described that CI levels declined rapidly with increasing male age in both *wMel*- and *wMelCS*-infected lines. Notably, 1-day-old males showed almost perfect CI, while 5-day-old males expressed no CI. Following the discovery of a male age effect, very young males have been used for CI tests in recent studies (VENETI *et al.* 2003; FRY *et al.* 2004). Unexpectedly, VENETI *et al.* (2003) observed weak CI in 3 different variants (25% with *wMel*, 0% with *wMelCS* and *wMelPop*) and FRY *et al.* (2004) observed no CI with *wMel*, despite using young males. Taken together, the large fluctuations of CI levels reported within single host lines under the same experimental conditions (SOLIGNAC *et al.* 1994; POINSOT *et al.* 1998) suggests that factors

other than male age are influencing CI levels in *D. melanogaster*.

In this study, we show for the first time, to our knowledge, an effect of male development time on CI expression. In *D. melanogaster*, consistently high levels of CI are expressed when the fastest developing males are used in crosses. The “younger brothers” of these males quickly lose their ability to express the CI phenotype as a function of development time. The observed younger brother effect may explain much of the reported variability in CI expression in *D. melanogaster*.

MATERIALS AND METHODS

Fly lines: Fly lines were kept on a standard corn diet at a constant temperature of 25°. The *D. melanogaster* strain designated BNE used in this work originated from field-caught flies collected in Brisbane, Queensland, Australia in 2004 (BNE) and 2006 (BNE2). BNE and BNE2 are both infected with the *wMel* Wolbachia strain (RIEGLER *et al.* 2005). Virgin females from *D. melanogaster* yw^{67c23} infected with the *wMel* Wolbachia strain were mated to males of the BNE line. Female offspring were then backcrossed to males of the BNE line for a total of five generations. The resulting *wMel*(BNE) line was maintained for a further five generations before performing CI assays. Cured *wMel*(BNE) and BNE2 lines were subsequently generated by tetracycline treatment following established protocols (HOFFMANN 1988) and designated BNE-T and BNE2-T. The following Wolbachia-infected *D. melanogaster* and *D. simulans* lines were used in this work: *D. melanogaster* Canton-S carrying the *wMelCS* Wolbachia strain, Harwich carrying the *wMel* (RIEGLER *et al.* 2005), *D. simulans* N7No carrying the *wNo* (MERCOT and POINSOT 1998b), Coffs Harbour carrying *wAu* (HOFFMANN *et al.* 1996), and DSH carrying the *wHa* infection (O’NEILL and KARR 1990). Wolbachia genotypes were characterized using polymorphic markers (RIEGLER *et al.* 2005; MILLER and RIEGLER 2006). Cured lines were generated and designated CS-T, Hw-T, No-T, Ha-T, and Au-T.

Rearing conditions: To standardize rearing conditions for CI tests, flies ($n = 100$ aged ~3–5 day, male and female mixed population) were grown under controlled low density conditions. One hundred flies (3–5 days old, male and female mixed population) were collected from stock bottles and placed into plastic bottle egg chambers. They were allowed to oviposit for 5 hr, and then 200 eggs were counted and transferred to fresh bottles containing 40 ml of diet. This ensured that all flies used in subsequent crosses had been reared under standardized conditions of low density. All flies were incubated at 25° with a 12-hr-light/dark cycle.

CI tests: Unless noted, male flies were used in CI tests within 24 hr of eclosing to avoid any complications arising from diminishing incompatibility with increasing male age. Female flies <5 days old were used in crosses. For *D. melanogaster*, single pairs of males and females were placed in empty vials and visually monitored for mating. Pairs that failed to mate were excluded. The Wolbachia infection status of mated males was confirmed by PCR of the *wsp* gene using primers 81F and 691R (BRAIG *et al.* 1998). Females were transferred to plastic bottles with molasses plate lids that were dotted with yeast suspension. Eggs were collected at 25° every 24 hr on molasses plates over a period of 3 days. Females that laid <30 eggs in the total of three plates were discarded from the experiment. The plates were placed at 25° for a further 36–48 hr and the number of total and unhatched eggs was counted. Statistical significance of hatch rates for various crosses was determined

using a Mann-Whitney *U*-test. For *D. simulans*, single pairs of virgin males and females were introduced to plastic bottles with molasses plate lids. They were given 24 hr to mate, then the males were removed and the females were allowed to lay eggs. The same procedure as above was followed to collect eggs.

