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Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*

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Summary

In *Drosophila melanogaster*, the maternally inherited endocellular microbe *Wolbachia* causes cytoplasmic incompatibility (CI) in crosses between infected males and uninfected females. CI results in a reduction in the number of eggs that hatch. The level of CI expression in this species has been reported as varying from partial (a few eggs fail to hatch) to nonexistent (all eggs hatch). We show that male age in this host species has a large impact on the level of CI exhibited and explains much of this variability. Strong CI is apparent when young males are used in crosses. CI declines rapidly with male age, particularly when males are repeatedly mated. *Wolbachia* from a Canton S line that was previously reported as not causing CI does in fact induce CI when young males are used in crosses, albeit at a weaker level than in other *D. melanogaster* strains. The strain differences in CI expression are due to host background effects rather than differences in *Wolbachia* strains. These results highlight the importance of undertaking crosses with a range of male ages and nuclear backgrounds before ascribing particular host phenotypes to *Wolbachia* strains.

1. Introduction

In *Drosophila* species, the maternally inherited bacterium *Wolbachia* induces cytoplasmic incompatibility (CI) (Binnington & Hoffmann, 1989; Louis & Nigro, 1989; Hoffmann *et al*., 1994; Giordano *et al*., 1995; Bourtzis *et al*., 1996). In diploid organisms, CI normally results in the death of embryos in crosses between uninfected females and infected males. All other crosses remain unaffected. In this way, the spread of *Wolbachia* in a population is promoted. Because mature sperm from infected males do not carry *Wolbachia*, it has been suggested that such sperm are altered in some way so that a 'rescue' factor present in infected eggs is required for fertilization to proceed normally. Crosses between uninfected males and infected females are successful because sperm are not altered and hence do not require this factor. Alternatively, sperm from uninfected males might be altered upon entry to an infected egg, thus rendering it compatible with that egg (Callaini *et al*., 1997). Although the exact mechanism of how *Wolbachia* achieves this is unknown, in incompatible crosses, the paternal chromosome set fails to condense and is eventually lost, rendering the embryo haploid and hence unviable (Callaini *et al*., 1997).

Within the *Drosophila* genus, a number of *Wolbachia* strains (based on phenotypic and genetic data) occur that vary in their ability to induce and rescue CI (Hoffmann *et al*., 1986; Montchamp-Moreau *et al*., 1991; Hoffmann *et al*., 1996; Nigro, 1991; Rousset & Solignac, 1995; Bourtzis *et al*., 1998; Zhou *et al*., 1998; James & Ballard, 2000). In particular, several strains with variable effects have been found in *Drosophila simulans*. Although some *Wolbachia* strains in *D. simulans* can reduce egg hatch to almost zero, others appear to induce only incomplete CI or to be incapable of causing CI at all. The existence of strains that appear to rescue the CI phenotype without inducing it has also been reported (Bourtzis *et al*., 1998; Mercot & Poinsot, 1998). In some cases the ability to induce or rescue CI is a feature of the particular *Wolbachia* strain. However, the host can also exert influence over *Wolbachia* and reduce CI levels (Boyle *et al*., 1993; Poinsot *et al*., 1998).

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In contrast to the different *Wolbachia* strains reported in *D. simulans*, only one strain, *w*Dm, has been found to date in *D. melanogaster*. This strain was originally found in Australian *D. melanogaster* populations (Hoffmann, 1988) and causes partial incompatibility under laboratory conditions, with reductions in egg hatch of ~15–30%. Other authors have subsequently reported a wide variety of CI levels for this strain, varying from zero to as high as 70% (Solignac *et al*., 1994; Holden *et al*., 1993; Bourtzis *et al*., 1996). Based on 16S rDNA (Bourtzis *et al*., 1994) and *ftsZ* (Werren *et al*., 1995) sequence data the strain appears to be similar to the Riverside *Wolbachia* strain (*w*Ri) that occurs in *D. simulans* (although differences in *wsp* gene sequences between these strains have been found (Zhou *et al*., 1998)). However, in *D. simulans*, the *w*Ri strain causes a much higher level of embryo mortality, with less than 5% of eggs hatching in crosses involving young males (Hoffmann *et al*., 1986). The lower CI levels in *D. melanogaster* might be a result of this hosts' ability to control the number of infected sperm cysts in testes. If the *w*Ri infection is transferred to *D. melanogaster* via microinjection, CI levels are similar to those usually reported for *D. melanogaster* (Boyle *et al*., 1993). Similarly, if the *w*Dm infection is transferred to *D. simulans*, strong CI expression occurs (Poinsot *et al*., 1998). In this latter case, *D. simulans* males were found to have 10 times more infected sperm cysts than the donor *D. melanogaster* line males.

