Naturally-occurring *Wolbachia* infection in *Drosophila simulans* that does not cause cytoplasmic incompatibility

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Microbes of the genus *Wolbachia* are transmitted by their hosts via the maternal parent and are responsible for cytoplasmic incompatibility among insect populations. This phenomenon can result in *Wolbachia* spreading through natural populations as previously demonstrated in *Drosophila simulans*. Here we describe another *Wolbachia* infection in *D. simulans* that does not cause cytoplasmic incompatibility. This is a property of the *Wolbachia* rather than the nuclear background. The infection occurs at a low frequency in natural populations from eastern Australia. The infection shows perfect maternal transmission in the field and does not cause any detectable deleterious effects on its host. These findings suggest that the *Wolbachia* infection behaves like a neutral variant in populations. The infection may represent an evolutionary outcome of interactions between *Wolbachia* infections and their hosts.

Keywords: cytoplasmic incompatibility, *Drosophila simulans*, host–parasite evolution, maternal effects.

Introduction

Wolbachia are vertically-transmitted microbes that are widespread in insects. These microbes can cause cytoplasmic incompatibility in their host organisms. Infected males become reproductively incompatible with uninfected females or females carrying another *Wolbachia* strain, as shown in a range of organisms including mosquitoes (Yen & Barr, 1973; Kambhampati *et al.*, 1993), *Drosophila* (O'Neill *et al.*, 1992; Rousset *et al.*, 1992; Solignac *et al.*, 1994), *Nasonia* (Breeuwer *et al.*, 1992) and *Tribolium* (Wade & Stevens, 1985). *Wolbachia* can also have other phenotypic effects on hosts including parthenogenesis in wasps (Stouthamer *et al.*, 1993).

By causing cytoplasmic incompatibility, *Wolbachia* can spread in natural populations. Infected females have an advantage in populations that are polymorphic for the infection as they do not suffer incompatibility when they mate with infected or uninfected males (Caspari & Watson, 1959; Fine, 1978; Hoffmann *et al.*, 1990; Stevens & Wade, 1990). Because the *Wolbachia* infection is almost always transmitted maternally, this advantage can result in an increase in the frequency of an infection. The spread of

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Wolbachia will also be influenced by other factors, including any effects that microbes have on host fitness other than cytoplasmic incompatibility, and any leakage in the transmission of the infection between generations.

The behaviour of *Wolbachia* infections in natural populations has been studied most intensively in *Drosophila*, particularly in *D. simulans*. Two *Wolbachia* infections causing strong incompatibility have previously been described in this species. One of these infections (type R) has spread rapidly through California and also occurs in other areas (Hoffmann *et al.*, 1990; Turelli & Hoffmann, 1991). The other infection (type S) appears to be restricted to Pacific islands (Montchamp-Moreau *et al.*, 1991). Strains with types R and S infections are bidirectionally incompatible (O'Neill & Karr, 1990).

The distribution of the type R infection in natural *D. simulans* populations can be largely described in terms of two parameters: the level of incompatibility observed under field conditions and the efficiency whereby the microbe is transmitted from females to progeny (Hoffmann & Turelli, 1988; Hoffmann *et al.*, 1990; Turelli & Hoffmann, 1991; Turelli *et al.*, 1992). In addition, the spread of this infection can be influenced by deleterious effects on its host and by curing when larvae encounter naturally-occurring

antibiotics (Stevens, 1989), although these effects have not been demonstrated under field conditions.

Previous work based on crosses between stocks indicate that neither the type R nor the type S infection is present in Australian populations of *D. simulans* (Hoffmann *et al.*, 1990) even though *Wolbachia* do occur in Australian *D. melanogaster* (Hoffmann *et al.*, 1994). However, we have recently detected another infection in *D. simulans* from Australia using *Wolbachia*-specific primers of an rDNA region (O'Neill *et al.*, 1992) rather than the progeny tests we normally use to test for incompatibility. The newly-discovered infection has been designated as type A.