Wolbachia density measurement: Virgin male flies were collected within 7 hr of eclosion and incubated overnight in standard food vials and then frozen at -80° . DNA of single flies was extracted using the Holmes-Bonner method (HOLMES and BONNER 1973). DNA of testes was extracted using the simplified STE method (O'NEILL *et al.* 1992). In brief, a single pair of testes was dissected into 20 μ l of STE (100 mM NaCl/10 mM Tris-HCl, pH 8.0/1 mM EDTA, pH 8.0) containing 1 mg/ml proteinase K and incubated for 30 min at 37° followed by 5 min at 95° . Samples were vortexed and briefly centrifuged, and 1 μ l of the supernatant was used as the template in subsequent quantitative PCR (Q-PCR) using the LightCycler system (Roche) with SYBR Green (Invitrogen, Carlsbad, CA). Primers were designed to amplify 69-bp regions of the single copy Wolbachia WD1063/*wsp* gene (444F 5'-AGCGTATATTAGCACTCCTTTGGAA and 512R 5'-TGACCA GCAAAACCAATTTACTTT). A temperature profile of 95° for 5 sec, 60° for 5 sec, and 72° for 10 sec was used for 50 cycles. Initial copy number was estimated by comparison to a standard curve using Roche LightCycler data analysis software v3.1.02. Three replicates were run and averaged for each sample. For each eclosion-day point, we collected measurement on five samples. Statistical analysis was performed using Mann-Whitney *U*-test.

Immunological studies: Testes were dissected in TBST (25 mM Tris, 137 mM NaCl, 5 mM KCl, 0.1% Tween, pH 7.5) and fixed with TBST containing 3.7% formaldehyde for 30 min. After washing with TBST, testes were incubated overnight at 4° in a 1:500 dilution of anti-WSP polyclonal antibody (DOBSON *et al.* 1999) in TBST with 1% BSA. After removing the primary antibody with TBST, testes were incubated for 1 hr at room temperature in a 1:500 dilution of Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (Molecular Probes, Eugene, OR; no. A11034) in TBST with 1% BSA. Testes were then washed in TBST and stained with 1 μ g/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min, washed again, and mounted with 80% glycerol. Individual cysts were removed from the testes and stained on poly-L-lysine-coated slides according to CLARK *et al.* (2002).

RESULTS

The complete genome sequence of the Wolbachia *wMel* strain was determined using Wolbachia isolated from the *D. melanogaster* *yw*^{67c23} line (WU *et al.* 2004). However, *yw*^{67c23} males failed to copulate within a 1-hr observation period in our lab, presumably due to mutations in the *yellow* and *white* genes (BURNET *et al.* 1973; SCIANDRA and BENNETT 1976; R. YAMADA, unpublished results), while wild-type *D. melanogaster* males typically copulate within 5 min. To facilitate standardized CI test conditions using visually monitored mating, we generated a field-caught wild-type *D. melanogaster* line carrying *wMel* originating from *yw*^{67c23}, designated *wMel*(BNE). We tested if male development time influenced the strength of CI expression in *D. melanogaster* using *wMel*(BNE) under controlled uncrowded conditions (200 eggs seeded per bottle). Adult fly emergence

under these conditions was very synchronous, occurring predominantly within a 2-day time interval (Figure 1A). In crosses between day 1-eclosing infected males and uninfected BNE-T females, the CI level (mean unhatched eggs) was 0.95 ($n = 17$) compared to 0.02 ($n = 12$) in crosses where both sexes were uninfected (Figure 2). The high CI induced by day 1 males declined with increasing larval development time. Day 2 males induced slightly weaker CI (0.85, $n = 19$) in comparison to that of the Day 1 males (Mann-Whitney, $P = 0.01$), while day 3 males induced greatly reduced CI (0.529, $n = 15$) when compared to day 1 males (Mann-Whitney, $P < 0.0001$).

Under crowded conditions (900 eggs seeded per bottle), flies eclosed over a longer time period with greater variability (Figure 1B). When males from these bottles were used in CI test crosses, it was found that initial CI strength from day 1 males was indistinguishable from equivalent males from uncrowded bottles. However, males collected on subsequent days showed significantly reduced CI expression compared to equivalent males reared under uncrowded conditions (Figure 2). Males eclosing on day 5 expressed no CI, hatch rates being indistinguishable from control crosses.

