Host age effect and expression of cytoplasmic incompatibility in field populations of *Wolbachia*-superinfected *Aedes albopictus*

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The Asian tiger mosquito, *Aedes albopictus* (Skuse), is a known vector of dengue in South America and Southeast Asia. It is naturally superinfected with two strains of *Wolbachia* endosymbiont that are able to induce cytoplasmic incompatibility (CI). In this paper, we report the strength of CI expression in crosses involving field-caught males. CI expression was found to be very strong in all crosses between field males and laboratory-reared uninfected or *w*AlbA infected young females. In addition, crossing experiments with laboratory colonies showed that aged superinfected males could express strong CI when mated with young uninfected or *w*AlbA infected females. These results provide additional evidence that the CI properties of *Wolbachia* infecting *Aedes albopictus* are well suited for applied strategies that seek to utilise *Wolbachia* for host population modification.
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Introduction

The Asian tiger mosquito, *Aedes albopictus* (Skuse), is native to Asia and the South Pacific and has been recently introduced into the continental United States and South America (Kambhampati and Rai, 1991; Kambhampati *et al*, 1991). *Ae. albopictus* is known to be an important vector of dengue in Southeast Asia (Gould *et al*, 1968; Chan *et al*, 1971) and has been implicated in a recent dengue outbreak in Mexico (Ibanez-Bernal *et al*, 1997). Maternally inherited *Wolbachia* infections were first found in the ovaries of this mosquito by Wright and Barr (1980). It is now known that nearly all populations of *Ae. albopictus* are superinfected with two different *Wolbachia* strains designated *w*AlbA (A group) and *w*AlbB (B group) (Sinkins *et al*, 1995b; Zhou *et al*, 1998; Kittayapong *et al*, 2002).

Cytoplasmic incompatibility (CI) is a common phenomenon in insects, caused by *Wolbachia* (Yen and Barr, 1971) and is known to occur in *Ae. albopictus* (Kambhampati *et al*, 1993). When a *Wolbachia*-infected male mates with an uninfected female, the eggs or embryos die. The net effect is a decrease in the fitness of uninfected females, which over time results in the spread of the infection through the host population (Turelli and Hoffmann, 1991). Individuals may be infected with more than one strain of *Wolbachia*. Among such superinfected

individuals, CI occurs if the female is uninfected with respect to the strain that the male carries. The net effect is a decrease in the fitness of single-infected females, and thus the superinfection spreads (Sinkins *et al*, 1995b). Through the action of CI and related phenotypes, *Wolbachia* is estimated to have successfully invaded approximately 15–20% of all insect species (Werren *et al*, 2000).

The development of transgenic mosquitoes that lack the ability to transmit disease has been suggested as a possible future method for the control of insect-transmitted diseases. This requires a method to introduce and spread transmission-blocking genes into natural vector populations. The phenomenon of CI makes *Wolbachia* a prime candidate in this endeavor (Sinkins *et al*, 1997; Curtis and Sinkins, 1998). The success of this long-term goal for disease control is critically dependent on the efficiency of CI expression and maternal transmission of *Wolbachia* under field conditions (Turelli and Hoffmann, 1999). While these factors have been well studied in *Drosophila*, little attention has been paid to the dynamics of *Wolbachia* infections in insects of medical importance. While maternal transmission rates have been examined recently in field populations of *Ae. albopictus* and shown to be very high (Kittayapong *et al*, 2002), no studies have examined the expression of CI in field-caught *Ae. albopictus*. Moreover, in published *Drosophila* studies a clear difference has been reported between laboratory and field-reared individuals, the latter displaying reduced CI expression (Hoffmann *et al*, 1990, 1998; Turelli and Hoffmann, 1995). Therefore, the main objective of this study was to investigate the strength of CI expression in field populations of the mosquito vector *Ae. albopictus*.

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We have also determined whether the age of male hosts has any effect on the expression of CI in this species.

Materials and methods

Field collection of mosquitoes
Aedes albopictus were collected from their natural habitats in two different localities approximately 300 km apart between August 1999 and January 2000. The collection sites were located in Plaeng Yao District, Chachoengsao Province, eastern Thailand and Sai Yok District, Kanchanaburi Province, western Thailand. Two different methods were used to collect *Ae. albopictus*. First, eggs were collected from several ovitraps placed near bamboo stumps. An ovitrap consisted of a black plastic container lined with a paper towel and half-filled with water. The paper towels containing mosquito eggs were collected from both locations and were brought to the laboratory in Bangkok within a 1-week period. Second, swarming male mosquitoes were collected from locations adjacent to bamboo forests in Chachoengsao and Kanchanaburi Provinces using a simple mosquito net. Live males were put in screen-top containers and were fed with a 10% sugar solution before being transported to the laboratory in Bangkok on the same day.

