Medical and Veterinary Entomology (2005) **19**, 60–65

Filarial susceptibility and effects of *Wolbachia* in *Aedes pseudoscutellaris* mosquitoes

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Abstract. The mosquito *Aedes pseudoscutellaris* (Theobald), a member of the *Aedes* (*Stegomyia*) *scutellaris* complex (Diptera: Culicidae), is an important vector of subperiodic *Wuchereria bancrofti* (Cobbold) (Spirurida: Onchocercidae), causing human lymphatic filariasis, on South Pacific islands. Maternal inheritance of filarial susceptibility in the complex has previously been asserted, and larval tetracycline treatment reduced susceptibility; the maternally inherited *Wolbachia* in these mosquitoes were suggested to be responsible. To investigate the relationship of these two factors, we eliminated *Wolbachia* from a strain of *Ae. pseudoscutellaris* by tetracycline treatment, and tested filarial susceptibility of the adult female mosquitoes using *Brugia pahangi* (Edeson & Buckley). Filarial susceptibility was not significantly different in *Wolbachia*-free and infected lines of *Ae. pseudoscutellaris*, suggesting that the *Wolbachia* in these mosquitoes do not influence vector competence. Crosses between *Wolbachia*-infected males and uninfected females of *Ae. pseudoscutellaris* showed cytoplasmic incompatibility (CI), i.e. no eggs hatched, unaffected by larval crowding or restricted nutrient availability, whereas these factors are known to affect CI in *Drosophila simulans*. Reciprocal crosses between *Ae. pseudoscutellaris and Ae. katherinensis* Woodhill produced no progeny, even when both parents were *Wolbachia*-free, suggesting that nuclear factors are responsible for this interspecific sterility.

Key words. *Aedes pseudoscutellaris*, *Ae. katherinensis*, *Ae. polynesiensis*, *Brugia pahangi*, *Wolbachia*, cytoplasmic incompatibility, filariasis vectors, maternal inheritance, vector competence, Fiji, Polynesia.

Introduction

Aedes pseudoscutellaris is a member of the *Aedes* (*Stegomyia*) *scutellaris* (Walker) complex of diurnally active mosquitoes, comprising around 30 species with scattered distributions across the South Pacific island groups, extending into south-east Asia and northern Australia. Several of these species, endemic to eastern parts of the range, particularly *Ae. pseudoscutellaris and Aedes polynesiensis* Marks, are vectors of subperiodic Bancroftian filariasis, whereas those to the west are not susceptible to *Wuchereria bancrofti*. Some species of the *Ae. scutellaris* complex can also transmit dengue viruses (Rosen *et al.*, 1954; Freier & Rosen, 1987). Partial or even complete fertility of hybrids has been observed in laboratory crosses between members of the *Ae. scutellaris* complex (Meek, 1988). Although difficult to control by conventional methods, vector species of the *Ae. scutellaris* complex have been discussed as promising targets for genetic control or replacement strategies, with renewed interest to reduce or eliminate their filarial vector competence (Macdonald, 1976; Meek, 1988).

Many species of the *Ae. scutellaris* complex are naturally infected with the intracellular bacterium *Wolbachia* (Wright & Barr, 1980; Meek, 1984; Behbahani *et al.*, 2005), a reproductive parasite that uses patterns of crossing sterility, known as cytoplasmic incompatibility (CI), to invade populations of mosquitoes and many other insects (O'Neill *et al.*, 1997; Werren, 1997). Trpis *et al.* (1981) found evidence of

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maternal inheritance of filarial susceptibility in the *Ae. scutellaris* complex, which suggested that the presence of the maternally inherited *Wolbachia* may influence filarial development. *Wolbachia* infections can be cured with antibiotics such as tetracycline, and it was reported that tetracycline treatment of larval *Ae. polynesiensis* reduced the infective filarial load of *Brugia malayi* in resulting adult mosquitoes (Duhrkopf & Trpis, 1981). However, Meek & MacDonald (1982) found no evidence of maternal inheritance of susceptibility in the *Ae. scutellaris* complex, and no further data has been reported on the issue.