The expression of very strong CI in the earliest eclosing males could be a function of the development time of the males used in test crosses. Alternatively, it might be due to effects associated with mothers of these males. In particular, the age at which females lay eggs might influence Wolbachia levels and development time of the males that were subsequently used in test crosses. To control for these possible effects, 50 *wMel*(BNE) virgin females were aged for 3 days in bottles and then mixed with 50 *wMel*(BNE) virgin males for 24 hr to mate. Consequently, flies were transferred to plastic bottles to lay eggs for 1 hr (early eggs). After egg collection, flies were transferred to fresh food bottles every day and allowed to lay eggs continuously. After 7 days, flies were transferred to plastic bottles again to collect eggs (late eggs). CI tests were performed with males that had developed from early or late eggs. There was no significant difference between these males (data not shown), suggesting that the loss of CI strength is independent of the age of female parents of the males and depends solely on development time of individual males. The males that develop fastest express strongest CI, while the younger brothers of these males lose their ability to induce CI as a function of development time.

Development time is known to correlate with body size and was examined as a possible explanation for the observed effect. To compare body size, we measured the wing length of male flies with differing development times and rearing densities (Figure 3). Late eclosing males had shorter wing lengths than early eclosing males in both crowded and uncrowded conditions. However, there was no direct correlation between body size and CI strength. For example, uncrowded day 3 males were larger than

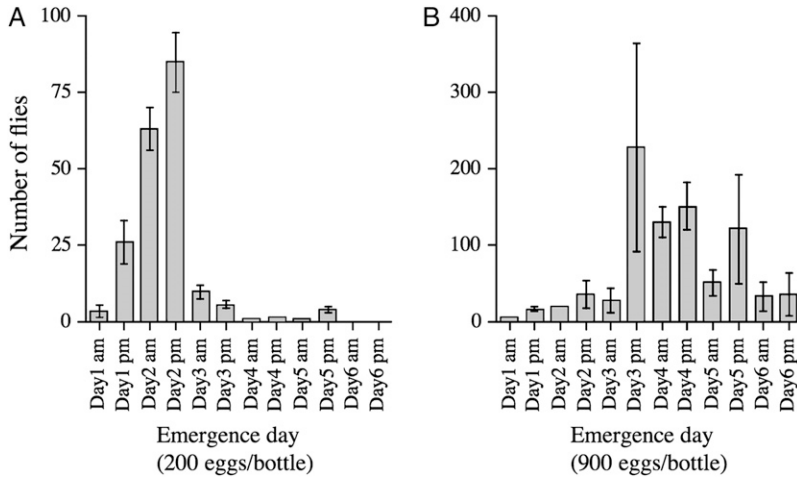


FIGURE 1.—Distribution of fly emergence in uncrowded conditions with 200 eggs per bottle (A) and crowded conditions with 900 eggs per bottle (B). *wMel(BNE)* flies were kept on a 12-hr-light/dark cycle and emerging flies were counted every morning (9:00 AM) and evening (5:00 PM). Bars, mean number of flies emerging per bottle ($n = 3$). Error bars indicate SEM.

crowded day 1 males, but CI data showed that the smaller flies eclosing earlier expressed stronger CI than the late eclosing larger flies. Therefore, the younger brother effect cannot be explained simply as a consequence of male size.

It is well known that CI strength is dependent on Wolbachia densities in insects (BREEUWER and WERREN 1993; BRESSAC and ROUSSET 1993; BOURTZIS *et al.* 1996; BORDENSTEIN *et al.* 2006) and it is possible that the observed effect could be explained by a relationship between male development time and Wolbachia density. We examined Wolbachia densities in flies collected on different emergence days by quantitative PCR (Q-PCR) using the single copy *wsp* gene. Initially *wsp* gene copy number was quantified from whole flies and no reduction was observed in absolute quantities of Wolba-

chia despite the smaller size of late eclosing males (Figure 4A). We then examined *wsp* copy number in dissected testes of males grown under both crowded and uncrowded conditions and again no correlation was found between Wolbachia density and male development time (Figure 4B), even under the more extreme crowded conditions. It is possible that Wolbachia distribution within sperm cysts, rather than density *per se*, might be a better predictor of CI levels (CLARK *et al.* 2002, 2003; VENETI *et al.* 2003). Therefore, we performed DAPI staining and immunostaining to visualize Wolbachia density and distribution in testes and cysts. Although day1-eclosed males induce almost perfect CI expression, we could not detect any difference in Wolbachia densities or distribution in cysts between day 1 and day 5 males (data not shown) consistent with the