One puzzling aspect of the *Wolbachia* infection in *D. melanogaster* is its maintenance in field populations. Despite causing CI in the laboratory, studies on field-caught *D. melanogaster* have failed to detect any incompatibility (Hoffmann *et al*., 1998). In the absence of CI, *Wolbachia* infections should eventually be lost because of imperfect maternal transmission, resulting in an increase in the relative number of uninfected individuals over time. Yet, despite the presumed absence of CI, *Wolbachia* is maintained in field populations, sometimes at very high frequencies (Hoffmann *et al*. 1994, 1998). One potential reason is that *Wolbachia* might have positive effects on host fitness. However, a previous study (Olsen *et al*., 2001) found no strong positive fitness effects under field conditions to account for the persistence of the infection in populations.

Another potential reason for its maintenance in field populations is that there is strong CI in *D. melanogaster* but has not been detected because it declines rapidly with male age in this species. Studies of *D. simulans* infected with the Riverside strain have shown that CI levels decline as males age (Hoffmann *et al*., 1990). In this case, incompatibility remains high (> 95%) for five days after emergence and then declines gradually over the subsequent two weeks. This decline has been correlated with a decrease in the number of infected sperm cysts over time (Binnington & Hoffmann, 1989; Bressac & Rousset, 1993). Age effects on CI also occur in males reared under field conditions (Turelli & Hoffmann, 1995).

Here, we show that the so-called weak CI strain in *D. melanogaster* can express CI almost as high as that found in young *D. simulans* males infected by *w*Ri. This high level of CI declines far more rapidly than in *D. simulans* and this factor, rather than host-strain differences, is likely to account for the variability of CI levels reported in *D. melanogaster*. In light of these findings, we show that an infected *D. melanogaster* line previously assumed not to express CI does, in fact, show strong CI, although the *Wolbachia* non-expressor strain *w*Au and the *w*Ma strain in *D. simulans* do appear to be a non-expressors. Strong CI was also exhibited by young *D. melanogaster* males obtained from the field and this explains why the *Wolbachia* infection increases rapidly in field-population cages. We discuss the implications of these findings for current hypotheses regarding the modification-rescue system of *Wolbachia* effects.

2. Materials and methods

(i) *Stocks*

The Australian *D. melanogaster* stocks used here originated from flies collected in the field in October–November 2000 in Innisfail (Queensland) and Wandin (Victoria). Infected and uninfected stocks representing each location were established from 15 isofemale lines following polymerase chain reaction (PCR) assays to establish infection status. For these assays and all others detailed below the *Wolbachia* primers '76–99 forward' and '1012–994 reverse' were used (O'Neill *et al*., 1992). Primers for the *Drosophila* nuclear gene *suppressor of sable su*(*s*) (Voelker *et al*., 1991) were also included in each reaction to ensure that negative results were not due to problems with either the DNA extraction procedure or the PCR. These lines were used in all experiments detailed below except where noted. The *D. simulans* line carrying the *w*Au *Wolbachia* strain is an isofemale line that was originally collected in Coffs Harbour (New South Wales; NSW) in 1999. The infected *D. melanogaster* Canton S line was kindly provided by S. O'Neill and was described in Holden *et al*. (1993). The *D. simulans* line carrying the *w*Ma infection was kindly provided by J. W. O. Ballard and was described in James & Ballard (2000). All stocks were reared on laboratory media containing sugar, yeast and agar, along with the preservatives Nipigin and propionic acid. Streptomycin and penicillin were added as antibacterial agents. These antibiotics do not influence *Wolbachia* expression. All flies were reared in bottles at low larval densities.