Here we show that the infection does not cause cytoplasmic incompatibility and that this is a property of the *Wolbachia* rather than the nuclear background. We also present data on the infection's distribution in natural populations of *D. simulans*. To understand why the infection is widespread in natural populations, we have considered its effects on host fitness and its maternal transmission efficiency in nature. Our findings suggest that the type A infection does not influence host fitness but shows perfect maternal transmission. The existence of infections with such properties has been predicted by theory (Turelli, 1994).

Materials and methods

Detection and infection levels

The newly-discovered *Wolbachia* infection was initially detected using the primers '76–99 forward' (5'-TTGTAGCCTGCTATGGTATAACT-3') and '1012–994 reverse' (5'-GAATAGGTATGATTTT-CATGT-3') of O'Neill *et al.* (1992) that amplify a 0.9 kb fragment of *Wolbachia* rDNA. As a positive control in PCR reactions, we used primers to amplify a single copy of a *Drosophila* nuclear gene, *suppressor of forked* (Langley *et al.*, 1993), using the '5812–5829 forward' (5'-ATTGACTACCTGTCC-CAT-3') and '6192–6174 reverse' (5'-ATAAG-TAAATTCGGCGGTT-3') primers that yield a fragment of around 400 bp. This procedure produces a 0.9 kb band if *Wolbachia* are present that can be readily distinguished from the control band on an agarose gel.

The presence of the microorganism was confirmed by DAPI staining of embryos (O'Neill & Karr, 1990). This staining method showed that the infection is localized in the outer cortex region, as is the case for other *Wolbachia* infections in *D. simulans* (O'Neill & Karr, 1990) and for the *Wolbachia* infec-

tion of Australian *D. melanogaster* (Hoffmann *et al.*, 1994). We also used embryo staining to quantify levels of infection in a type A strain collected from Coffs Harbour in December 1993. For this purpose, we scored the density of bacteria in the outer cortex of embryos (Boyle *et al.*, 1993). *Wolbachia* were visualized by epifluorescence microscopy of fixed embryos stained by indirect immunofluorescence. Embryos were collected and fixed following Hoffmann *et al.* (1994). They were then incubated at room temperature for 1 h using the primary, an anti-type R *Wolbachia* IgM (supplied by Hroyuki Kose & Tim Karr), diluted 1:2 in PBST (0.1 per cent Triton X-100 in phosphate buffered saline) then rinsed three times in PBST. The embryos were incubated in the secondary, a goat anti-mouse IgM-TRITC (Southern Biotechnology Associates, Birmingham, AL 35226, U.S.A.) diluted 1:200 in PBST, for 1 h at room temperature in the dark, then rinsed three times in PBST. The nuclear stain DAPI (1–2 μg mL^{-1} for 8–12 min) was used to assay their developmental stage, and the stained embryos were mounted as described in Hoffmann *et al.* (1994).

Only embryos displaying less than five nuclei were scored because densities can only be determined in young embryos (Boyle *et al.*, 1993). The cortex region was scored to a depth of 11–12 microns from the surface over an area of 94 microns (along long axis) $\times$ 65 microns. As well as examining 14 type A embryos (from an infected strain from Coffs Harbour), we also considered infection levels in 20 embryos from a type R strain from Ottawa (Hoffmann & Turelli, 1988) and 13 embryos from infected *D. melanogaster* from Coffs Harbour (Hoffmann *et al.*, 1994).

Distribution and maternal transmission

We used the PCR assay to examine the frequency of the type A infection along the east coast of Australia. In total, 1641 flies were collected from 12 sites along the coast (Fig. 1) between August 1993 and April 1994 by baiting with rotting fruit. *D. simulans* females were sorted from *D. melanogaster* females by abdominal banding patterns, cheek width (Gallo, 1973) and the appearance of the genital arch in male offspring. Flies were collected from two of the sites (Coffs Harbour, Gold Coast) on three separate occasions (8/93, 12/93, 4/94).

