Population Dynamics of the Wolbachia Infection Causing Cytoplasmic Incompatibility in *Drosophila melanogaster*

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ABSTRACT

Field populations of *Drosophila melanogaster* are often infected with Wolbachia, a vertically transmitted microorganism. Under laboratory conditions the infection causes partial incompatibility in crosses between infected males and uninfected females. Here we examine factors influencing the distribution of the infection in natural populations. We show that the level of incompatibility under field conditions was much weaker than in the laboratory. The infection was not transmitted with complete fidelity under field conditions, while field males did not transmit the infection to uninfected females and Wolbachia did not influence sperm competition. There was no association between field fitness as measured by fluctuating asymmetry and the infection status of adults. Infected field females were smaller than uninfecteds in some collections from a subtropical location, but not in other collections from the same location. Laboratory cage studies showed that the infection did not change in frequency when populations were maintained at a low larval density, but it decreased in frequency at a high larval density. Monitoring of infection frequencies in natural populations indicated stable frequencies in some populations but marked fluctuations in others. Simple models suggest that the infection probably provides a fitness benefit for the host in order to persist in populations. The exact nature of this benefit remains elusive.

THE vertically transmitted microorganism, Wolbachia, causes cytoplasmic incompatibility (CI) in many insects (Wade and Stevens 1985; Hoffmann *et al.* 1986; O'Neill and Karr 1990; Breeuwer and Werren 1993; Giordano *et al.* 1995; Kambhampati *et al.* 1993) and is present in a substantial fraction of all insect species (Werren *et al.* 1995a). Under unidirectional incompatibility, females lacking the infection produce no progeny or a reduced number of progeny when they mate with infected males. Levels of incompatibility can vary with the type of Wolbachia infection, genetic background of the host, age of the males or environmental conditions. This has been particularly well demonstrated in Drosophila, where some species carry infections causing strong incompatibility (Hoffmann *et al.* 1986; O'Neill and Karr 1990; Rousset *et al.* 1992) while others carry infections causing only partial incompatibility (Hoffmann 1988; Giordano *et al.* 1995). Infections in the same species may also have diverse effects; for instance, in *Drosophila simulans*, some infections cause strong incompatibility whereas others cause no detectable incompatibility (O'Neill and Karr 1990; Turelli and Hoffmann 1995; Hoffmann *et al.* 1996). Nasonia and Drosophila studies suggest that CI levels may vary because of the density of bacteria carried by insects (Breeuwer and Werren 1993; Clancy and Hoffmann 1997) although any relationship with density breaks down when different species or different infections are considered (Giordano *et al.* 1995; Hoffmann *et al.* 1996).

The population dynamics of Wolbachia infections was first modeled by Caspari and Watson (1959) who showed that infections are expected to spread rapidly in natural populations under unidirectional incompatibility. Such a spread has been shown for the *w*Ri Wolbachia infection of *D. simulans* (Turelli and Hoffmann 1991, 1995). In this system, simple population models have predicted the rate of spread and equilibrium frequency of the infection, based on the level of incompatibility under field conditions and on the transmission fidelity of the infection. Estimates for both these parameters differ in the field compared to the laboratory environment. CI is strong in laboratory crosses with young males, but weaker in the field, while transmission is perfect in the laboratory but not in the field (Hoffmann *et al.* 1990; Turelli and Hoffmann 1995). Such findings highlight the need for detailed field studies to understand the distribution of Wolbachia in natural populations.

Unlike the *w*Ri infection of *D. simulans*, the Wolbachia infection in *D. melanogaster* populations is associated with weak incompatibility in the laboratory (Hoffmann 1988). This infection appears widespread, having

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been isolated from populations around the world (Solignac *et al.* 1994). In Australia, most populations are polymorphic for the infection but can differ markedly in infection frequency (Hoffmann *et al.* 1994). Because the infection causes only weak laboratory CI, its persistence is difficult to explain if there is incomplete maternal transmission (Hoffmann *et al.* 1994).

To understand the population dynamics of the Wolbachia infection of *D. melanogaster,* field estimates of relevant parameters are needed and frequencies in natural and laboratory populations need to be monitored. Here we address a number of questions aimed at understanding Wolbachia persistence.

1. Is CI expressed under field conditions? If laboratory CI is lowered in the field to the same extent as observed for the *w*Ri infection of *D. simulans,* field CI in *D. melanogaster* should be extremely weak.
2. Can fitness effects be associated with the infection under field conditions? While it is difficult to determine the fitness of Drosophila in nature, measurements of fluctuating asymmetry (FA) and size may provide an indirect measure. Low levels of FA have been associated with high fitness in insects (*e.g.*, McKenzie and Clarke 1988; Thornhill and Sauer 1992), while size can influence the development time and fecundity of Drosophila (Robertson 1957; Partridge and Fowler 1993).
3. Are infection frequencies stable in populations? We provide data on changes in infection frequencies in laboratory populations, as well as in several natural populations sampled repeatedly.
4. What is the rate of transmission of the infection? Imperfect transmission of the infection will influence persistence in populations, as shown in *D. simulans* (Hoffmann *et al.* 1990; Turelli and Hoffmann 1995). We provide estimates of transmission from females to progeny in nature. We also test for paternal transmission in infected field males because this type of transmission could explain the persistence of the infection even in the absence of fitness effects. Paternal transmission is rare in *w*Ri *D. simulans* (Turelli and Hoffmann 1995).
5. Is there evidence for Wolbachia effects on sperm usage by females? Indirect evidence from Tribolium suggests that Wolbachia may influence sperm usage in females (Wade and Chang 1995) although this is not the case in *w*Ri *D. simulans* (Hoffmann and Turelli 1988).

Answers to these questions can be used to predict changes in infection frequencies in *D. melanogaster* populations. They show that the Wolbachia infection in *D. melanogaster* is likely to have beneficial effects on its host to account for its persistence.