(ii) *CI tests*

Unless otherwise noted, CI was tested using the following protocol. Single males were placed with single virgin females in vials containing laboratory media. In all cases, the vials were monitored for mating. Any pairs that failed to mate were excluded. Following mating females were transferred to vials containing spoons holding $\sim$1·5 ml of a yeast–treacle–agar media. The media contained food dye to facilitate egg counts. The females were then allowed to lay for 24 h, after which they were removed and the eggs counted within a 5 h period. The eggs were left for a further 24 h at 25 °C and the number of unhatched eggs counted. In some cases, the female was transferred to a second spoon and the procedure repeated in order to obtain sufficient eggs for an accurate assessment of CI. Very rarely, the appearance of the eggs suggested that they were infertile. In these cases, the spoons were discarded. In all cases, females that laid fewer than ten eggs were excluded from analyses. To compare levels of CI among treatments, nonparametric Mann–Whitney tests were used and probabilities were adjusted for multiple comparisons by the Dunn–Sidak method (Sokal & Rohlf, 1995). Confidence limits for CI levels were determined from angular transformed data and these were then transformed back to proportions.

(iii) *Effects of male age and temperature on CI*

The effects of male age on CI in *D. melanogaster* were examined at 19 °C and 25 °C. Flies were reared at either temperature. Virgin males were collected and held for aging in vials containing laboratory media at the same temperature at which they were reared. Males were then mated to virgin 1–4-day-old uninfected females and CI assessed as above. Males were assessed when they were 1, 3 and 5 days old (Australian stocks) and 1, 2, 3 and 4 days old (Canton S).

(iv) *Repeat-mating effects*

Males were collected as virgins and allowed to mate up to three times when 1 day old with uninfected virgin females. Females were mated only once, with each female being removed and replaced with another virgin female after mating. This ensured that these males were sperm depleted. Males that did not mate were removed from the trial. CI for 1-day-old males was then scored using the first female with which the male had mated. Males were then held alone for one day before being mated once to new virgin females. This procedure was repeated for four days with each male being exposed to a new female on each day.

(v) *Effects of female age on CI*

If *Wolbachia* infection levels decline in females as they age then CI might occur when older females are crossed to young males. To test this, infected virgin females were collected and held in vials containing laboratory media for 7 days at 25 °C. These were then mated to virgin 1-day-old infected males and the egg hatch rate determined. Control crosses with uninfected 7-day-old females and young uninfected males were included to test the effect of female age on egg hatch independently of *Wolbachia* effects.

(vi) *CI in the field*

To obtain young males of known age, pupae and late-instar larvae were collected from discarded bananas at a plantation in Coffs Harbour (NSW) in April 2001. This location was chosen because there is polymorphism for *Wolbachia* infection there (Hoffmann *et al*., 1998), increasing the likelihood of collecting infected and uninfected males. Each larva and pupa was placed singly into 1·5 ml Eppendorf tubes that contained $\sim$1 ml of laboratory medium. Each tube was then covered with gauze. This procedure ensured that all flies that emerged remained virgin. The pupae and larvae were then transported to Melbourne and held at 25 °C until emergence. CI tests were carried out as described above using 1-day-old males and laboratory-reared uninfected females. The males were then tested for infection status using PCR. Any females that emerged from the pupae were crossed to laboratory-reared uninfected males to test whether these had the same hatch rate as crosses with uninfected field males, as would be expected if *Wolbachia* alone influences hatch rate.

(vii) *Field-cage experiment*

This experiment was set up at Red Rock (NSW) (30° 7′ S, 153° 12′ E), where *Wolbachia* infection frequencies fluctuate (Hoffmann *et al*., 1998). Field cages were constructed from cylindrical plastic containers 7 cm in diameter and 4·5 cm high. The top and bottom of these containers were removed and covered with a fine mesh material. 50 flies (with an approximately equal sex ratio) were placed into each cage, of which 25 of the 50 flies were infected with *Wolbachia*. Cups containing laboratory media were also placed in each cage. These cages were then suspended with a metal hook inside larger rectangular plastic boxes (five cages per box) that were 55 cm long and 41 cm high. The boxes provided shade and protection against disturbance, and suspension of the cages inside boxes prevented predation by ants. Boxes were placed on the ground in a shaded position and maintained temperatures similar to ambient conditions (see Mitrovski & Hoffmann, 2001). After 4 days, food cups were removed and placed into new cages, and the eggs laid in the media left to develop. Following eclosion, 50 flies from each cage were transferred to new cages to initiate the next generation. This procedure was repeated for 18 generations between April 1999 and September 2000. Infection frequencies in the cages were assessed by collecting 10–30 flies per cage at generations 4, 5, 9, 10 and 18. The infection status of each individual was then determined by PCR.