Laboratory-reared mosquito strains
There were three colonies of *Ae. albopictus* mosquitoes used in the crossing experiments. The superinfected colony (KLPP) was generated in 1997 from crosses between males and females collected from Kanchanaburi Province, western Thailand and the Phi Phi Islands off the west coast of Thailand. The superinfected status of this colony for *w*AlbA and *w*AlbB *Wolbachia* was confirmed by both RFLP analysis of *ftsZ* PCR products and group-specific *wsp* primers as described in Kittayapong *et al* (2002). The single-infected *w*AlbA colony (KOH) was established before 1970 from mosquitoes collected at Samui Island (Koh Samui), off the east coast of Thailand and provided by Dr KS Rai, University of Notre Dame, USA. The uninfected colony (UJU) was artificially generated by tetracycline treatment and was provided by Dr Yasushi Otsuka, Oita Medical University, Japan. All these colonies were maintained in the insectary at $27 \pm 2°C$ and with $70 \pm 10\%$ relative humidity.

Crossing experiments
Eggs collected from individual ovitraps were placed in separate rearing trays filled with 1 litre of distilled water and larvae were fed fish food. To avoid evaluating individuals from the same cohort, only one male per ovitrap was used in this study. Individual adult males were isolated and allowed to mate for 3 days in a confined cup with either three, 1 to 3-day-old, uninfected females from the UJU laboratory colony, or three, 1 to 3-day-old, *w*AlbA infected females from the KOH colony. After mating, each female was blood-fed with a hamster and allowed to oviposit F1 eggs. The same individual males were then mated again with three, 1 to 3-day-old, superinfected females from the laboratory reared KLPP colony to serve as a control cross. After 3 days, superinfected females were isolated for blood-feeding and egg-laying. Eggs laid by individual females were counted and then allowed to hatch for 24 h in deoxygenated water. CI

expression of the superinfected phenotype was determined by the average number of eggs hatched from each crossing experiment. Single pair crosses between field-collected adult males and either UJU or KOH females were also done as described above.

Several crosses were done between laboratory-reared uninfected, *w*AlbA infected and *w*AlbA + *w*AlbB superinfected *Ae. albopictus* to investigate the effect of host age on CI expression, since it was not possible to determine the exact age of field-collected mosquitoes. In our experiments, the age of males was varied in three different incompatible crosses. These crosses were uninfected females × superinfected males, *w*AlbA infected females × superinfected males, and uninfected females × *w*AlbA infected males. Normal control crosses were performed with 2 to 5-day-old individuals. In these experiments, the age of males was varied from 2–5 days old to 10, 20, 30, 40, 50 and 60 days old. Individual males were first aged then mated to young individual females (1–3 days old) using an artificial force-mating technique to ensure that individual pairs were successfully mated. After mating, each female was allowed to oviposit in a confined cup. Eggs laid on each paper towel were left in the wet container to mature, then dried and hatched in distilled water. Spermatheca of individual females were dissected and the presence of sperm was identified by microscopy to confirm insemination. Eggs obtained from mating pairs with no sperm insemination were discarded. CI expression was determined as the average egg hatch rate of each cross. Statistical comparisons of egg hatch rate were performed between methods of collection and between locations using the Mann-Whitney U test from SPSS for windows version 7.5. *P* values at or below alpha 0.05 were considered significant.

PCR detection of *Wolbachia*
After crossing experiments, individual field-collected males were checked for their infection status of *Wolbachia* (Kittayapong *et al*, 2002). In this study, we used A and B group specific *wsp* primers, which were designed from the conserved regions of the outer surface protein gene, to determine the type of infection of individual field-collected mosquitoes (Braig *et al*, 1998; Zhou *et al*, 1998). The universal eukaryotic 12S rDNA primers (12 SAI and 12 SBI) were also used for checking the quality of DNA extraction (O'Neill *et al*, 1992). DNA extraction of individual gonad tissues from a colony of laboratory-reared superinfected *Ae. albopictus* was used as a positive control in the PCR reaction.