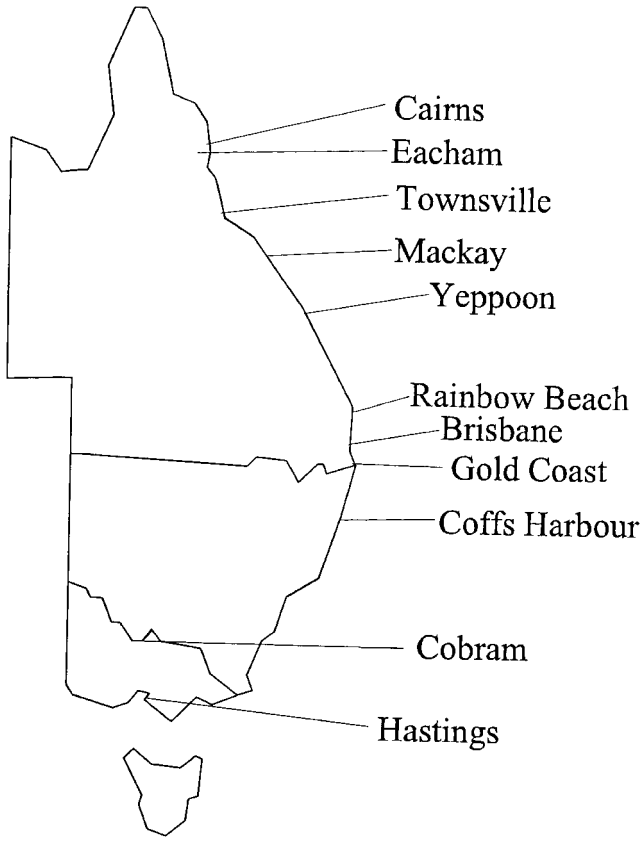

Figure 1.—Map of eastern Australia showing collection sites.

MATERIALS AND METHODS

Stocks and infection status: Reference stocks of infected and uninfected flies were obtained from Coffs Harbour (Figure 1) in November 1994. These stocks had been established after pooling the progeny of infected or uninfected field females. The infection status of these reference stocks was checked prior to the start of each experiment. We used a PCR assay involving Wolbachia-specific primers developed by O'Neill *et al.* (1992) to determine the infection status of all flies. This method and its validation in *D. simulans* are described elsewhere (Turelli and Hoffmann 1995). In *D. simulans,* the presence of Wolbachia in 323 lines from the field was (1) visualized with DAPI staining of Wolbachia in embryos, and (2) tested with the PCR assay. Both assays provided consistent results in 319 lines; inconsistencies in the remaining four lines could be explained by leakage during maternal transmission. A more limited validation has been undertaken in *D. melanogaster* (Hoffmann *et al.* 1994); results from the PCR and DAPI staining assays were in complete agreement for the 60 lines tested. Both DAPI staining and PCR results were also in complete agreement with the infection status of *D. melanogaster* lines as assessed by progeny testing (Hoffman *et al.* 1994; Solignac *et al.* 1994).

Infection frequency in natural populations: We monitored infection frequencies at a site on the Gold Coast (Figure 1) from February 1995 until February 1997. Flies with hardened cuticles (*i.e.*, flies that had not recently emerged from resources) were collected from 20–25 buckets with bananas along transects of at least 3 km. Collections were made every 1–2 months. By collecting mature flies from several buckets

away from resources, we ensured that flies were more likely to represent independent samples rather than sib groups. *D. melanogaster* and *D. simulans* females were separated by abdominal banding patterns and cheek width, while males were separated by genital arch morphology. Flies were frozen at −20° until PCR analysis. Infrequent collections with buckets were also made from the same sites near Coffs Harbour, Cobram and Hastings (Figure 1). Flies at the latter two sites were usually collected directly from rotting fruit rather than banana baiting. Finally, flies were obtained from several sites north of the Gold Coast (see Figure 1) between October 1994 and September 1995. Infection frequencies at all sites were usually determined from field females. However, in a few collections we obtained frequencies from males if only a few females were collected in a sample. For three collections with the largest number of females and males (at least 15 per sex), we statistically compared frequencies in the sexes; because there were no sex differences (contingency tests with 1 d.f.: $G = 1.42$, $P = 0.23$; $G = 1.12$, $P = 0.29$; $G = 0.88$, $P = 0.35$), data were pooled across sexes.

Field incompatibility: Field incompatibility levels were estimated following the approach outlined elsewhere (Hoffmann *et al.* 1990; Turelli and Hoffmann 1995). Flies were collected from natural populations (Gold Coast, Coffs Harbour and Cairns and Yeppoon) polymorphic for the infection. Except for the Cairns population, flies were collected by banana baiting using at least 20 buckets per site spread over transects of 3–15 km. The Cairns flies came from 12–15 banana traps as well as a giant pile of rotting bananas (around 30 m long, 5 m wide and 1 m deep) with many thousands of Drosophila (mostly *D. melanogaster*).

Females were separated from males directly after they had been collected. They were then placed on spoons with laboratory medium at 25° within 2 days of collecting. Females were transferred daily to fresh spoons for the next 2 days and then frozen (some were used to measure asymmetry as described below). The hatch rate of eggs from the first two spoons after 24 hr at 25° was used as a measure of incompatibility. Larvae from the third spoon were transferred to vials with food and eclosing adults were scored for the presence of Wolbachia using the PCR assay to ascertain infection status of the female parent.

We also collected males from Cairns and the Gold Coast to test CI in crosses between field males and uninfected females. Males were crossed to females from the uninfected reference stock within 1–2 days after being collected from the field. Hatch rates for each cross were obtained from eggs laid on spoons over 2 days. Males were then frozen and the infection status determined. This approach tests CI more directly than the field female approach because hatch rates from incompatible crosses can be scored directly; in contrast, for female data, hatch rates depend on the Wolbachia frequency in the field which is estimated with some error (Turelli and Hoffmann 1995). However, the female estimate does not suffer from the possibility that there is a substantial gap between the time females are inseminated and the time they are tested for CI. This factor could result in male data underestimating CI.