3. Results

(i) *CI levels in* D. melanogaster *and* D. simulans

Our initial test to ascertain the strength of CI in *D. melanogaster* with 1-day-old males was performed using Australian *D. melanogaster* stocks. To ascertain whether differences in CI levels occurred in different populations, two mass-bred lines that originated from separate locations (Innisfail (Queensland) and Wandin (Victoria)) were assayed. Very high levels of CI were found in both populations, flies from Innisfail producing eggs with a mean hatch failure rate of 96% (confidence limits 0·86 to 1·00, $N = 19$) and those from Wandin 93% (confidence limits 0·86 to 0·99, $N = 7$). A Mann–Whitney test indicated that there were no significant differences between the populations for CI ($P > 0{\cdot}05$) and hence data from both were combined to obtain a CI level for Australian *D. melanogaster* (Table 1). These results demonstrate that young male *D. melanogaster* are capable of inducing strong CI.

CI was also expressed by 1-day-old Canton S *D. melanogaster* males when mated to uninfected

Table 1. *Mean reduction in egg hatchability (CI) for crosses involving Australian and Canton S* D. melanogaster *lines*

Crosses[a]	*N*	Mean egg hatch failure rate (95% confidence limits)
a) UA × IA	26	0·95 (0·88–0·99)
b) UA × UA	10	0·12 (0·06–0·19)
c) UA × IC	23	0·51 (0·35–0·67)
d) IC × UA	5	0·01 (0–0·05)
e) UA × UA	19	0·01 (0–0·02)
Comparisons	*z*	Probability[b]
1) a & b	−4·305	< 0·001[c]
2) c & e	−5·217	< 0·001[c]
3) d & e	−1·938	0·053
4) a & c	−3·427	< 0·001[c]

[a] Crosses are listed as female × male.
[b] Probabilities and *z* values are for Mann–Whitney tests.
[c] Remains highly significant after correction for multiple comparisons.
Abbreviations: IA, infected Australia; IC, infected Canton S; UA, uninfected Australia.

Table 2. *Mean reduction in egg hatchability (CI) expressed by F1 male offspring from reciprocal crosses between Canton S and Australian* D. melanogaster *lines*

Crosses[a]	*N*	Mean egg hatch failure rate (95% confidence limits)
a) UA × F1 (IC × UA)	23	0·80 (0·64–0·93)
b) UA × F1 (IA × UC)	18	0·87 (0·77–0·96)
c) UA × IC	28	0·31 (0·21–0·42)
d) UA × IA	20	0·89 (0·76–0·97)
e) UA × UA	22	0·01 (0–0·03)
Comparisons	*z*	Probability[b]
1) a & b	−0·844	0·398
2) a & c	−4·209	< 0·001[c]
3) b & d	−0·784	0·443
4) a & e	−5·443	< 0·001[c]
b & e	−5·445	< 0·001[c]
c & e	−5·344	< 0·001[c]
d & e	−5·619	< 0·001[c]

[a] Crosses are listed as female × male.
[b] Probabilities and *z* values are for Mann–Whitney tests.
[c] Remains highly significant after correction for multiple comparisons.
Abbreviations: IA, infected Australia; IC, infected Canton S; UA, uninfected Australia.

Australian females (Table 1), although the reduction in egg hatch was around half that observed in crosses with infected Australian males. The difference in hatch rates between these lines was significant (comparison 4). No differences were found between the reciprocal cross and the control cross (comparison 3), suggesting that the lowered hatch rate was due to *Wolbachia* rather than to other differences between the two host strains.

To determine whether the difference in CI expression was due to different *Wolbachia* strains or to host effects on CI, the lines were reciprocally crossed to each other so that they carried either the Australian or Canton S infection. Infected F1 males from each of these crosses were crossed to uninfected females. There was no significant difference between crosses involving the reciprocal F1 males (comparison 1, Table 2), indicating that differences in CI expression are due to host- rather than *Wolbachia*-strain effects. This was supported by the high level of CI induced by the Canton S female × Australian male F1s in comparison to that induced by Canton S males (comparison 2, Table 2). Both types of male carry the Canton S infection but males with a mixed nuclear background induced a much higher level of CI than males with a complete Canton S nuclear background.