As described here, we have undertaken the first reported antibiotic curing of *Ae. pseudoscutellaris* to create a *Wolbachia*-uninfected line, in order to address several questions. The first was to examine whether *Wolbachia* has any influence on filarial susceptibility in this species, using *Brugia pahangi* which has been demonstrated to be a good model for *Wuchereria bancrofti* in laboratory susceptibility studies (Macdonald, 1976; Trpis, 1981). The second question addressed was the extent to which *Wolbachia* is responsible for interspecific sterility in crosses with *Aedes katherinensis*, a filaria-refractory species in the *Ae. scutellaris* complex. The final aim was to examine whether *Wolbachia* causes CI within *Ae. pseudoscutellaris*. Crossing experiments incorporated an examination of the potential CI effects of larval rearing conditions, since, in *Drosophila simulans*, larval crowding reduces penetrance of CI produced by the resulting males (Sinkins *et al.*, 1995).

Materials and methods

Mosquitoes and Wolbachia

Three species of the *A. scutellaris* complex were used. Filaria-refractory (i.e. non-susceptible) *Ae. katherinensis* originated from near Darwin in Northern Territory, Australia; this mosquito colony was established in 1979 by P. Whelan, K. Hodder and G. Davis. Filaria-susceptible *Ae. pseudoscutellaris* and *Ae. polynesiensis* were collected by T.J.D. in the vicinity of Suva in Fiji, November 2001, and colonies were established at the Liverpool School of Tropical Medicine.

The presence of *Wolbachia* was determined with generic PCR assays, using primers *Wsp*81F and *Wsp*691R, under conditions described by Zhou *et al.* (1998) with 1 μL of template DNA extracted from individual adults by the Livak buffer method (Collins *et al.*, 1987) and re-suspended in 100 μL of water. PCR products were visualized on 1% agarose gels.

A *Wolbachia*-free (uninfected) colony of *Ae. pseudoscutellaris*, which will be referred to as *pseudo*-tet, was created by feeding the adult mosquitoes on 1 mg/mL tetracycline hydrochloride in 10% sucrose solution on a pad of cotton wool (Dobson & Rattanadechakul, 2001), with no other source of water or sucrose supplied. At each generation of treatment, the *Wolbachia* PCR assay was performed on a sample of adult females. If no bands could be detected, a nested PCR was performed, using 1 μL of template from the *Wsp*81F/*Wsp*691R PCR reaction with the internal primers *Wsp*183F and *Wsp*522R (Zhou *et al.*, 1998) and the same reagent/PCR cycle conditions but 50 °C annealing temperature. After four generations of *Ae. pseudoscutellaris* treatment, no amplification was detected with the nested PCR and, in subsequent generations, repeated nested PCRs confirmed that the line was fully cured of *Wolbachia*.

Filarial susceptibility testing

Brugia pahangi was maintained by cyclical passage through Mongolian Jirds, *Meriones unguiculatus* (Milne-Edwards), infected by intraperitoneal inoculation of infective larvae. Mosquitoes were given an infective bloodmeal, 3–6 days after emergence, via chick-skin membrane. Using Hank's balanced buffered saline for flushing, microfilariae were extracted from the peritoneal cavity of a jird and counted in saline, added to a known quantity of rabbit blood and pipetted into the blood chamber of a glass artificial feeding unit that maintained the blood at 37 °C. *Aedes katherinensis* was given an infective bloodmeal with 5 microfilariae/μL, whereas *Ae. polynesiensis* and *Ae. pseudoscutellaris* (normal and *pseudo*-tet lines) were provided with an infective bloodmeal having 3 microfilariae/μL. On day 10 postinfective feed, mosquitoes were dissected in saline and examined microscopically for presence of filarial larvae in each body section (head, thorax, abdomen): the numbers were counted and stages of development assessed.