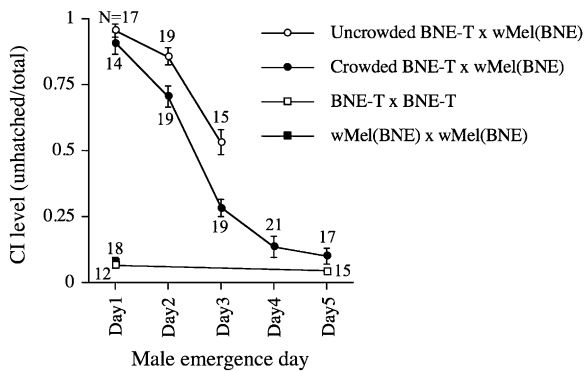


FIGURE 2.—Younger brother effect on the level of CI in *wMel*-infected *D. melanogaster*. Crosses were performed as follows: open circles, BNE-T females \times *wMel(BNE)* males grown under uncrowded conditions of 200 eggs per bottle; closed circles, BNE-T females \times *wMel(BNE)* males grown under crowded conditions of 900 eggs per bottle; open square, BNE-T females \times BNE-T males compatible cross control; closed square, *wMel(BNE)* females \times *wMel(BNE)* males compatible cross control. Values beside circles and squares represent number of single-female replicates. Error bars indicate SEM.

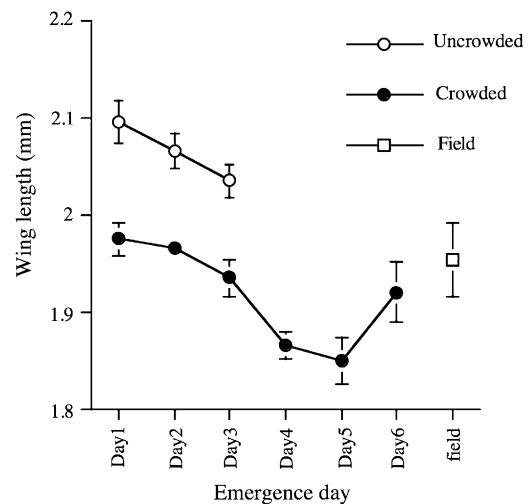


FIGURE 3.—Mean wing length of male flies. Open circles, *wMel(BNE)* males grown under uncrowded conditions of 200 eggs per bottle. Closed circles, *wMel(BNE)* males grown under crowded conditions of 900 eggs per bottle. Open square, field-collected (Brisbane) male *D. melanogaster*. Error bars indicate SEM ($n = 10$).

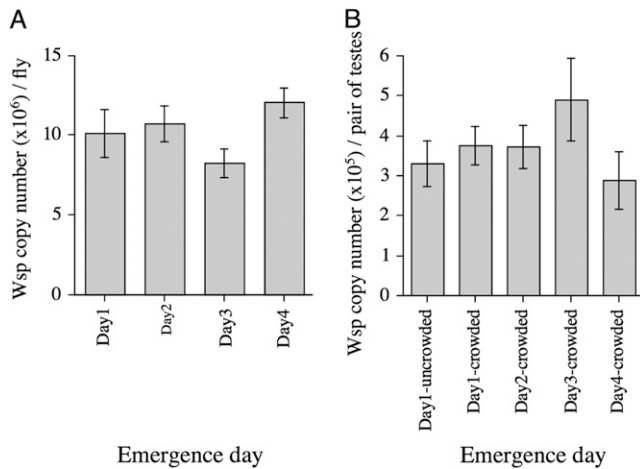


FIGURE 4.—Mean Wolbachia density as determined by quantitative PCR. (A) Mean Wolbachia density for *wMel*(BNE) adult males eclosing over different days and grown under crowded conditions of 900 eggs per bottle. (B) Mean Wolbachia density for *wMel*(BNE) testes taken from males eclosing over different days and grown under crowded conditions of 900 eggs per bottle and uncrowded conditions with 200 eggs per bottle. Error bars indicate SEM ($n = 5$).