Following Turelli and Hoffmann (1995), relative hatch rates (H) were defined as H_I/H_C, where H_I is the hatch rate of incompatible crosses and H_C the hatch rate of compatible crosses. When hatch rates are determined from field females, H is defined as $[H_U - (1-p)H_W]/pH_W$ to compensate for the presence of compatible crosses for uninfected females. H_U and H_W are hatch rates from uninfected and Wolbachia-infected females, respectively, while p is the infection frequency. Confidence intervals were determined by bootstrapping (see Turelli and Hoffmann 1995).

Finally, we tested if the infected field flies eventually produced lines under laboratory culture showing CI, as expected from previous experiments demonstrating partial CI in laboratory stocks of *D. melanogaster* (Hoffmann 1988). Females were collected from the Gold Coast and crossed to infected males to determine incompatibility levels as described above. They were then used to establish isofemale lines. Field females were checked for infection status after they had produced progeny. Twenty-one infected lines (*i.e.*, lines established from PCR-positive females) were maintained at 20° for five generations before testing for incompatibility again. This was done by mating males from each line to uninfected females from the reference stock. Three of the males (3–4 days old) from each line were paired with three uninfected females and hatch rates of eggs on spoons were scored after 24 hr at 25°. While this experiment provides a test of incompatibility after laboratory culture, it is not clear if CI is due to Wolbachia infection or unrelated changes taking place as a consequence of the laboratory environment. We therefore crossed males from the lines to females from the infected reference stock (*i.e.*, set up known compatible crosses) and compared hatch rates in the two sets of crosses.

Asymmetry: Fluctuating asymmetry (FA) was measured for infected and uninfected flies from three collections (two from the Gold Coast and one from Cairns). We initially measured the fluctuating asymmetry of six bilateral traits: the numbers of sternopleural, inner and outer orbital bristles, the wing width and the length of the inner and outer wing cross veins. Wing width was measured from the intersection of the outer wing margin with vein II to the intersection with vein IV. Repeated measurements on 20 females indicated that FA in one of these traits was unreliable (Pearson's correlation coefficient, $r = 0.32$ in the case of inner crossvein length). The other quantitative FA measures had a high reliability ($r > 0.98$ in each case). A complete analysis of the repeatability of these FA measures following the procedures outlined in Palmer (1994) is presented elsewhere (R. Woods, M. Newman and A. A. Hoffmann, unpublished data).

Individual measures of FA were combined to obtain an overall score of developmental instability. FA in each trait was first standardized by taking the mean away from each score and dividing by the standard deviation. Scores were then summed to estimate FA for each individual.

Effects of infection status on size: We monitored the size of females from the Gold Coast collections by measuring thorax length. Measurements were made with an ocular micrometer and were highly repeatable ($r = 0.99$, $N = 20$, $P < 0.001$).

Transmission fidelity and paternal transmission: To score transmission fidelity, females were collected from the Gold Coast in February 1995 when the infection frequency was high. Field females were placed in vials and up to 10 progeny per female as well as the field females themselves were tested for infection status with the PCR assay.

To determine if paternal transmission occurred, we tested the infection status of one or two progeny from each cross involving a field male and uninfected laboratory female. This follows the suggestion of a low (<1%) level of paternal transmission in the *D. simulans* *w*Ri infection (Hoffmann and Turelli 1988; Turelli *et al.* 1992) although a direct estimate of paternal transmission in field males has not previously been obtained for any species. Field males from the Cairns (4/95) and Gold Coast (10/95) collections were used in these tests.

Frequency changes in laboratory populations: Infected *D. melanogaster* from Coffs Harbour were reared for a generation on 0.03% tetracycline (Hoffmann *et al.* 1986) to produce an uninfected stock. The infection status of the cured stock was confirmed by PCR assay. Lines were set up at an infection frequency of 50% using these stocks and then maintained at 25°.

Lines were initiated and maintained in one of two ways. For the high density treatment, 15 pairs of infected males and females along with 15 uninfected pairs (all 3–6 days old) were left in an empty bottle containing five small spoons each containing 2 ml of laboratory medium covered with a live yeast suspension. Flies were left overnight to lay on spoons. The medium on the spoons along with the eggs were transferred to a vial containing 13 ml of laboratory medium. For the low-density treatment, the same procedure was followed except that medium with eggs from the spoons was transferred to a bottle containing 90 ml of medium. In this manner we set up eight replicate high-density lines and an equivalent number of low-density lines. The next generation, flies were collected from vials or bottles and aged in bottles for 3–4 days. Sixty flies from each line were then used to repeat the egg laying procedure. This process was followed for another four generations, at which stage 30 adult females were sampled from each line and tested for infection status. We also tested a sample of flies after four generations to confirm that flies from the high-density treatment were smaller than those from the low-density treatment.

Sperm competition: The design is similar to one described in Hoffmann and Turelli (1988) for testing sperm effects in *w*Ri *D. simulans*. Four generations of backcrossing were used to generate an infected stock of ebony flies carrying the nuclear background of the infected reference stock. The mutant stock was exposed for a generation to medium containing 0.03% tetracycline, which is known (Hoffmann *et al.* 1986) to eliminate the infection. The infected reference stock was also exposed to tetracycline for a generation to produce an uninfected wild-type stock.

We set up four sets of crosses with uninfected ebony females at 25°. Virgin females were aged for 2–3 days and then mated individually to infected or uninfected wild type males (2–6 days old). By observing the flies, males could be removed within a minute of completing copulations to ensure that females had only mated once. Females were then transferred daily to fresh yeasted vials for 4 days before they were remated. This procedure stimulates remating in *D. melanogaster*, which occurs more readily when females have utilized some of their stored sperm and when food is present (*e.g.*, Harshman *et al.* 1988). Females from both types of first matings were remated with either infected or uninfected ebony males to generate the four sets of crosses. Females that remated over a 10-hr period (>60% of those set up) were placed in yeasted vials and transferred every second day until they stopped producing progeny.