We also ascertained whether CI could be induced by young *D. simulans* males harbouring the previously described strains *w*Au and *w*Ma. The *w*Au strain has

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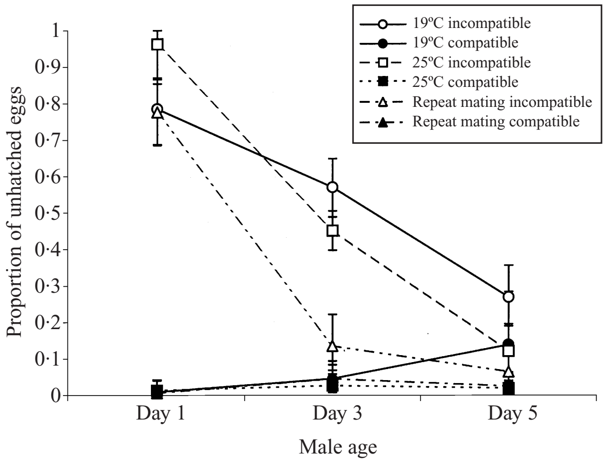

Fig. 1. Effects of temperature, male age and repeated mating on mean CI levels in Australian *D. melanogaster*. Compatible crosses involved crosses between uninfected males and females. Error bars indicate 95% confidence intervals.

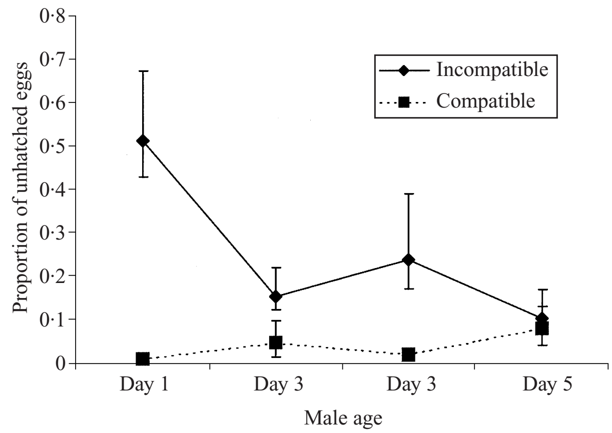

Fig. 2. Effects of male age on mean CI levels in the Canton S line of *D. melanogaster*. Compatible crosses were between uninfected males and females. Error bars indicate 95% confidence intervals.

been described as a non-expressor, whereas there is some confusion about the status of the *w*Ma strain. In both cases, crosses between 1-day-old males and 2–3-day-old uninfected females did not produce evidence of CI. In the case of the *w*Au infection, the mean proportion of eggs that failed to hatch was 0·03 (95% confidence intervals of 0·01 to 0·07, $N = 26$) compared to 0·06 (0·01 to 0·15, $N = 10$) for the control cross between uninfected males and females. A Mann-Whitney test indicated that this difference was not significant ($P = 0{\cdot}36$). This confirms the earlier findings that the *w*Au strain does not induce CI (Hoffmann *et al.*, 1996).

In the case of the *w*Ma infection, the mean proportion of eggs that failed to hatch was 0·10 (95% confidence intervals of 0·02 to 0·25, $N = 22$) for the incompatible cross, compared to 0·08 (0 to 0·30, $N = 10$) for the control cross between uninfected males and females. A Mann–Whitney test indicated that this difference was not significant ($P = 0{\cdot}87$), suggesting that CI is not induced by this *Wolbachia* strain. This is in contrast to an earlier report by Nigro (1991) but in agreement with a report by Rousset & Solignac (1995).