Mosquito crossing experiments

Mosquitoes were maintained in standard climatic conditions as described by Meek & Macdonald (1982, 1984). Larvae were maintained at densities of 150–200 per tray and, prior to adult emergence, males and females were separated by examination of the pupal terminalia to ensure virgin adults. As single pair crosses suffered a high rate of mortality, mass crosses were set up with 80 individuals of each sex in 30 cm cubic cages. Females were blood-fed via membrane ~72 h after the introduction of males. Moist filter paper was provided for oviposition; after eggs were laid the filter paper was removed, dried overnight and stored in an airtight plastic bag. The eggs were hatched in de-oxygenated water. Hatch rates were calculated by counting total number of eggs and the number of resulting larvae. To check for effective mating, spermathecae of the females from each cross were dissected and examined for presence of sperm.

Possible effects of larval rearing conditions on CI were investigated for three different conditions. Uncrowded control larvae were reared at a density of one per 5 mL of water, fed with yeast ~1 g/L provided daily. Crowded larvae were reared at density of 3 per 1 mL water and fed yeast, either 2 g/L high nutrient diet or 0.35 g/L low nutrient diet daily.

Results

Wolbachia *infection*

By PCR assay the *Ae. katherinensis* colony was found to lack *Wolbachia*, confirming previous reports that this species is *Wolbachia*-free, based on electron microscopy (Meek, 1988). Adult tetracycline treatment proved to be an effective method for removing *Wolbachia* infection in *Ae. pseudoscutellaris*, with complete curing achieved within four generations, obviating the need for single-pair selection.

Comparative filarial susceptibility

Figures 1 and 2 show the relative susceptibility to *B.pahangi* of *Wolbachia*-infected *Ae. polynesiensis*, *Wolbachia*-free *Ae. katherinensis* and *Ae. pseudoscutellaris* with or without (*pseudo*-tet line) *Wolbachia*. The mean numbers of *B. pahangi* larvae in *Ae. pseudoscutellaris* with and without *Wolbachia* were not significantly different by independent *t*-tests ($P = 0.162$). Figure 3 shows the distribution of *B. pahangi* larvae within dissected mosquitoes (head, thorax, abdomen), expected to reveal any contrasts in their rate of development in each of the mosquito colonies. The proportional distributions of *B. pahangi* larvae were very similar in *Ae. polynesiensis* and *Ae. pseudoscutellaris*, with and without *Wolbachia*, the majority of filaria larvae (all L3 infective-stage) being in the head.

Crossing relationships

Table 1 shows results of reciprocal crosses between *Ae. katherinensis* and *Ae. pseudoscutellaris and* within the latter. Females of *Ae. pseudoscutellaris* crossed with males of *Ae. katherinensis* (cross 1) were consistently incompatible, without egg production despite 63% insemination, while the reciprocal (cross 2) yielded only infertile eggs. *Aedes katherinensis* females crossed with *pseudo*-tet males, both lacking *Wolbachia* (cross 3), yielded only infertile eggs, showing that removal of *Wolbachia* did not increase the compatibility between these two populations.

Both tables show results of crosses between *Wolbachia*-infected and uninfected lines of *Ae. pseudoscutellaris*. The cross between *Wolbachia*-free females (*pseudo*-tet) and normal *Ae. pseudoscutellaris* males with *Wolbachia* showed complete incompatibility (Table 1, line 7; Table 2, line 1), indicating that the strain of *Wolbachia* present is capable of inducing CI with very high penetrance in this host. The reciprocal cross between normal *Wolbachia*-infected *Ae. pseudoscutellaris* females and *Wolbachia*-free males (*pseudo*-tet) was compatible (Table 1, line 8; Table 2, line 4), although both this and the control cross between *Wolbachia*-free (*pseudo*-tet) males and females (Table 1, cross 6) showed lower hatch rates than from the normal *Wolbachia*-infected males and females of *Ae. pseudoscutellaris* (Table 1, cross 5); this might be a consequence of genetic bottlenecking during creation of the *pseudo*-tet line.