In the second remating experiment, the same procedure was followed, except that crosses were undertaken with infected rather than uninfected ebony females. Sperm competition in this experiment was not confounded by CI because infected females are compatible with both infected and uninfected males.

RESULTS

Infection frequency in natural populations: The infection frequency at the Gold Coast site fluctuated markedly over an 18-month period (Figure 2). Contingency analysis indicated that frequencies differed significantly between collections ($G = 281.41$, d.f. $= 14$, $P < 0.001$), Confidence intervals were computed following Zar (1996) and often do not overlap for adjacent collections (Figure 2). Changes in the infection frequency were marked between some of the monthly intervals, exceeding 20%. There is no seasonal pattern evident from the results because the lowest infection frequencies were observed in summer, autumn and winter.

Fluctuations in the infection frequency were also evident at Coffs Harbour (Figure 2) despite the limited sampling undertaken. Frequencies differed significantly between collections ($G = 74.25$, d.f. $= 4$, $P < 0.001$). The low infection frequency in April 1995 matches the Gold Coast data for that time, but this was not the case for the December 1995 collection.

In contrast to these results, the Wolbachia frequency did not differ significantly between collections from Cobram ($G = 3.03$, d.f. $= 4$, $P = 0.55$; two collections with low infection frequencies were pooled) and Hastings ($G = 2.02$, d.f. $= 3$, $P = 0.73$). The infection frequency was stable and always low. We also found that collections north of the Gold Coast did not differ significantly in frequency ($G = 13.44$, d.f. $= 9$, $P = 0.14$) and were uniformly high (Figure 3) even though collections were undertaken at different times and spanned a wide region (Figure 1). These data and previous results (Hoffmann *et al.* 1994) suggest that the infection is always common in northern regions of the east coast of Australia and always rare in the southernmost areas.

Field incompatibility: For all five collections, infected and uninfected females produced eggs with similar hatch rates (Table 1). These were compared with nonparametric Mann-Whitney tests because the high hatch rates in most crosses produced a skewed distribution. The tests indicated that the infection status of the females did not influence hatch rate. Confidence intervals for the hatch rates of infected and uninfected females (determined by bootstrapping) always overlap (Table 1).

Our ability to detect differences between females may have been hampered by the high infection frequency in most collections, reducing the sample sizes for uninfected females. However, a high infection frequency makes it more likely that uninfected field females would have mated with infected field males (*i.e.*, participated in incompatible crosses). Estimates of the relative hatch rate (H) for all collections are close to 1. Confidence intervals were not computed for the Coffs Harbour collection because there were only three infected females with hatch rates of 100%. For the remaining collections, intervals are large in three collections but fairly small in two others.

When field males were crossed to uninfected laboratory females, hatch rates were similar for the infected and uninfected males (Table 1). The only suggestive difference ($P < 0.10$) for the Gold Coast collection was in the opposite direction to that expected because H is greater than 1. The Wolbachia infection therefore does not appear to induce CI in field flies, in contrast to laboratory results (*cf.* Hoffmann 1988; Hoffmann *et al.* 1994).