(ii) *Effects of male age and temperature on CI*

The level of hatchability was variable, as indicated by the large standard deviations in (Figs 1, 2) but patterns associated with age were nevertheless apparent. In the Australian *D. melanogaster* stocks, CI levels declined rapidly as males aged (Fig 1). By 5 days after eclosion, the number of eggs that failed to hatch in the incompatible crosses was close to that in the uninfected × uninfected control crosses, although Mann–Whitney tests showed that the difference between the incompatible and control crosses was still significant ($P = 0{\cdot}001$ for both temperatures). The rates of decline were similar at both 19 °C and 25 °C, although significant differences were found between these temperatures on all days tested (Mann–Whitney results: day 1, $P = 0{\cdot}01$; day 3, $P = 0{\cdot}007$; day 5, $P = 0{\cdot}007$) (Fig. 1). This reflected a higher initial CI for the 25 °C crosses on day 1 and lower CI on the other two days. The decline in CI with male age suggests that the high level of incompatibility exhibited by 1-day-old males is unlikely to be due to problems with male sterility or sperm transfer.

The level of egg hatch in the incompatible cross with the *D. melanogaster* Canton S line approached that of the control cross when males were 3 days old (Fig. 2), although differences for all days except day 4 were significant by Mann–Whitney tests ($P < 0{\cdot}001$). It is not clear whether CI declines more rapidly in this strain than in *D. melanogaster* or whether the differences reflect an initially lower CI level in crosses with 1-day-old males.

(iii) *Effects of repeat mating on CI*

The repeated mating of Australian *D. melanogaster* males affected the expression of CI (Fig. 1). By day 3, the mean level of incompatibility was similar to that of the control cross (Mann–Whitney, $P = 0{\cdot}059$), whereas this level was not reached until 4–5 days for males mated once. Significant differences were found between males mated once at 25 °C and repeatedly mated males on day 3 ($P < 0{\cdot}001$) but not on day 5 ($P = 0{\cdot}166$). The day 3 result remains significant after correction for multiple comparisons.

(iv) *Effects of female age on CI*

Crosses between 7-day-old infected females and 1-day-old infected males had a mean egg hatch failure

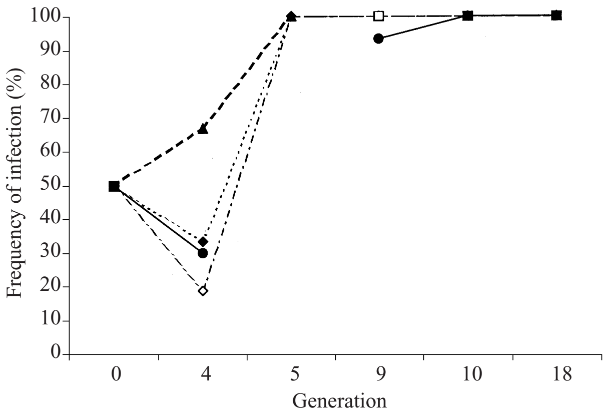

Fig. 3. Infection frequencies in replicated flied cages measured over 18 generations. Each line represents a single field cage, initiated at a frequency of 50%.

rate of 0·14 (95% confidence intervals of 0·05 to 0·26, $N = 25$) compared with 0·04 (0·01 to 0·09, $N = 11$) in crosses between 1-day-old infected females and males. Although these data suggest that older females have developed some a degree of CI in crosses with males carrying the same infection, a Mann–Whitney test showed that the difference between these ages was not significant ($P = 0{\cdot}278$) because there was a high level of variability in the data. Of the 25, 7-day-old females assayed, eight had egg-hatch failure rates of $>30\%$ but there were five females that produced eggs that all hatched. The control crosses between 7-day-old uninfected females and 1-day-old uninfected males had a mean hatch failure rate of 0·04 (95% confidence intervals of 0 to 0·14, $N = 8$) and were not significantly different by a Mann–Whitney test to crosses between 1-day-old males and females ($P = 0{\cdot}615$), indicating that egg-hatch rate was not influenced by female age *per se*.

(v) *CI in the field*

Although more than 300 larvae and pupae were collected in the field, most did not reach the adult stage. The reasons for this are unknown. Of the survivors, 16 were male, 14 of which were infected with *Wolbachia*. CI levels varied widely amongst the infected flies, ranging from nearly all eggs hatching to no egg hatch. A total of nine males exhibited CI of greater than 0·25 and, of these, five exhibited CI of greater than 0·90. Overall, the 14 crosses involving infected males yielded a mean CI level of 61% (95% confidence intervals of 0·34 to 0·85), whereas 100% of eggs hatched in crosses involving the two uninfected males. Differences between these crosses are significant by a Mann–Whitney test ($P = 0{\cdot}012$). Control crosses were also carried out using eight field-collected females mated to uninfected laboratory-reared males. These females exhibited a mean egg hatch failure rate of 0·02 (95% confidence intervals of 0 to 0·05) and were not significantly different by a Mann–Whitney test ($P = 0{\cdot}140$) from the crosses with uninfected field males. These data indicate that CI can be expressed in field-reared males when the males are mated at a young age.