Reproductive tissues from individual mosquitoes were used for DNA extraction. The extraction procedure followed the crude boiling method of O'Neill *et al* (1992). Gonad tissues from individual mosquitoes were dissected under distilled water on a sterile microscope slide and were then homogenised in 100 μl of STE buffer. Homogenates were heated at 95°C for 10 min and were centrifuged at 14 000 rpm for 1 min. Supernatant of 1 μl from individual mosquitoes was used in each 20-μl reaction volume. Each PCR reaction mixture contained 2 μl 10× buffer (Promega), 2 μl 25 mM $MgCl_2$, 0.5 μl dNTPs (10 mM each), 0.5 μl of each primer and 1 unit of *Taq* DNA polymerase (Promega). The temperature profile for PCR was as follows: 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min per cycle. Ten μl of each PCR product was electrophoresed

Table 1 Results of crossing experiments between field-collected adult males and 1–3 days old, uninfected (UJU), single *w*AlbA infected (KOH) or superinfected (KLPP) laboratory-reared females

Cross	No.	Treatment crosses		Control crosses (with KLPP ♀)	
		No. eggs laid (Mean ± S.E.)	% egg hatch (Mean ± S.E.)	No. eggs laid (Mean ± S.E.)	% egg hatch (Mean ± S.E.)
1. UJU ♀ × CH ♂	40	40.69 ± 3.11	0.30 ± 0.19	42.70 ± 2.87	88.90 ± 0.82
2. KOH ♀ × CH ♂	40	39.03 ± 2.64	0.19 ± 0.09	43.02 ± 2.92	88.40 ± 0.86
3. UJU ♀ × KL ♂	40	45.92 ± 3.92	0.40 ± 0.19	46.05 ± 3.12	89.38 ± 0.70
4. KOH ♀ × KL ♂	40	43.55 ± 3.21	0.38 ± 0.17	51.48 ± 3.70	90.48 ± 0.56

CH = Chachoengsao; KL = Kanchanaburi.

on a 1% agarose gel and was visualised by ethidium bromide staining. The size of the PCR product was determined using a 1 Kb DNA ladder (Gibco). Expected sizes of the PCR products with A and B group-specific *wsp* primers were 397 bp and 501 bp respectively.

Results

Expression of CI in field-collected males
For each cross, a total of 35–40 field-collected adult males from each location were sequentially crossed with either 1 to 3-day-old, laboratory-reared uninfected (UJU) or *w*AlbA infected (KOH) females and then superinfected (KLPP) females. The cross to UJU females was able to measure the combined CI effect of the superinfection. The cross to KOH enabled CI due to the presence of the *w*AlbB infection in field males to be evaluated independently of the *w*AlbA infection. The final cross was a control to verify that the males were fertile, only data from fertile males was subsequently used. Table 1 shows the number of eggs laid by individual females and the percentage of egg hatch from all crosses. CI expression, as determined by egg hatch rate, was very strong (0.19–0.40% hatching) in all of the crosses between field-collected males and young UJU or KOH females.

In addition to field-caught adults, eggs were collected from field ovitraps and reared in the insectary. The resulting adult males were crossed as above to either UJU or KOH females, which in all cases resulted in low egg hatch rates (0.02–0.74% hatching) as shown in Table 2. Males derived from ovitraps or caught as adults from swarms show no significant differences in the rate of egg hatch when crossed with either UJU ($P > 0.05$) or KOH ($P > 0.05$) females. In addition, males collected from different locations, eastern or western Thailand, did not show significant differences in the strength of CI expression when crossed with either UJU ($P > 0.05$) or KOH ($P > 0.05$) females. In control crosses between field males and superinfected females, the mean percent egg hatch ranged between 87.91% to 91.01%.

Host age effect on CI expression
Results of egg hatch rates from the crosses that varied male age are shown in Table 3. In these crosses, strong CI was expressed at all ages for superinfected KLPP males regardless of whether their young female partners were uninfected or *w*AlbA infected. There were no viable eggs produced at all male age classes. In the crosses between single infected KOH males and uninfected females, strong CI expression was observed until the males were 10 days old. The *w*AlbA infected males that were 20 to 60 days old produced some viable offspring ranging from 18.27% to 54.44% egg hatch.