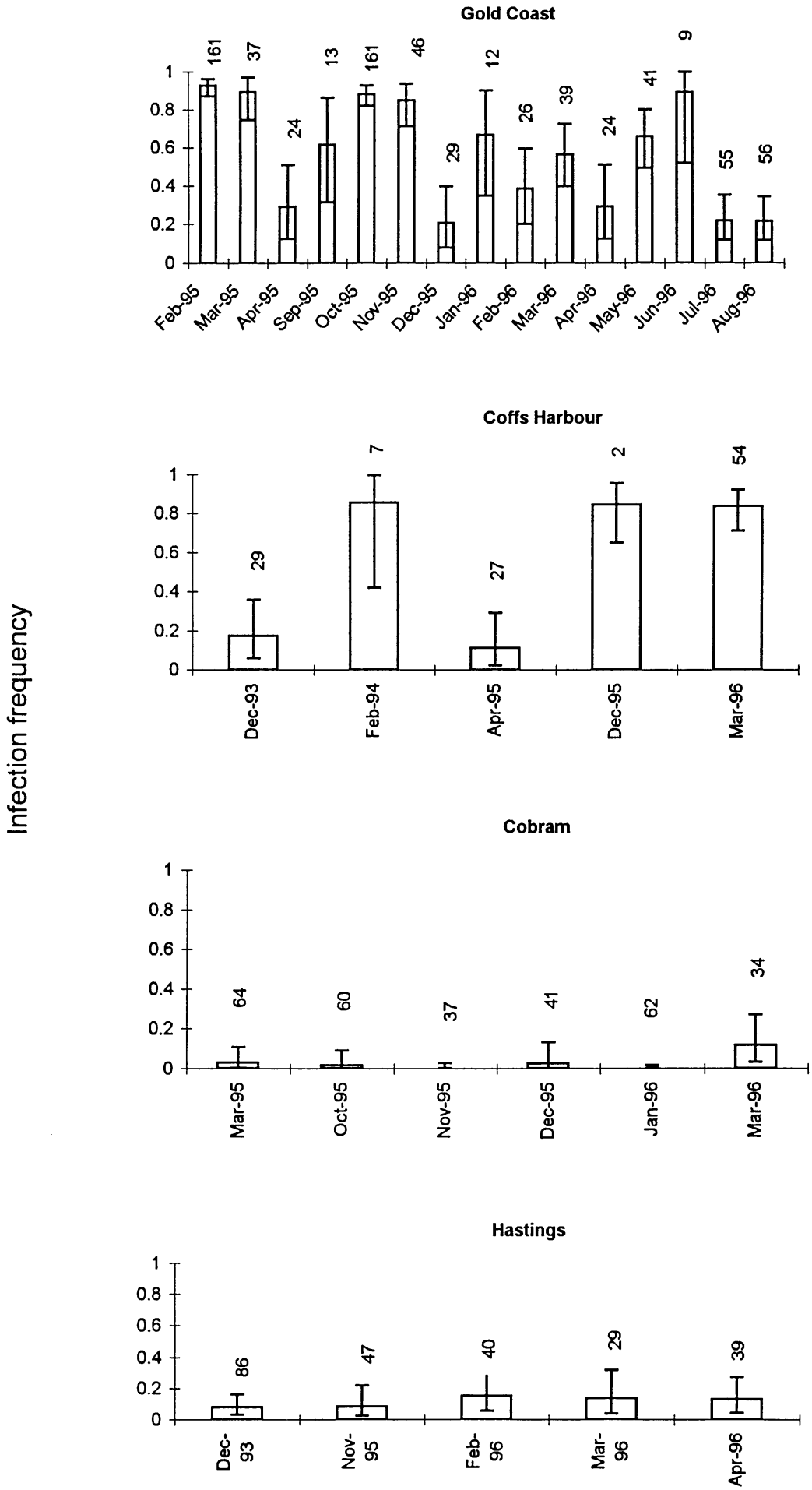

Figure 2.—Frequency of the Wolbachia infection in samples collected from four sites. For site locations, see Figure 1. Error bars indicate 95% confidence intervals; numbers indicate sample sizes.

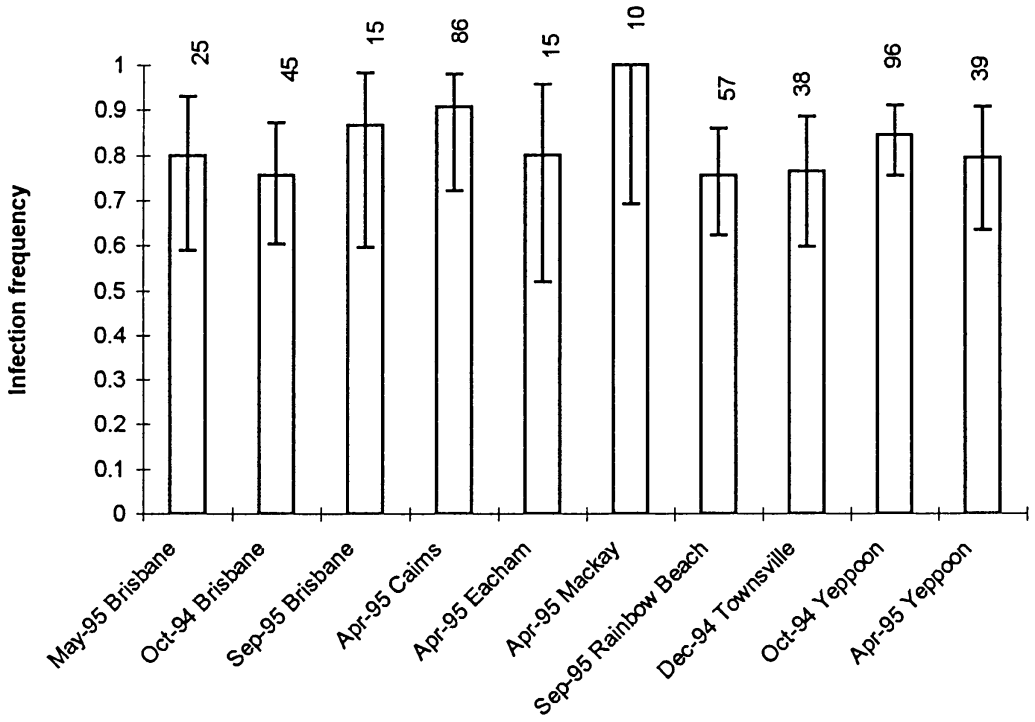

Figure 3.—Infection frequencies in collections from locations north of the Gold Coast. Error bars indicate 95% confidence intervals; numbers are sample sizes. For site locations, see Figure 1.

To test the hypothesis that incompatibility levels increase under laboratory culture, we examined CI of males from infected isofemale lines after five generations. As controls, we undertook crosses between males from the lines and infected females from the reference stock. Spoons producing only unhatched eggs (<2% of total number of spoons) were excluded from further analysis because eggs may have been laid by unmated females. Paired *t*-tests were used to compare the hatch rates of each of the 21 lines in these two sets of crosses. When males were crossed to uninfected females, the mean hatch rate for the 21 lines was 0.88 (SD = 0.088) compared to a mean hatch rate of 0.94 (SD = 0.0435) for crosses to infected females. These results differed significantly ($t = 4.22$, $P < 0.001$) indicating the presence of CI after five generations of laboratory culturing. The level of incompatibility is somewhat lower than previously observed in crosses between laboratory stocks (Hoffmann 1988). This could reflect the variability in incompatibility levels commonly observed in

TABLE 1

Hatch rates of eggs obtained from field-collected *D. melanogaster* and estimates of the relative hatch rates of compatible and incompatible crosses

	Infected flies			Uninfected flies				
Collection	*N*	$\bar{x}$	95% CI	*N*	$\bar{x}$	95% CI	H^a	*P*
Field females								
Yeppon (10/94)	71	0.950	0.910–0.980	13	0.903	0.880–0.994	0.941 (0.903–1.079)	0.924
Gold Coast (2/95)	149	0.944	0.922–0.961	12	0.952	0.925–0.979	1.009 (0.973–1.047)	0.356
Coffs Harbour (4/95)	3	1.000	—	27	0.971	0.938–0.999	0.710	0.381
Cairns (4/95)	76	0.942	0.900–0.959	7	0.933	0.884–0.970	0.990 (0.936–1.056)	0.254
Gold Coast (10/95)	118	0.963	0.938–0.987	16	0.904	0.771–0.993	0.930 (0.783–1.040)	0.677
Field males								
Cairns (4/95)	50	0.961	0.934–0.979	6	0.972	0.933–1.000	0.989 (0.951–1.035)	0.513
Gold Coast (10/95)	86	0.934	0.927–0.974	24	0.846	0.732–0.934	1.104 (0.998–1.306)	0.061

Hatch rates were obtained directly from inseminated females from the field. For field males, flies were crossed to virgin uninfected females to obtain hatch rates. Probabilities are from Mann-Whitney tests comparing rankings of infected and uninfected crosses. The relative hatch rate of infecteds compared to uninfecteds (H) is defined H_I/H_C where H_I is the hatch rate of incompatible crosses, and H_C is the hatch rate of compatible crosses.