Field females were frozen at $-5°$C within 4 days of being collected, and eventually subjected to PCR to determine their infection status. If positive controls failed, extracts from females were retested. The infection status of all females collected from the
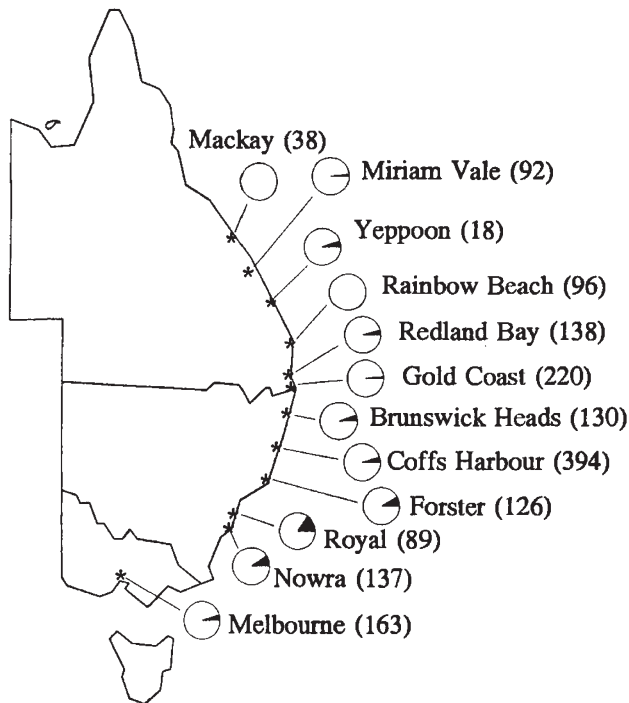
Fig. 1 Approximate location of *Drosophila simulans* collection sites in eastern Australia and the frequency of the type A *Wolbachia* infection. Shaded parts of the pie diagrams indicate infection frequencies determined by PCR analysis using *Wolbachia*-specific primers from the variable region of the 16S gene. Numbers in brackets indicate sample sizes. Coffs Harbour and Gold Coast frequencies are based on three pooled samples. Frequencies and confidence intervals are: Melbourne: 0.052 (0.028, 0.089); Nowra: 0.008 (0.059, 0.149); Royal: 0.187 (0.118, 0.276); Forster: 0.096 (0.075, 0.154); Coffs Harbour: 0.051 (0.034, 0.072); Brunswick Heads: 0.057 (0.027, 0.107); Gold Coast: 0.018 (0.006, 0.041); Redland Bay: 0.045 (0.020, 0.086); Rainbow Beach: 0 (0, 0.047); Yeppoon: 0.059 (0.017, 0.252); Miriam Vale: 0.022 (0.008, 0.068); Mackay: 0 (0, 0.120).

field was determined. The use of PCR to determine infection levels in natural populations has previously been verified on type R *D. simulans* where results were consistent with those obtained using progeny testing and DAPI staining (Turelli & Hoffmann, 1995).

We tested for the efficiency of maternal transmission in field flies using the method of Hoffmann *et al.* (1990), except that the infection was detected by PCR assay rather than progeny testing. We used females from Coffs Harbour collected on 12/93, 4/94 and 8/94. We tested these for infection status with the PCR assay after they had produced eggs. We identified 21 infected females in this manner. Between 15 and 40 progeny from each of the females were tested for infection status using the PCR assay to determine transmission rates.

Incompatibility and fecundity

Four experiments were undertaken to investigate the effects of the infection on incompatibility. In each experiment, pairs of males and females (2–3 days posteclosion) were set up in vials containing a spoon with 5 mL of a blackcurrant medium (61 per cent blackcurrant syrup, 5 per cent agar) covered with a live yeast suspension. Eggs were removed after 24 h and hatch rates were scored after 24 h at 25°C. Spoons with less than 10 eggs were not considered. In all experiments, type A flies were obtained from an infected strain collected from Coffs Harbour in December 1993.