Discussion

A number of studies have documented a reduction in the strength of *Wolbachia*-mediated CI expression when old males are used in crosses. This was first characterised for *Culex* (= *fatigans*) *quinquefasciatus* (Singh *et al*, 1976) and has since been reported for *Drosophila simulans* (Hoffmann *et al*, 1986) and *Armigeres subalbatus* (Jamnongluk *et al*, 1999). The underlying mechanistic hypothesis is that *Wolbachia* densities decrease with age of males (Binnington and Hoffmann, 1989; Bressac and Rousset, 1993). However, in the studies reported here, no evidence could be found for this effect in aged superinfected males. When KOH *w*AlbA males were aged and

Table 2 Results of crossing experiments between laboratory-raised adult males derived from field ovitrap eggs and 1–3 days old, uninfected (UJU), single *w*AlbA infected (KOH) or superinfected (KLPP) laboratory-reared females

Cross	No.	Treatment crosses		Control crosses (with KLPP ♀)	
		No. eggs laid (Mean ± S.E.)	% egg hatch (Mean ± S.E.)	No. eggs laid (Mean ± S.E.)	% egg hatch (Mean ± S.E.)
1. UJU ♀ × CH ♂	40	43.99 ± 3.55	0.02 ± 0.02	48.48 ± 3.40	88.94 ± 0.89
2. KOH ♀ × CH ♂	38	56.08 ± 4.01	0.74 ± 0.46	49.00 ± 3.15	87.91 ± 0.91
3. UJU ♀ × KL ♂	40	41.15 ± 3.27	0.05 ± 0.06	48.07 ± 2.97	91.01 ± 0.78
4. KOH ♀ × KL ♂	40	60.60 ± 4.88	0.49 ± 0.25	54.55 ± 4.09	88.78 ± 0.82

CH = Chachoengsao; KL = Kanchanaburi.

Table 3 Results of egg hatch between incompatible crossing types that should show strong CI expression only if males were young. In this experiment, the age of individual males was varied from 2–5 days old to 10, 20, 30, 40, 50, and 60 days old

Cross	Percent egg hatch when male age varied[a]						
	2–5d	*10d*	*20d*	*30d*	*40d*	*50d*	*60d*
UJU ♀ × KLPP ♂	0.00 [0.30]	0.00 [0.23]	0.00 [0.27]	0.00 [0.27]	0.00 [0.27]	0.00 [0.27]	0.00 [0.27]
No. of pair (*n*)	(10)	(13)	(11)	(11)	(11)	(11)	(11)
KOH ♀ × KLPP ♂	0.00 [0.30]	0.00 [0.27]	0.00 [0.27]	0.00 [0.30]	0.00 [0.30]	0.00 [0.30]	0.00 [0.23]
No. of pair (*n*)	(10)	(11)	(11)	(10)	(10)	(10)	(13)
UJU ♀ × KOH ♂	0.00 [0.30]	18.27 ± 9.72	30.60 ± 10.28	23.74 ± 4.13	54.44 ± 9.07	33.47 ± 10.44	43.58 ± 11.02
No. of pair (*n*)	(10)	(11)	(12)	(10)	(10)	(10)	(10)

[a]Mean ± S.E. or mean (95% upper confident limit). KOH = single *w*AlbA infected; KLPP = *w*AlbA + *w*AlbB superinfected; UJU = uninfected.

then mated, a pronounced weakening of CI was detected which mirrored studies in other species. The KOH strain has been shown to be infected at much lower densities than superinfected strains (Sinkins *et al*, 1995a) and as such might be more susceptible to density related aging effects. Considering that nearly all individuals sampled from field populations to date have been shown to be superinfected (Kittayapong *et al*, 2002), it would indicate that male age effects should not be influencing CI dynamics in the field.

Adult males caught from the field, transported back to the lab and mated to colony females all produced strong CI, indicating that field rearing does not appear to influence the strength of CI expression in this mosquito species. This is in contrast to studies with *Drosophila* that have indicated that CI expression can be much stronger in the laboratory than the field (Hoffmann *et al*, 1998). In our study, no difference was seen between laboratory or field-reared individuals. Turelli and Hoffmann (1999) have suggested that in order for *Wolbachia* to be used effectively in an applied setting to invade target insect populations, maternal transmission rates and CI expression should both be high. The results presented in this and previous studies (Kittayapong *et al*, 2002) indicate that *Wolbachia* infections of *Ae. albopictus*, a known dengue vector, have CI properties in the field that suggest *Wolbachia* may be a better candidate for applied strategies to modify insect vector competence than parameter estimates from *Drosophila* would initially indicate.

Acknowledgements

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