[a] 95% confidence intervals (in parentheses) for H and the means were determined by bootstrapping.

TABLE 2

Overall fluctuating asymmetry levels of infected and uninfected *D. melanogaster* females from field collections

	Infected flies			Uninfected flies			
Collection	*N*	$\bar{x}$	SD	*N*	$\bar{x}$	SD	*P*
Gold Coast (2/95)	123	−0.19	2.26	9	−0.13	1.89	0.941
Cairns (4/95)	50	−0.14	2.18	5	1.11	1.06	0.058
Gold Coast (10/95)	106	−0.01	2.29	15	−0.67	1.19	0.277

P values are for *t*-tests comparing overall asymmetry of infected and uninfected females. Overall asymmetry was determined by standardizing and summing asymmetry levels of five traits (see text).

D. melanogaster, which may be associated with bacterial density (Solignac *et al.* 1994). The lower CI values in our current experiments are probably environmentally based because a previous attempt to select for the altered expression of CI in lines from Coffs Harbour was unsuccessful (Hoffmann *et al.* 1994).

Asymmetry: There was no evidence that infected females had higher levels of fluctuating asymmetry than uninfected females although sample sizes for uninfecteds were small (Table 2). Comparisons based on *t*-tests were all nonsignificant; there was suggestive evidence of a difference in FA in the Cairns collection ($P < 0.10$), but uninfected flies had a relatively higher level of FA in contrast to expectations.

Effects of infection status on size: We measured the thorax length of 64 infected and 128 uninfected females from Gold Coast collections. An ANOVA indicated significant effects of collection date and infection status, as well as an interaction between these factors (Table 3). The differences among collections are not surprising; as expected, flies likely to have developed in the coldest months (*i.e.*, collected in July and August) tended to be larger than those likely to have developed in the warmer months (Figure 4). The overall effect of infection status is due to the fact that uninfected flies tended to be larger than infected flies. This difference is evident in some months but not in others; *t*-tests indicate significant differences for May ($t = 3.03$, d.f. = 17, $P = 0.007$) and August ($t = 2.66$, d.f. = 54, $P = 0.013$) but not for the other months. The Wolbachia infection therefore influences the size of field females on some occasions.

Transmission fidelity and paternal transmission: To examine transmission of Wolbachia, progeny were obtained from 37 females from the Gold Coast. Four of these were uninfected and progeny were therefore not scored. Three infected females produced a single uninfected progeny (out of 7–10 tested), while another female produced two uninfected and eight infected progeny. This led to an overall rate of maternal leakage of 5 out of 194 tested, or 2.6%, with confidence intervals of 0.8 and 5.9%. Wolbachia are therefore not transferred with complete fidelity by field *D. melanogaster*.

We scored one or two progeny from 60 crosses involving infected males collected at the Gold Coast to test for paternal transmission. None of the 74 progeny were infected. The 95% confidence intervals for transmission are 0 and 3.9%. A second estimate was obtained from 45 males collected from Cairns. Again, none of the 90 progeny were infected, leading to an upper confidence limit of 3.3%. When these two estimates are combined, they suggest a paternal transmission rate of 0 with an upper confidence limit of 1.8%. Paternal transmission of Wolbachia is therefore rare or absent in the field.

Frequency changes in laboratory populations: We expected flies from the high-density treatment to be relatively smaller. To test this, we compared the wing and thorax lengths of 30 flies from each treatment. For the high-density population, the mean wing length was 1.27 mm (SD = 0.04) and the mean thorax length was 0.90 mm (SD = 0.04). These values are significantly lower ($P < 0.001$) than wing ($\bar{x} = 1.34$ mm, SD = 0.03) and thorax ($\bar{x} = 0.95$ mm, SD = 0.02) lengths of flies from the low-density treatment.

Wolbachia frequencies were estimated after five generations in both sets of populations. These indicate that the mean proportion of infected flies was greater in the low-($\bar{x} = 0.418$, SD = 0.165) compared to the high-density ($\bar{x} = 0.158$, SD = 0.260) treatment based on population means. Four high-density populations had lost the infection completely. The others had infection frequencies of 0.77, 0.2, 0.17 and 0.13. In contrast, low-

TABLE 3

Analysis of variance on thorax length data for females collected from the Gold Coast

Term	d.f.	MS	*P*
Collection	4	0.018	0.005
Infection status	1	0.030	0.013
C × I interaction	4	0.013	0.026
Error	182	0.005	

MS, mean squares.