(vi) *Field-cage experiment*

We were unable to score one of the samples (Cage E, generation 5). In the other cages, there was a sharp increase in *Wolbachia* frequency by generation 5, whereas frequencies were variable at generation 4 (Fig. 3). Because infection frequencies were based on a sample of 10–30 flies per cage, it is not clear whether *Wolbachia* has gone to fixation or if rare uninfected individuals remained in the cages. Nevertheless, given that all individuals were also infected in the later generations, the *Wolbachia* frequency appeared to be stable at a frequency close to 100% in all populations.

4. Discussion

Why has the high level of CI not been found previously in crosses with *D. melanogaster* infected lines? The rapid decline in CI with male age is probably the main reason for this; unless 1-day-old males are used, high CI levels will not be detected. Only one other study, by Solignac *et al.* (1994), has used 1-day-old males in CI assays. However, in this study, males remained with females for several days and eggs were also collected over several days. Thus, an initial mating that might have resulted in a low hatchability would have been masked by subsequent matings with older males. All other studies have used males 2–5 days old and CI levels in these studies range from 0% to 40%.

The variability in levels of CI previously reported for *D. melanogaster* strains might also be partly or largely attributable to age effects. For instance, Solignac *et al.* (1994) found, in a survey of 23 infected *D. melanogaster* lines, CI levels varying from 10% to 77%. Although some of this variability might have been due to host-strain differences, much of it might be due to experimental procedure. Given that CI was determined by holding males and females together over several days until at least 150 eggs were scored, CI would have been influenced by both the time of mating and the rate of oviposition. If sufficient eggs were produced on the first day, a high level of CI should have been detected based on our data.

The initially high CI in the Australian *D. melanogaster* stock suggests that, prior to emergence from pupation, all spermatocysts are infected with *Wolbachia*. As reviewed in Lindsley & Tokayusu (1980), spermatogenesis in *Drosophila* is an ongoing

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process involving the continuous production of spermatocysts. Stem cells in the testes divide to give rise to daughter spermatogonia cells, each of which undergoes mitosis to produce 16 spermatocysts. Each spermatocyst then undergoes meiosis to produce a total of 64 spermatids, which mature to form spermatozoa. Thus, at any one time, there are cohorts of cells at similar stages of development in the testes. This process begins before the hatching of the embryo and the first spermatocysts produced enter meiosis just before pupation. It is thus possible that the first cohort(s) of spermatocysts produced during these early stages are all infected. All sperm produced from the first sperm cysts would then be modified, accounting for the very high CI levels induced by 1-day-old males.

As declining CI has been correlated with decreasing numbers of infected spermatocysts (Binnington & Hoffmann, 1989; Bressac & Rousset, 1993), differences in CI aging effects between strains and hosts might arise from a lower initial level of infection or from a more rapid loss of *Wolbachia* from spermatocysts. In *D. simulans* infected with *w*Ri, a strong CI-expressing strain, ~87% of sperm cysts are infected in 1-day-old males (Bressac & Rousset, 1993). Karr *et al*. (1998) found no uninfected sperm cysts in young *D. simulans* males. In a recent study, Clark *et al*. (2002) found that not all spermatocytes within a cyst contained *Wolbachia* in *D. simulans* males aged 3 days and older. It appears that *Wolbachia* multiplication does not match the production rate of new spermatocytes and that, as result, *Wolbachia* becomes depleted. *D. melanogaster* males were found to have far fewer *Wolbachia* in their testes, possibly resulting in an earlier and more rapid production of uninfected spermatocytes and cysts. It is thus possible that *D. simulans* males produce mostly modified sperm for several days after emergence, accounting for the persistence of high CI levels in virgin males of up to 5 days old in this species, whereas *D. melanogaster* males appear to produce mostly unmodified sperm after emergence.