Q-PCR data. As such, the younger brother effect cannot be explained by an interaction between development time and Wolbachia densities in flies.

We examined the generality of the younger brother effect by examining different *D. melanogaster* lines. Canton-S and Harwich are long-established inbred laboratory strains naturally infected with *wMel*CS and *wMel*, respectively (RIEGLER *et al.* 2005). In *wMel*CS-infected Canton-S, we observed a weaker younger brother effect (Figure 5A). Under uncrowded conditions, day 2 males induced a similar level of CI (0.92, $n = 10$) compared to day 1 males (0.88, $n = 21$), while day 3 males induced reduced CI (0.72, $n = 15$) when compared to day 1 males (Mann-Whitney, $P = 0.03$). Under crowded condition, the initial CI strength from day 1 males (0.71, $n = 16$) was distinguishable from equivalent males from uncrowded bottles (Mann-Whitney, $P = 0.02$), whereas day 2 and day 3 males induced similar CI strength (0.68, $n = 19$; 0.679, $n = 39$, respectively) when compared to day 1 males. The CI strength declined in day 4 males (0.47, $n = 38$) compared to day 3 males under crowded conditions (Mann-Whitney, $P < 0.001$). In *wMel*-infected Harwich, the younger brother effect was obvious in both uncrowded and crowded conditions (Figure 5B). In addition, the recently field-caught BNE2 line infected with *wMel* was also examined for the effect. In this line, a strong younger brother effect was observed in both uncrowded and crowded conditions (Figure 5C). To examine the influence of male age effects, day 1 males were aged for 5 days and then used for CI tests. Five-day-old males that had originally eclosed on day 1 showed a reduced ability to express CI (0.19, $n = 30$) when compared to 1-day-old

males (Mann-Whitney, $P < 0.001$) (Figure 5C). Seven-day-old males expressed no CI in the Canton-S and Harwich lines, although equivalent males induced high CI if they were only 1 day old (Figure 5, A and B).

We also examined Wolbachia strains in *D. simulans*. As observed for *wMel* in *D. melanogaster*, CI levels associated with the *wNo* strain in *D. simulans* have been reported as quite variable, making it a potential candidate for the younger brother effect (MERCOT *et al.* 1995; MERCOT and POINSOT 1998a; JAMES *et al.* 2002; VENETI *et al.* 2003; MERCOT and CHARLAT 2004). Under our experimental conditions, *wNo* induced strong CI expression. In crosses between day 1 *wNo*-infected males and uninfected females, mean egg hatch failure was 0.86 ($n = 21$) compared to 0.09 ($n = 21$) in crosses where both sexes were uninfected (Table 1). While there was some variability in CI levels between males with different development time, there was no clear younger brother effect. We also examined *wHa*- and *wAu*-infected *D. simulans*. The *wHa* infection expressed strong CI regardless of male development time. The *wAu* line expressed no CI even when day 1 males were used in the test cross (Table 1). This is consistent with the previous report that the *wAu* strain does not induce CI (JAMES and BALLARD 2000; REYNOLDS and HOFFMANN 2002; CHARLAT *et al.* 2003, 2004).

DISCUSSION

In studies of the Wolbachia symbiosis of insects during the last 20 years, *D. simulans* has been used more heavily as a model host than *D. melanogaster*, despite the wealth of genetic tools available in the latter. One of the reasons for this is the highly variable expression of the CI phenotype in *D. melanogaster*, making its study difficult. In different papers, a variety of CI levels have been reported, varying from 0 to almost 100%. Recent work from REYNOLDS and HOFFMANN (2002) indicated that almost 100% CI could be obtained with *wMel*-infected 1-day-old males but not with older males, indicating a strong male age effect in this species. However, even when this factor has been controlled in subsequent experiments, variability in CI expression has still been seen. For example, FRY *et al.* (2004) observed no CI expression in *wMel*-infected lines, even though they used ~16- to 40-hr posteclosion males. In a study reported by VENETI *et al.* (2003), 1-day-old males were used in CI crosses; however, *wMel*-infected lines showed low CI levels (25%), while *wMel*CS and *wMel*Pop showed no CI. Similarly, MCGRAW *et al.* (2002) detected weak CI using *wMel*-infected 1- to 2-day-old males.