In the first experiment, we examined the ability of type A males to cause incompatibility when mated with uninfected *D. simulans*. To provide controls, crosses were carried out between uninfected females and uninfected males or type R males. The former were expected to be compatible and the latter were expected to be incompatible. We set up a large number of replicates (93) for crosses with uninfected males and type A males so that we could detect low levels of incompatibility.

In the second experiment, we tested incompatibility in type A females. Females were crossed to type A males, strains of type R males from Riverside (Hoffmann *et al.*, 1986) or Ottawa (Hoffmann & Turelli, 1988) and type S males from a Hawaii strain. The infected Hawaii strain was collected in 1991 from Volcano.

The final two experiments tested the inheritance of type A incompatibility. Reciprocal crosses were carried out between the Riverside strain and the type A strain from Coffs Harbour. In the third experiment, F_1 males from the reciprocal crosses were mated to uninfected females. Crosses between Riverside males and uninfected females were also set up. In the fourth experiment, Riverside males were crossed to F_1 females from the reciprocal crosses. Riverside males were also crossed to type A females and uninfected females.

Apart from causing incompatibility, *Wolbachia* may have deleterious fitness effects on its host. In the case of type R *D. simulans*, infected females have a lower fecundity than uninfected females, at least under laboratory conditions (Hoffmann *et al.*, 1990). Deleterious effects have also been associated with *Wolbachia* infections of *Tribolium* (Stevens & Wade, 1990). To test if fecundity costs were associated with the type A infection, we cured an infected

line from Coffs Harbour by culturing it for a generation on medium supplemented with 0.03 per cent tetracycline (Hoffmann *et al.*, 1986), followed by a generation on unsupplemented medium to remove any effects of tetracycline exposure. The infected and uninfected lines differ in their infection status but not in their nuclear background. The fecundity of the two lines was measured following Hoffmann *et al.* (1994). For each line, 71 pairs of males and females (3–4 days old) were set up in vials with a small spoon containing laboratory medium covered with a live yeast suspension. Spoons were replaced daily for three days and egg numbers were summed to give an estimate of fecundity for each pair of flies.

Results

Infection levels

We anticipated that if microbe numbers were correlated with incompatibility levels, numbers for the type A infection should be lower than for the type R infection and the *D. melanogaster* infection. We therefore undertook one-tailed tests to compare the means. Because the two comparisons between the type A infection and either type R or *D. melanogaster* were not orthogonal, we corrected for the number of comparisons (2) using the Bonferonni technique. Counts between the R and A infections differed significantly at the 5 per cent level ($F_{1,32} = 4.79$), and numbers were substantially higher for the R infection. However, differences between type A and *D. melanogaster* were in the wrong direction (Fig. 2).

Distribution and maternal transmission

The A infection is widespread along the east coast of Australia and present at a low frequency in most populations (Fig. 1). Contingency tests indicated no differences in infection frequencies in the three collections from Coffs Harbour ($G = 0.314$, d.f. $= 2$) and the two largest collections from the Gold Coast (Fisher's exact test, $P = 0.63$). Data were therefore pooled from these sites before making comparisons between populations.

To compare infection frequencies among populations, collections north of Rainbow Beach were pooled to overcome the problem of having too many expected values less than five. Infection frequencies differed significantly among populations by a contingency test ($G_9 = 40.24$, $P < 0.001$). Population differences were also significant if the pooled localities were excluded ($G_8 = 40.78$, $P < 0.001$).
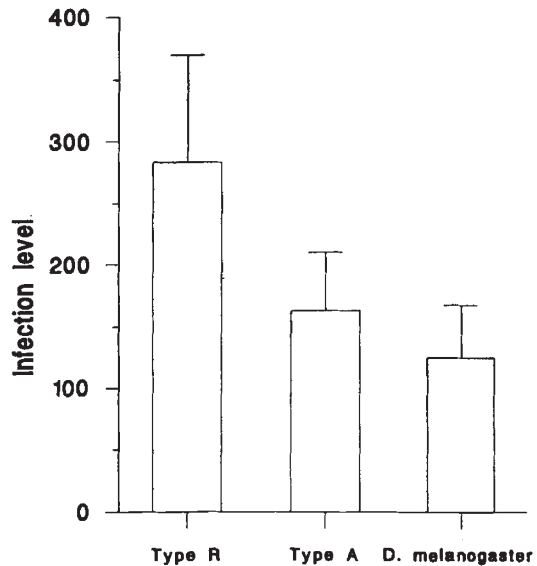