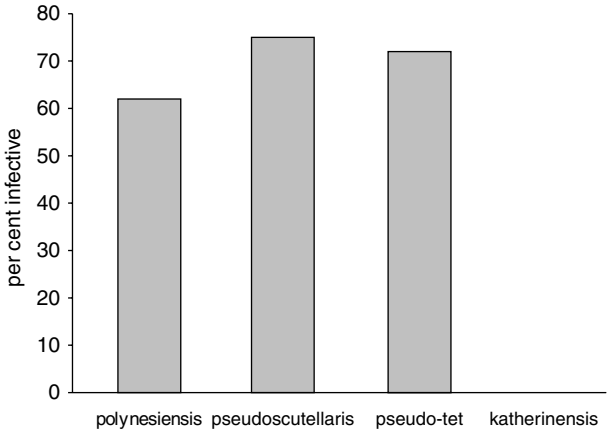

Fig. 1. Percentage of female mosquitoes containing infective (third stage, L3) *Brugia pahangi* larvae when dissected 10 days postinfection: *Ae. katherinensis* ($N = 33$), *Ae. polynesiensis* ($N = 37$) and *Ae. pseudoscutellaris* with ($N = 28$) or without (*pseudo*-tet, $N = 50$) *Wolbachia* infection.

Effects of larval stress on penetrance of CI produced by the resulting males were also examined for *Ae. pseudoscutellaris*, as crowding and limited nutrition are likely to occur under natural conditions. Results of crosses 2 and 3 in Table 2 showed no significant variation in penetrance of CI when *Wolbachia*-infected males are reared at different densities, some simulating crowded larval conditions, with either high or low nutrient levels provided.

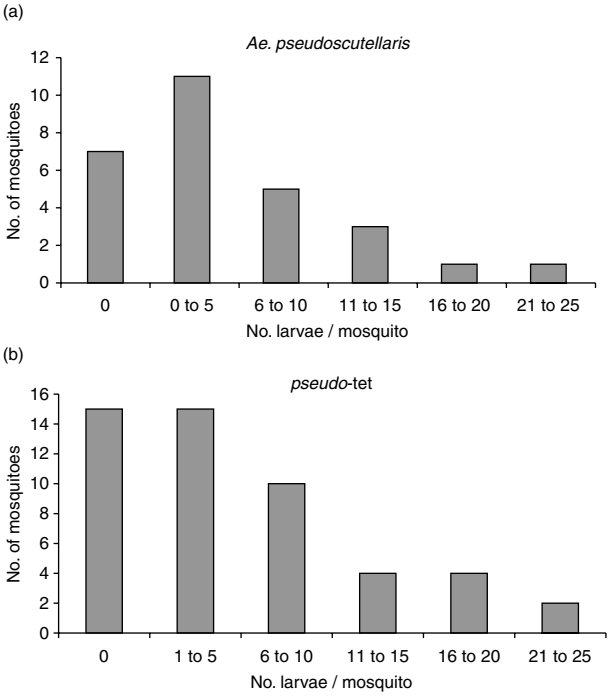

Fig. 2. Mean number of *Brugia pahangi* larvae/mosquito dissected 10 days postinfective bloodmeal for *Ae. pseudoscutellaris* females with (a) or without (*pseudo*-tet, (b) *Wolbachia* infection.

