

# Interspecific transfer of *Wolbachia* into the mosquito disease vector *Aedes albopictus*

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Intracellular *Wolbachia* bacteria are obligate, maternally inherited endosymbionts found frequently in insects and other invertebrates. The evolutionary success of *Wolbachia* is due in part to an ability to manipulate reproduction. In mosquitoes and many other insects, *Wolbachia* causes a form of sterility known as cytoplasmic incompatibility (CI). *Wolbachia*-induced CI has attracted interest as a potential agent for affecting medically important disease vectors. However, application of the approach has been restricted by an absence of appropriate, naturally occurring *Wolbachia* infections. Here, we report the interspecific transfer of *Wolbachia* infection into a medically important mosquito. Using embryonic microinjection, *Wolbachia* is transferred from *Drosophila simulans* into the invasive pest and disease vector: *Aedes albopictus* (Asian tiger mosquito). The resulting infection is stably maintained and displays a unique pattern of bidirectional CI in crosses with naturally infected mosquitoes. Laboratory population cage experiments examine a strategy in which releases of *Wolbachia*-infected males are used to suppress mosquito egg hatch. We discuss the results in relation to developing appropriate *Wolbachia*-infected mosquito strains for population replacement and population suppression strategies.

**Keywords:** *Wolbachia*; *Aedes albopictus*; *Drosophila*; cytoplasmic incompatibility

## 1. INTRODUCTION

Intracellular *Wolbachia* bacteria are estimated to naturally infect approximately 20% of insect species and up to 28% of surveyed mosquito species (Werren *et al.* 1995; Kittayapong *et al.* 2000; Ricci *et al.* 2002). In mosquitoes and other insects, naturally occurring *Wolbachia* infections can cause cytoplasmic incompatibility (CI), which results in the developmental arrest of early embryos (O'Neill *et al.* 1997; Tram & Sullivan 2002). CI occurs when a *Wolbachia*-infected male mates with an uninfected female or a female with a different *Wolbachia* type. The ability of *Wolbachia* to manipulate host reproduction has led to considerable scientific attention directed at better understanding the impact of *Wolbachia* infections on the evolution and ecology of host populations (Charlat *et al.* 2003).

In medically important mosquitoes, CI-induced sterility has led to the proposal of applied strategies for the suppression of mosquito populations (Laven 1967; Sinkins & O'Neill 2000; Dobson *et al.* 2002a) and for the use of *Wolbachia* as a vehicle for driving desired genotypes into medically important disease vectors (Sinkins & O'Neill 2000; Dobson 2003). However, application of proposed strategies to mosquito populations has been restricted by an inability to identify appropriate naturally occurring *Wolbachia* infection types. Thus, realization of the proposed strategies will require a technique for generating artificial *Wolbachia* infections.

A technique for artificially generating incompatible CI types has been available in *Drosophila* for more than a decade. The artificial transfer of *Wolbachia* ('transfection') is accomplished via microinjection of *Wolbachia*-infected cytoplasm in early embryos (Boyle *et al.* 1993; Xi & Dobson 2005). Recently, a transfection technique has been developed for *Aedes albopictus* and used to segregate the naturally occurring *Wolbachia* superinfection, resulting in an artificial *wAlbB* single infection ('HTB' strain) (Xi *et al.* 2005). Although the prior report demonstrates an ability to transfect *Ae. albopictus*, the HTB strain is not useful for applied strategies, because it will be wiped out by the naturally superinfected *Ae. albopictus* after release. Thus, in order to generate an infection that is incompatible with target mosquitoes, including *Anopheles gambiae*, an ability of the interspecific transfer of *Wolbachia* is required.

Here, we use embryonic microinjection to transfer *Wolbachia* infection from *Drosophila simulans* to embryos of *Ae. albopictus* (Asian tiger mosquito). We selected *Ae. albopictus* for initial transfection experiments, since it is known to naturally support *Wolbachia* infection (Sinkins *et al.* 1995; Zhou *et al.* 1998; Kittayapong *et al.* 2002) and due to prior development of a transfection technique (Xi *et al.* 2005). Furthermore, *Ae. albopictus* is medically important as an invasive pest species and vector of multiple arboviruses and filaria (Moore & Mitchell 1997). The *wRi* *Wolbachia* infection from *D. simulans* was selected due to its phylogenetic similarity to the *wAlbA* infection that occurs naturally in *Ae. albopictus* (Ruang Areerate *et al.* 2003), and prior research demonstrating it to be bidirectionally incompatible with the *Ae. albopictus* infection in *D. simulans* hosts (Braig *et al.* 1994). Prior transfection experiments suggest that the *wRi*

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infection is well suited for adaptation to distantly related hosts (Kang *et al.* 2003). Furthermore, prior direct experience of the researchers with horizontal transfers of *wRi* reduces concern with *Wolbachia* viability during microinjection (Xi & Dobson 2005).