Fig. 2 Levels of infection in embryos from type A or type R *Drosophila simulans* or from infected *D. melanogaster*. Numbers represent *Wolbachia* counts in an area of 6110 μm^2 from the outer cortical region of embryos. Means and standard deviations (error bars) are based on 14 (type A), 20 (type R) or 13 (*D. melanogaster*) embryos.

Confidence limits for infection frequencies were determined following Zar (1984) and are given in the legend of Fig. 1. These indicate that the main difference between sites is the high frequency of infection in the Royal National Park, just south of Sydney. There is no obvious geographical pattern which suggests that infection frequencies are not related to climatic variables that vary latitudinally.

In the experiments on maternal transmission efficiency, all 572 of the progeny that were tested carried the *Wolbachia* infection. When 95 per cent confidence intervals are computed, the upper bound for the proportion of uninfected progeny produced by infected mothers is 0.01. Maternal transmission therefore seems to be perfect or near-perfect, in contrast to transmission of the type R infection of *D. simulans* (Hoffmann *et al.*, 1990) and a *Wolbachia* infection in *D. melanogaster* (A. A. Hoffmann & J. Duncan, unpublished data) where 3–9 per cent of progeny from infected females do not carry the infection.

Incompatibility and fecundity

Hatch rates from crosses were compared with nonparametric Kruskal–Wallis tests because the data had skewed distributions. These tests indicated significant differences ($P < 0.001$) among crosses in all four experiments. Differences between individual

crosses were examined using the STP approach for unplanned nonparametric comparisons outlined in Sokal & Rohlf (1981).

Results from experiments 1 and 2 indicate that the infection did not cause incompatibility. Males from *D. simulans* strains infected with *Wolbachia* are normally incompatible with females from uninfected strains (Hoffmann et al., 1986; O'Neill & Karr, 1990) and this is evident in the crosses with type R and type S males (Table 1). However, hatch rates in crosses between uninfected individuals are indistinguishable from those in crosses between type A males and uninfected females, despite the fact that we set up 93 replicates in one of the experiments. Males with the A infection therefore behaved like uninfected individuals. Females from strains with the A infection also behaved like those from uninfected strains. These females were incompatible with both type R and type S males, but compatible with males that carried the A infection (Table 1). We have obtained similar results with three other infected *D. simulans* strains from eastern Australia (data not given).

The F_1 data from experiments 3 and 4 indicate that the inability of type A males to cause incompatibility resides in the genome of *Wolbachia* or another cytoplasmic factor rather than in the genome of its host. When F_1s were obtained from reciprocal crosses between strains with R and A infections, males behaved the same as their maternal infection type when they were crossed to uninfected females (experiment 3, Table 1). The susceptibility of

females to incompatibility caused by the R infection is also inherited maternally because F_1 females behaved like their maternal parent (experiment 4). We have carried out successive generations of backcrossing to an R strain to demonstrate that the type A infection is inherited via the maternal parent. For instance, after the progeny from a cross between A females and R males were backcrossed to R males for three generations, the mean hatch rate from matings between backcross males and uninfected females was 0.843 (SD = 0.147, $N = 29$) compared with a mean hatch rate for controls (uninfected × uninfected) of 0.869 (SD = 0.192, $N = 30$).