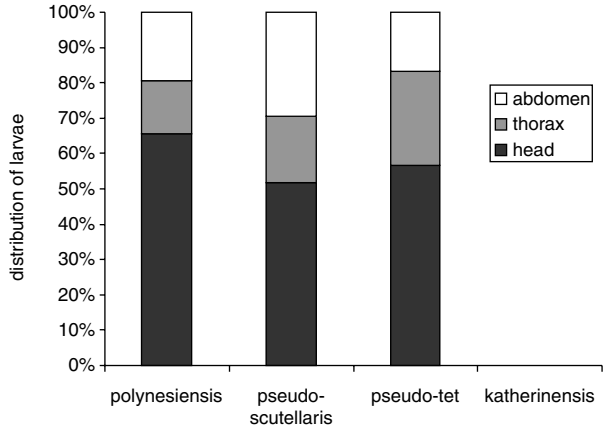

Fig. 3. Distribution of *Brugia pahangi* larvae in female mosquitoes (head, thorax, abdomen) dissected on day 10 after feeding with microfilaria-infected blood: *Ae. katherinensis* ($N = 33$), *Ae. polynesiensis* ($N = 37$) and *Ae. pseudoscutellaris* with ($N = 28$) or without (*pseudo*-tet, $N = 50$) *Wolbachia* infection.

Discussion

Filarial susceptibility

Results of our experiments suggest that presence or absence of *Wolbachia* in *Ae. pseudoscutellaris* has no effect on the infection of this mosquito with *Brugia* and, by inference, *Wuchereria*. This finding contrasts with that of Duhrkopf & Trpis (1981), who found that tetracycline treatment of *Ae. polynesiensis* produced some refractory individuals and reduced the yield of infective *Brugia* (mean number of L3 per mosquito). They added tetracycline hydrochloride to the rearing medium of mosquitoes, giving 24 h exposure during the fourth larval instar, then tested the resulting adult mosquitoes for filaria susceptibility. In contrast, our experiments assessed filaria susceptibility of the mosquitoes several generations after cessation of antibiotic treatment. Even though the activity of tetracycline is probably short-lived, the most likely explanation for the difference is that residual tetracycline in the adult mosquitoes had directly affected the *Brugia* larvae in the study by Duhrkopf & Trpis (1981). Filarial nematodes are also infected with a separate clade of *Wolbachia*, apparently having a mutualistic association (Bandi *et al*., 1998). The *Wolbachia* of filarial nematodes are susceptible to antibiotic treatment and the development of the host nematode is slowed or prevented by the loss of *Wolbachia*, a finding that has stimulated research into antibiotic therapy as a filarial control method (Taylor *et al*., 2000).

Results reported here for *Ae. pseudoscutellaris* are consistent with findings by Curtis *et al*. (1983) on susceptibility of *Culex quinquefasciatus* to *Wuchereria bancrofti*. Their mosquito larvae were reared in tetracycline hydrochloride and, in some of the resulting adults, no *Wolbachia* could be detected by electron microscopy (less sensitive than PCR). When susceptibility of the cured (or partially cured) lines was compared with *Wolbachia*-infected controls, the cured mosquitoes were found to be fully susceptible to *Wuchereria*.

Table 2. Egg hatch rates from crosses between *Aedes pseudoscutellaris* with (control) or without (*pseudo*-tet) *Wolbachia* infection, using mosquito adults reared under different larval conditions: uncrowded control larvae (density one per 5 mL water, fed yeast ~1 g/L daily); crowded larvae (density 3 per 1 mL water) fed with high nutrient (HN: yeast 2 g/L daily) or low nutrient (LN: yeast 0.35 g/L daily).

Cross (female × male)	% inseminated (no. dissected)	No. eggs	% hatch
1. *pseudo*-tet × control	74 (40)	482	0
2. *pseudo*-tet × crowded HN	58 (42)	160	0
3. *pseudo*-tet × crowded LN	64 (45)	225	0
4. control × *pseudo*-tet	72 (38)	577	65.9
5. crowded HN × *pseudo*-tet	78 (40)	297	79.1
6. crowded LN × *pseudo*-tet	50 (35)	448	44.0

Crossing relationships

From interspecific crosses conducted previously between *Ae. pseudoscutellaris and Ae. katherinensis*, variable results have been obtained. Some workers reported that both reciprocal crosses produced no viable eggs or a very low percentage hatch (Woodhill, 1950; Dev & Rai, 1982; Meek & Macdonald, 1984), whereas other researchers found the two species to be unidirectionally compatible (Wade &

Table 1. Egg hatch rates and female insemination resulting from crosses involving *Aedes pseudoscutellaris*, with or without (*pseudo*-tet) *Wolbachia* infection, and the filaria-refractory species *Ae. katherinensis*.