TABLE 4

Effects of infection status of *D. melanogaster* on sperm condition

	First male	Second male	*N*	Proportion ebony (total)[a]	Proportion ebony (mating)[b]
Crosses with uninfected females					
1.	Infected	Uninfected	36	0.55 (0.17)	0.91 (0.09)
2.	Uninfected	Infected	30	0.34 (0.15)	0.71 (0.27)
3.	Infected	Infected	37	0.47 (0.14)	0.88 (0.11)
4.	Uninfected	Uninfected	33	0.40 (0.20)	0.74 (0.23)
Crosses with infected females					
1.	Infected	Uninfected	36	0.47 (0.19)	0.88 (0.21)
2.	Uninfected	Infected	41	0.37 (0.22)	0.75 (0.21)
3.	Infected	Infected	39	0.40 (0.15)	0.84 (0.23)
4.	Uninfected	Uninfected	44	0.38 (0.17)	0.75 (0.23)

Values are the mean proportion of offspring that were ebony, with standard deviations in parentheses.
[a] Proportion determined from all emerging progeny.
[b] Proportion determined only from offspring emerging after second mating.

density populations had frequencies of 0.65, 0.6, 0.59, 0.56, 0.38, 0.28, 0.26 and 0.19. The difference between treatments was significant by a Kruskal Wallis test ($\chi^2 = 5.91$, d.f. = 1, $P = 0.015$) when the proportion of the flies infected in each population was used as a single data point. Infection frequencies were therefore influenced directly or indirectly by the larval density treatments. There was no evidence that the infection was altered from its initial frequency in the low-density treatment; following arcsine transformation, the mean frequency of the populations did not differ significantly from 0.5 by a *t* test ($t = 1.42$, d.f. = 7, $P = 0.20$).

Sperm competition: Experiments were conducted using laboratory strains showing cytoplasmic incompatibility. The number of ebony flies produced by each female was expressed as a proportion of the total number of progeny she produced; the larger the proportion, the greater the contribution made by the second male. Proportions were analyzed using ANOVAs and two contrasts (Tables 4 and 5). For matings with uninfected females, differences between treatments were highly significant. The first contrast compared the effects of the first mating on the proportion of ebony progeny. This was significantly higher for matings involving infected rather than uninfected first males (Table 5). The second contrast tested the effect of the second mating, and this contrast was also significant due to the higher proportion of ebony produced when the second male was uninfected. These results can be explained by incompatibility. When uninfected females first mate with infected males, CI results in a reduction in the number of wild-type progeny. Because the infection status of the second male also had an effect, CI is also expressed when uninfected females remate, leading to a greater proportion of ebony when second males are uninfected. Similar patterns were evident when the proportion of ebony produced only after the second mating was compared (Table 4).

To examine the extent to which the infection caused CI in the first and second matings, we compared the relative reduction in the number of wild-type flies produced in crosses with infected and uninfected males (first mating) with the equivalent reduction in ebony flies (second mating). Before remating, the mean number of wild-type flies produced from compatible crosses was 177.8, compared to 125.2 for incompatible matings, or a reduction of 29%. After remating, the number of ebony from crosses with uninfected ebony was 163.1, compared to 136.1 for crosses with uninfected males, or a reduction of 17%. Thus CI is substantial in both matings and comparable to values in Hoffmann (1988) and Hoffmann *et al.* (1994), although CI may have been reduced somewhat in the second mating even though males of a similar age were used in both cases.

TABLE 5

Mean squares and *t* values for ANOVAs and contrasts on the proportion of offspring that were ebony after the second mating had occurred

	d.f.	MS or *t*	*P*
Crosses with uninfected females			
Treatment	3	0.474	<0.001
Error	132	0.033	
Contrasts			
(i) (1 + 3) − (2 + 4)	1	5.63	<0.001
(ii) (1 + 4) − (2 + 3)	1	3.11	0.002
Crosses with infected females			
Treatment	3	0.150	0.028
Error	156	0.048	
Contrasts			
(i) (1 + 3) − (2 + 4)	1	2.99	0.003
(ii) (1 + 4) − (2 + 3)	1	0.56	0.575

Numbers in contrasts refer to treatment are listed in Table 5.

Data from these crosses were used to test if infected sperm displaced uninfected sperm. If there is displacement, fewer wild-type progeny should be produced when crosses involve an uninfected first male and the remating ebony male is infected rather than uninfected. The data provide no evidence for this. In rematings with infected males, a mean of 42.1 (SD = 30.2) wild-type progeny were produced after the second mating, compared to a mean of 44.0 (SD = 41.7) for rematings with uninfected males.

In the second experiment with infected females, the proportion of flies that were ebony differed significantly between treatments (Table 5). The contrasts indicate an effect of infection status of the first but not the second male. Because the second male has no effect, there is no evidence that infected sperm carrying the ebony marker were able to displace sperm from the first male to a greater extent than ebony uninfected sperm. The same conclusion is reached when considering only those progeny produced after remating (see means in Table 4). The effect of the first male on ebony numbers was surprising. Means indicate that proportions were relatively greater when females first mated with infected males rather than uninfected males (Table 4), suggesting that fewer wild-type flies were produced when matings took place with infected males. This difference is also apparent when the number of wild-type progeny produced by females is considered. In total, when the first mating was with an uninfected male, the mean number of wild-type progeny was 260.1 (N = 85, SD = 103.3) compared to 205.3 (N = 75, SD = 66.2) for matings with infected males. These means differ significantly ($F_{(1,158)} = 15.5$, $P < 0.001$). Perhaps infected sperm were lost more readily than uninfected sperm, regardless of the infection status of the remating male. In any case, there is no evidence for sperm displacement by infected sperm or increased retention of infected sperm.

DISCUSSION

Can we explain the distribution of Wolbachia in natural populations? In *D. simulans*, the spread of the *w*Ri infection is largely determined by its ability to induce incompatibility under field conditions. Although field incompatibility levels are lower than those observed in the laboratory because matings appear to take place with older males (Hoffmann *et al.* 1990; Turelli and Hoffmann 1995), the incompatibility is still sufficiently strong to explain the rapid spread of the *w*Ri infection in natural populations (Turelli and Hoffmann 1991, 1995). The *w*Ri infection continues to increase until an equilibrium is reached between imperfect maternal transmission and incompatibility, and populations then remain at around 94% infected.