The fecundity data indicated that the infection was not associated with deleterious effects. The mean number of eggs produced by the infected line ($\bar{x} = 273.8$, SD = 61.1, $N = 71$) did not differ significantly from the mean produced by the cured line ($\bar{x} = 265.2$, SD = 80.5, $N = 71$). *D. simulans* females carrying the A infection therefore did not suffer a detectable decrease in fecundity under laboratory conditions such as found in type R strains (Hoffmann et al., 1990). We have also failed to find deleterious effects for other traits related to fitness, including egg-to-adult development time, body size, larval viability and the resistance of adults and eggs to environmental stresses.

Discussion

The Australian *Wolbachia* infection of *D. simulans* does not appear to cause incompatibility under

Table 1 Hatch rates in four experiments involving crosses between a *Wolbachia*-infected *Drosophila simulans* strain from Australia and other uninfected and infected *D. simulans* strains

Crosses between strains				Crosses involving reciprocal F_1s			
Cross ♀ × ♂	$\bar{x}$	SD	N	Cross ♀ × ♂	$\bar{x}$	SD	N
Experiment 1				Experiment 3			
U × R	0.139[a]	0.116	12	U × R	0.010[a]	0.015	15
U × U	0.876[b]	0.245	93	U × F_1 (R × A)	0.048[a]	0.060	10
U × A	0.859[b]	0.284	93	U × F_1 (A × R)	0.865[b]	0.272	15
Experiment 2				Experiment 4			
A × A	0.811[a]	0.322	10	A × R	0.010[a]	0.014	13
A × R	0.089[b]	0.153	10	U × R	0.007[a]	0.012	15
A × O	0.013[b]	0.017	10	F_1 (R × A) × R	0.861[b]	0.060	10
A × S	0.033[b]	0.064	20	F_1 (A × R) × R	0.007[a]	0.009	12

Abbreviations: U is an uninfected *D. simulans* strain from Melbourne; R is an infected type R *D. simulans* strain from Riverside; O is an infected type R *D. simulans* strain from Ottawa; A is an infected *D. simulans* strain from Coffs Harbour, Australia, S is an infected *D. simulans* strain from Hawaii; and F_1 refers to F_1 males or females produced from reciprocal crosses between R females and A males (R × A) or between A females and R males (A × R).
Means followed by the same letter do not differ significantly by the nonparametric STP procedure.

laboratory conditions. Power tests indicate that we could have detected incompatibility levels of around 4 per cent or more in the comparison of crosses involving uninfected males and type A males, so if any incompatibility is present it must be extremely weak. Because studies with the type R infection of *D. simulans* indicate that incompatibility levels in the field tend to be lower than in the laboratory (Hoffmann *et al.*, 1990), the type A infection is also unlikely to cause incompatibility under field conditions. Low levels of incompatibility have previously been found in laboratory studies on *D. melanogaster* (Hoffmann, 1988; Holden *et al.*, 1993) and Giordano *et al.* (1995) have recently described a *Wolbachia* infection in *D. mauritiana* that does not appear to cause incompatibility. *Wolbachia* therefore may have variable effects on cytoplasmic incompatibility in *Drosophila* and probably in other insects as well.

Expected changes in the frequency of an infection in a population have previously been modelled with simple equations (Caspari & Watson, 1959; Fine, 1978; Hoffmann *et al.*, 1990; Turelli *et al.*, 1992). These equations have successfully predicted the spread of type R *Wolbachia* in Californian populations of *D. simulans* (Turelli & Hoffmann, 1991). In the absence of incompatibility, the frequency of a *Wolbachia* infection (p) in an isolated population at generation $t+1$ is given by the equation

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

where $1-s_f$ is the relative fecundity of infected females and μ is the proportion of progeny produced by an infected female that is no longer infected. The μ term is included in this equation because previous work has shown that maternal transmission of the infection is not perfect (i.e. $\mu>0$) (Hoffmann *et al.*, 1990). It has also been shown previously that *Wolbachia* infections can have deleterious effects on the fecundity of *D. simulans* (Hoffmann & Turelli, 1988; Hoffmann *et al.*, 1990; Nigro & Prout, 1990). In the equation, p_{t+1} will be less than p_t if μ is greater than 0 and/or if s_f falls between 0 and 1. If the type A infection is to persist in populations, maternal transmission must therefore be perfect in the field and there should be no deleterious effects on fecundity (or other fitness components). On the other hand, the type A infection will only spread if infected females have a fitness advantage (i.e. $s_f<0$).