One possible confounding factor in these different studies is the possibility of multiple matings by males. REYNOLDS and HOFFMANN (2002) separated males and females after mating to avoid repeated copulation. In contrast, other studies have left males and females

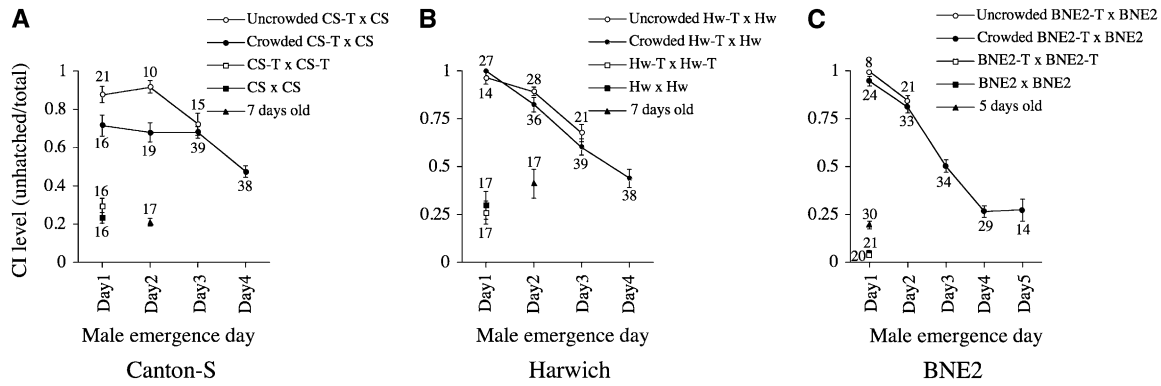


FIGURE 5.—Younger brother effect on the level of CI in different *D. melanogaster* lines. (A) Canton-S line. (B) Harwich line. (C) BNE2 line. Crosses were performed as follows: open circles, uninfected females \times infected males grown under uncrowded conditions of 200 eggs per bottle; closed circles, uninfected females \times infected males grown under crowded conditions of 900 eggs per bottle; open square, uninfected females \times uninfected males compatible cross control; closed square, infected females \times infected males compatible crosses control; closed triangle, uninfected females \times 7-day-old infected males (A and B) and uninfected females \times 5-day-old infected males (C). Values beside circles and squares represent number of single-female replicates. Error bars indicate SEM.

together during the egg collection period. Repeated copulation is a factor that is known to reduce CI in *D. simulans* (KARR *et al.* 1998). If males remained with females for several days, CI expression could be diminished by both repeated copulation and subsequent matings with older males. In contrast, isolated females produce constant CI levels for 5 days after copulation (R. YAMADA, unpublished results). However, in the report of FRY *et al.* (2004), eggs were collected from females kept with males for one 24-hr period, limiting the possibility of either male age or multiple mating effects. CI levels would have been predicted under these conditions to be around 50%, but no CI was found.

Although some of these contradictions might have been due to repeated copulation and/or host line differences, much of it might be due to an undescribed factor influencing CI levels, independently of male age effects. In this study, we found that a rapid decline in CI levels is correlated with male development time. This

effect is independent of male age. For example, 1-day-old males expressed high CI levels if they had undergone fast development, whereas no CI was detected with 1-day-old males that had undergone slow development. However, males that develop fastest lose their ability to express CI as they age. These results suggest that male development time and male age influence CI expression independently. Male development time influenced CI expression in all *D. melanogaster* lines examined, including North American inbred lab lines (Canton-S, Harwich) and a recently caught Australian (BNE2) wild-type strain as well as across two Wolbachia genotypes (*wMel* and *wMelCS*), indicating that this is a general effect in *D. melanogaster*. We refer to this observation as the younger brother effect, and it may explain much of the reported variability in CI expression in *D. melanogaster*.