Cross (female × male)	% inseminated (no. dissected)	No. eggs	% hatch
1. *pseudoscutellaris* × *katherinensis*	63 (48)	0	0
2. *katherinensis* × *pseudoscutellaris*	53 (30)	655	0
3. *katherinensis* x *pseudo*-tet	90 (38)	257	0
4. *pseudo*-tet × *katherinensis*	50 (32)	93	0
5. *pseudoscutellaris* × *pseudoscutellaris*	94 (47)	1203	80
6. *pseudo*-tet × *pseudo*-tet	82 (49)	1995	69
7. *pseudo*-tet × *pseudoscutellaris*	74 (40)	482	0
8. *pseudoscutellaris* × *pseudoscutellaris*	72 (38)	577	66

Macdonald, 1977; Hoyer & Rozeboom, 1977). By conducting crosses between *Ae. katherinensis* females and *pseudo*-tet males it was possible here, for the first time, to examine how the presence of *Wolbachia* in *Ae. pseudoscutellaris* males affected the interspecific crossing relationship. All crosses between the two species were completely incompatible in both directions, and removal of *Wolbachia* from *Ae. pseudoscutellaris* did not increase compatibility. It appears therefore that nuclear factors can be responsible for the bidirectional interspecific sterility observed; the variable results obtained by different researchers would suggest that these nuclear factors show intraspecific variation between different colonies or populations.

Unidirectional CI can allow *Wolbachia* to spread rapidly through uninfected insect populations, because uninfected females are only able to produce progeny successfully when they mate with uninfected males, whereas infected females are able to mate with both infected and uninfected males. *Wolbachia* spread has been directly observed in the field in *Drosophila simulans* (Turelli & Hoffmann, 1991). The penetrance of CI influences the speed of population invasion and also, when maternal transmission is imperfect or there are *Wolbachia*-associated fitness costs, affects the unstable threshold equilibrium population frequency that must be exceeded before spread can be initiated, or maintained through fragmented populations (Turelli & Hoffmann, 1995; Hoffmann & Turelli, 1997). These dynamics are very important considerations in the assessment of the potential utility of *Wolbachia* as a method of spreading useful transgenes through target populations, in order to reduce their ability to transmit disease (Turelli & Hoffmann, 1999; Sinkins & O'Neill, 2000). Very high penetrance of CI was observed in *Ae. pseudoscutellaris*, even when males were raised under crowded and nutritionally stressed larval rearing conditions, as would often occur in field populations.

These results contrast with observations in *D. simulans*, where the penetrance of CI produced by infected males was reduced when the males were reared under crowded larval conditions in the laboratory (Sinkins *et al*., 1995). It would appear that with respect to penetrance of CI, *Ae. pseudoscutellaris* shows more favourable *Wolbachia* invasion dynamics than the *D. simulans* system that is often used as a model. High penetrance of CI, with nearly 100% mortality, has also been reported in *Aedes albopictus* when males were field-caught (Kittayapong *et al*., 2002) or raised under crowded conditions in the laboratory (Dutton & Sinkins, 2004).

Acknowledgements

We thank Arun Raju and Gyan Prakash for their assistance with the collection of *Aedes polynesiensis* and *Aedes pseudoscutellaris* in Fiji, and Amy Lynd for help in the laboratory. T.J.D. was supported by a research studentship from the U.K. Medical Research Council and by a Jean Clayton travel award from the Liverpool School of Tropical Medicine.

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Accepted 3 July 2004