The lack of CI elicited by young *D. simulans* males carrying the *w*Au strain suggests that *w*Au is a true non-expressor strain. Hoffmann *et al*. (1996) found that *D. simulans* embryos with this infection carried similar bacterial loads to *D. melanogaster*, suggesting that the inability to cause CI is probably a function of the *Wolbachia* strain rather than bacterial density. It is possible that this host removes *Wolbachia* from spermatocysts at an earlier stage than *D. melanogaster*, or prevents spermatocysts from being infected, but it is also probable that the *w*Au strain is unable to modify male chromosomes. The inability of the *w*Ma strain to induce CI in very young males suggests that the conflicting reports about its status are probably not due to differences in the ages of males used in tests James & Ballard (2000) found variable results for this *Wolbachia* strain, with incompatibility being exhibited in some cases but not in others. In contrast to Holden *et al*. (1993), we found that the Canton S *D. melanogaster* line does exhibit CI but at a reduced level. Solignac *et al*. (1994) also found CI in Canton S, albeit at a lower level than found here. This might reflect the fact that some females mated with 1-day-old males in their experiments.

The difference between the Australian and the Canton S *D. melanogaster* lines in CI levels and the results of the F1s from crosses between these two lines suggests that the host can control *Wolbachia* levels. CI suppression by the host can evolve under some circumstances depending on infection effects on host fitness as well as infection transmission rates (Turelli, 1994). In contrast to the effects of aging in males, female age had no detectable effect on CI levels. Selection is not expected to reduce *Wolbachia* loads in females, in which CI is the only consequence of infection. A reduction in *Wolbachia* density could produce eggs that are incompatible with infected sperm (Turelli & Hoffmann, 1995).

An early reduction in the number of infected cysts in *D. melanogaster* might explain the effect of repeated mating in decreasing CI, which also occurs in *D. simulans* (Karr *et al*., 1998). In *D. simulans*, the reduction in CI occurs during periods when no uninfected sperm cysts are found in the testes of young males. Sperm might be produced more quickly in mated adult males, and the shorter exposure time of sperm could prevent modification. However, as mentioned previously, by the time of emergence, adult *D. melanogaster* males produce sperm from mostly uninfected cysts. One potential explanation for the effect of mating on CI in this species is that repeated mating rapidly removes modified sperm from the testes, and that this is replaced by unmodified sperm.

The presence of CI in the field can help to explain how *Wolbachia* persists in natural *D. melanogaster* populations. Some of the matings that contribute offspring to the next generation are likely to involve young males, causing CI and an increase in *Wolbachia* frequency. The field-cage experiment simulated a population in which young males were involved in matings and, in these experimental populations, the infection quickly increased to close to fixation. Thus, *Wolbachia* frequencies should be high in populations in which young males are relatively successful. Perhaps this helps to explain the high incidence of *Wolbachia* in the tropics (Hoffmann *et al*., 1998), assuming that male longevity is reduced in this climate relative to temperate areas, where *Wolbachia* is less common. This issue could be resolved by obtaining CI estimates from field-collected females. Such data have been collected by Hoffmann *et al*. (1998) and provide no evidence of CI. However, only a few females were scored from areas where *Wolbachia* was at a particularly high or low frequency, owing to the difficulty of obtaining uninfected females that had taken part in matings with infected males in such areas. Accurate estimates of field CI would require repeated collections of numerous females at several locations, taking care to remove females immediately from males to prevent matings with older males.

Our findings bring into question the phenotypes of *Wolbachia* strains in terms of their ability to rescue sperm modifications (resc$^+$ or resc$^-$) and to cause CI themselves (mod$^+$ or mod$^-$). To date, four *Wolbachia* strains have been proposed as being resc$^+$ and mod$^-$ (Bourtzis *et al.*, 1998; Mercot & Poinsot, 1998). These strains have been used to support the hypothesis that different genes control the sperm-modification and rescue functions of *Wolbachia*. However, rigorous testing taking into account host effects such as age are needed before concluding that strains are true non-CI expressers. Even then, a lack of CI might be attributable to host factors rather than to the strain of *Wolbachia*. Perhaps the ability to induce CI should be viewed as a quantitative rather than a qualitative trait, with host and environmental factors determining the level of expression of CI and rescue.

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