The results of this paper indicate that μ is likely to be 0 or very close to 0. In addition, s_f appears to be near 0 in the laboratory. A power test indicates that

we could have detected a fecundity effect of around 7 per cent. If any deleterious effects on host fecundity are present, they must be small. The type A infection could therefore persist in natural populations of *D. simulans* even without causing incompatibility, behaving as a neutral variant whose frequency is determined by random processes. Differences in infection frequencies among *D. simulans* populations along the east coast of Australia may reflect random events, such as the recolonization of an area following unfavourable conditions.

It has previously been postulated that there is an association between levels of incompatibility and the density of *Wolbachia* in cells (Breeuwer & Werren, 1993). High densities of *Wolbachia* may be associated with high levels of incompatibility. Supporting evidence has come from *Wolbachia* infections in different strains of *Nasonia* (Breeuwer & Werren, 1993) and *D. melanogaster* (Solignac *et al.*, 1994) and in comparisons of *Wolbachia* numbers in *D. simulans* vs. *D. melanogaster* (Boyle *et al.*, 1993). However, our data indicate that embryo infection levels are not the sole cause of incompatibility. Infection levels in the type A strain were similar to those in *D. melanogaster*. Because infected *D. melanogaster* show partial incompatibility (Hoffmann *et al.*, 1994), the results indicate that levels of incompatibility in *Drosophila* are not strictly correlated with the density of *Wolbachia* in embryos. The intermediate density of *Wolbachia* in type A embryos also indicates that maternal transmission efficiency is not necessarily related to *Wolbachia* density as postulated elsewhere (Turelli, 1994) because maternal transmission of the type R infection is less efficient than that of the type A infection.

How might this infection have become widely distributed in Australian *D. simulans* populations? The reason why the R *Wolbachia* infection has spread in California is because it causes incompatibility when infected males mate with uninfected females. However, evolutionary changes in the genome of *Wolbachia* and in the genome of their hosts do not necessarily favour increasingly higher levels of incompatibility. Instead, theoretical models (Turelli, 1994) show that infections associated with lower levels of incompatibility can be favoured when they have a higher rate of maternal transmission and when they have a reduced deleterious effect on their host organism.

The A infection of *D. simulans* may represent the outcome of such an evolutionary process. Once the A infection had spread and supplanted the ancestral type, it may have decreased in frequency because of random events, unidentified deleterious effects on

the host or perhaps occasional curing of larvae exposed to antibiotics produced by naturally-occurring microorganisms. The low frequency of this infection in extant *D. simulans* populations from Australia could therefore reflect a relict distribution of an infection that was once widespread and predates the colonization of Australia by *D. simulans* because infected *D. simulans* strains that do not cause incompatibility have recently been found at a low frequency in populations from North and South America (Turelli & Hoffmann, 1995).

Other explanations also need to be considered. For instance, infection levels may represent an equilibrium between environmental curing and reinfection by parasites carrying *Wolbachia*. It is possible that infected individuals become completely cured after exposure to natural antibiotics even though environmental curing does not appear to influence maternal transmission of the A infection. Reinfection rates mediated by parasites have not been measured. However sympatric sibling species such as *D. melanogaster* and *D. simulans* that share the same parasites do not share the same infections (e.g. Hoffmann *et al.*, 1990), suggesting that parasite infection rates may be low. The presence of *Wolbachia* in *Drosophila* parasites such as mites and parasitic wasps could be screened to provide evidence on this hypothesis.

Acknowledgements

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