We examined three possibilities to explain the relationship between CI levels and observed development

TABLE 1
Effect of male development time on CI in Wolbachia-infected *D. simulans*

| Female | Male | No. of crosses | Total eggs counter | Mean CI (unhatched/total) | SE |
|-------------|------------------|----------------|--------------------|---------------------------|-------|
| No-T | <i>w</i> No-Day1 | 21 | 1387 | 0.862 | 0.022 |
| No-T | <i>w</i> No-Day2 | 23 | 2262 | 0.779 | 0.025 |
| No-T | <i>w</i> No-Day3 | 22 | 2361 | 0.798 | 0.042 |
| No-T | <i>w</i> No-Day4 | 25 | 2770 | 0.835 | 0.031 |
| No-T | No-T | 21 | 1530 | 0.089 | 0.011 |
| Ha-T | <i>w</i> Ha-Day1 | 9 | 734 | 0.994 | 0.006 |
| Ha-T | <i>w</i> Ha-Day2 | 10 | 726 | 0.987 | 0.007 |
| Ha-T | <i>w</i> Ha-Day3 | 3 | 243 | 0.870 | 0.065 |
| Ha-T | Ha-T | 10 | 920 | 0.096 | 0.009 |
| Au-T | <i>w</i> Au-Day1 | 19 | 2118 | 0.346 | 0.058 |
| <i>w</i> Au | <i>w</i> Au-Day | 20 | 2028 | 0.363 | 0.045 |
| Au-T | Au-T | 10 | 928 | 0.302 | 0.092 |

time differences. First, slower developing males might lose the infection, resulting in the presence of uninfected males in CI crosses. This can be excluded by the observation that the Wolbachia infection in males was present in nearly 100% of individuals across all development times. The infection status of males was confirmed by PCR after copulation and data from PCR-negative males were excluded in our analysis. Second, fastest developing males might have originated from eggs laid earlier in the life of females, which in turn may have influenced CI. Larvae from these eggs may develop faster and contain higher levels of Wolbachia. If a female effect such as this existed, then males that develop from eggs laid by older females should express lower CI than males that develop from eggs laid by younger females. No difference in CI levels of sons derived from either young or old females was observed.

Third, a relationship might exist between Wolbachia density and development time such that highly infected larvae develop faster than larvae infected at low levels. There are a number of reports suggesting a positive correlation between Wolbachia density and strength of CI in many insect species, including *Drosophila*. In an earlier study, BRESSAC and ROUSSET (1993) found a decrease in the frequency of infected sperm cysts with age, which might correlate with the reduction of CI levels in older males. Following this discovery, CLARK *et al.* (2002) found that fewer cysts are infected in *wMel*-infected *D. melanogaster* than *wRi*-infected *D. simulans*. Recently, VENETI *et al.* (2003) showed a relationship between the percentage of infected cysts and CI levels in a variety of Wolbachia strains. In their report, *mod+* strains, including *wRi*, *wHa*, *wNo*, and *wMel* showed a positive correlation between infected cysts and CI levels. On the basis of this hypothesis, testes of fast developing males should carry higher infection densities than slower developing males. However, we failed to detect any difference in either Wolbachia density in testes or frequency of infected cysts between fast and slow developing males. In our data, all *wMel*-infected males showed a low infection frequency of cysts (<10%). This observation is consistent with previous studies (VENETI *et al.* 2003). It is possible that Wolbachia are lost after eclosion, although sperm chromosomes are fully modified in the early stages of development. The loss of Wolbachia-infected cysts happens around day 3 posteclosion in *D. simulans* (CLARK *et al.* 2002). It is possible that slower developing males lose their Wolbachia, whereas fast developing males maintain a Wolbachia density necessary for high CI induction. To test this, we examined the infection status of newly eclosed males prior to the standard time allowed for maturation before being used in crossing studies. Again, we saw no difference between fast and slow developing males by both Q-PCR of testes and DAPI staining of sperm cysts (data not shown).

The effect of crowding on expression of the younger brother effect was quite pronounced, suggesting that

nutritional stress may play a role in its expression. However, neither nutritional condition nor body size *per se* is known to directly influence CI strength (CLANCY and HOFFMANN 1998; our data). While the younger brother effect appears to be quite strong in *D. melanogaster*, the mechanism by which it acts seems independent of bacterial density. It also seems to be largely absent in *D. simulans*. Understanding this effect and controlling for it in experiments allows consistently high levels of CI to be expressed, which will greatly facilitate the use of *D. melanogaster* as a model organism to determine the molecular mechanisms by which Wolbachia is so successfully able to manipulate the reproduction of its host.

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