In contrast, there is no detectable incompatibility associated with the *D. melanogaster* infection under field conditions despite partial incompatibility in the laboratory as evident from earlier work (Hoffmann 1988) and the incompatibility detected in the present study after lines were reared under laboratory conditions. This suggests that the dynamics of the *D. melanogaster* infection are different to those governing *w*Ri. In the absence of incompatibility, the frequency of the infection (p) in an isolated population at generation $t + 1$ is given by

$$p_{t+1} = \frac{p_t(1-\mu)(1-s_f)}{1-s_f p_t},$$

where $1 - s_f$ is the relative fitness of infected females and μ is the transmission efficiency defined by the proportion of uninfected progeny produced by an infected female (see Caspari and Watson 1959; Turelli and Hoffmann 1995). If μ is greater than 0 and/or s_f falls between 0 and 1, then the infection will be lost because p_{t+1} will be less than p_t. Our only estimate of μ suggests that it is around 2.5%, which is similar to values obtained for the wRi infection of *D. simulans* (Hoffmann *et al.* 1990; Turelli and Hoffmann 1995). This level of imperfect transmission would lead to a gradual loss of the infection in the absence of fitness effects. For instance, in 50 generations the infection would decrease from 50 to 14%. The laboratory population data suggest that such a decrease may be more rapid when populations are cultured at a high level of crowding.

These arguments are based on simple models that ignore quantitative variation in parameter estimates. Changing intracellular Wolbachia densities can lead to variation in both expression of CI and transmission efficiency. Incompatibility levels induced by *D. melanogaster* males from different lines have previously been related to Wolbachia density (Solignac *et al.* 1994). In addition, the effect of male age on CI (in *D. simulans*) is related to the fact that older males carry fewer Wolbachia (Binnington and Hoffmann 1989; Bressac and Rousset 1993). Finally, larval density effects on incompatibility levels and transmission efficiency in *D. simulans* may be mediated via Wolbachia density in cells (Sinkins *et al.* 1995; Clancy and Hoffmann 1997).

However, as in the case of *D. simulans*, it is doubtful if complex models incorporating density effects would alter the main predictions of the simple models. One reason is that variability in CI in *D. melanogaster* seems to be low; no CI was detected in any of our seven samples. Variability in maternal leakage rates may also be low as in *D. simulans* (Turelli and Hoffmann 1995). Without CI but in the presence of maternal leakage, Wolbachia frequencies are expected to decrease in the absence of other factors. One possibility is that Wolbachia is transmitted paternally as well as maternally, increasing its transmission rate in a population, particularly under multiple mating. However we have found no evidence of Wolbachia transfer when infected field males mated with uninfected laboratory females. Another possibility

is that horizontal transmission of Wolbachia occurs at a relatively high frequency. While the absence of a phylogenetic association between Wolbachia and its hosts indicates that such transmission can occur (O'Neill *et al.* 1992; Werren *et al.* 1995b), it is likely to be extremely rare and has never been detected experimentally.

Our experiments provide no evidence for an increased ability of infected sperm to displace uninfected sperm, consistent with data for the *D. simulans w*Ri infection (Hoffmann *et al.* 1990). The frequency of the infection is therefore unlikely to be influenced by this factor. However, infected sperm may be at a disadvantage in *D. melanogaster* because they are lost more readily from the first mating. This effect could be important at times when females remate infrequently. These results contrast with those of Wade and Chang (1995), suggesting that infected sperm fertilize more females than expected in multiply mated females.

Because paternal transmission and sperm competition effects are absent and because CI appears weak or absent, host fitness is likely to be influenced by Wolbachia to account for its distribution in *D. melanogaster.* Our attempts to identify fitness effects have met with mixed success. Infection frequency changes in laboratory populations provided no evidence that infected individuals have a fitness advantage at 25°. The field asymmetry data suggested that there was no association between Wolbachia and developmental stability.

In contrast, we did detect infection effects on body size in some collections, in that infected females were smaller than uninfected individuals. Size can have a marked influence on the fitness of Drosophila. It is well known that female fecundity is positively correlated with size (*e.g.*, Robertson 1957; Tantawy and Rahka 1964) although we have not found a fecundity disadvantage associated with the *D. melanogaster* infection under laboratory conditions (Hoffmann *et al.* 1994). Large size can also result in a slower larval development and influence competitive ability (Partridge and Fowler 1993). Size effects associated with the Wolbachia infection could therefore influence the fitness of flies in the field in different ways. Clinal patterns indicate selection on size in field *D. melanogaster.* For instance, in eastern Australia, size increases both phenotypically and genetically with latitude (James *et al.* 1995). Therefore relatively larger flies are expected to have a fitness advantage in southern states. This coincides with the low incidence of Wolbachia in the southernmost populations, compared with a high frequency at low latitudes. Perhaps factors contributing to the cline in size are the same as those influencing infection frequency. In addition, the Wolbachia infection could partly account for the cline in body size.

These possibilities remain to be tested, and fitness traits associated with size need to be identified under field conditions. The Gold Coast data indicate that any selective factors influencing Wolbachia frequency should be identifiable because they are likely to be large. Infection levels in this population increased more than 20% between a monthly interval, which probably corresponds to a single field generation (see Turelli and Hoffmann 1995). Infected females will need to have a fitness at least 1.2 times that of uninfected females to account for such increases (although movement of flies between areas differing in infection frequency may also play a role).

In conclusion, we have shown that the Wolbachia infection in *D. melanogaster* is stable in some populations but changes rapidly in others. Transmission data suggest that the infection should be lost from populations in the absence of paternal transmission and effects on sperm competition, particularly under crowded conditions. The persistence of the Wolbachia infection in *D. melanogaster* therefore may be associated with positive fitness effects on the host insect. One possibility is that fitness effects are mediated via size. However, there are also others. For instance, it is possible that positive effects occur because of interactions between Wolbachia and other fungal or bacterial infections. Perhaps the presence of Wolbachia prevents other infections becoming established.

In future work, we will examine such fitness effects, particularly under field conditions. We will also consider the association between Wolbachia density and the current findings. For instance, does the absence of CI in the field and subsequent increase in the laboratory reflect changes in infection density? Are variation in transmission frequency and size effects associated with this factor? Is CI induced when infected field females mate with infected laboratory males that presumably carry a higher infection load?

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