The results demonstrate that the *wRi* infection has been successfully transferred from *Drosophila* into *Ae. albopictus* and is stably maintained in the transfected line ('HTR'). The HTR line displays a unique CI pattern and is bidirectionally incompatible with both natural infections and the previously generated artificial infection (Xi *et al.* 2005). Generation of the HTR strain provides a model system for examining proposed population suppression strategies. As an initial test, releases of HTR males into laboratory cages of naturally infected populations result in reduced egg hatch. We discuss the results in relation to the potential applied use of the HTR strain.

## 2. MATERIAL AND METHODS

### (a) Insect strains

Individuals within the *Ae. albopictus* Houston (Hou) strain are naturally superinfected with both the *wAlbA* and *wAlbB* *Wolbachia* types (Sinkins *et al.* 1995). The aposymbiotic HT1 strain was generated by tetracycline treatment of the Hou strain (Dobson & Rattanadechakul 2001). The Koh Samui strain (Koh) is naturally single infected with the *wAlbA* *Wolbachia* type (Sinkins *et al.* 1995). The HTB strain is single infected with *wAlbB* and was artificially generated via microinjection of HT1 with Hou cytoplasm (Xi *et al.* 2005). *Drosophila simulans* Riverside (DSR) are naturally infected with the *wRi* infection (Zhou *et al.* 1998). Mosquito and *Drosophila* strains were maintained following standard procedures as described previously (Roberts 1998; Dobson *et al.* 2001).

### (b) Microinjection

Embryo injection was based upon techniques successfully used for mosquito and *Drosophila* transfection (Xi *et al.* 2005; Xi & Dobson 2005). Microinjection needles were prepared from quartz microcapillaries (#QF100-70-7.5; Sutter Instrument Co., Novato, CA) using a P2000 micropipette puller (Sutter Instrument Co.; Novato, CA).

DSR embryos were used as the donor of *wRi*-infected cytoplasm. DSR embryos were collected up to 30 min post-oviposition, using apple juice agar plates with yeast paste (Roberts 1998). Embryos were dechorionated in 50% bleach for 2 min, rinsed, aligned on agar plate and transferred onto a glass slide with double-sided tape (Scotch 665; St Paul, MN), and covered with water-saturated halocarbon 700 oil (Sigma-Aldrich Co.). Donor DSR embryos were not desiccated.

HT1 embryos (recipient embryos) were aligned on wet filter paper, transferred onto a cover-slip with double-sided tape, briefly desiccated, and covered with water-saturated halocarbon 700 oil. Embryos were injected up to 90 min post-oviposition.

Cytoplasm was withdrawn from the posterior of donor DSR embryos and injected into the posterior of recipient HT1 embryos using an IM300 microinjector (Narishige Scientific; Tokyo, Japan) similar to prior descriptions (Xi *et al.* 2005; Xi & Dobson 2005).

Following injection, HT1 embryos were incubated at 80% relative humidity and 27°C for approximately 40 min. Subsequently, the embryos were removed from oil and

transferred onto wet filter paper, where they were allowed to develop for 5 days. The eggs were then submerged in deoxygenated water to hatch. Resulting larvae ( $G_0$ ) were reared using standard conditions as described previously.

### (c) Crosses of transfected lines

Eclosing  $G_0$  females were isolated as pupae to assure virginity and were subsequently mated with HT1 males. Following blood feeding and oviposition,  $G_0$  females were assayed for *Wolbachia* infection via PCR (described in §2d).  $G_0$  males were assayed for *Wolbachia* infection approximately 2 days post-eclosion.  $G_0$  females testing negative for *Wolbachia* infection were discarded along with their progeny. One *wRi*-infected line was selected for subsequent experiments and designated as the 'HTR' strain: Houston strain, Tetracycline treated, with *wRi* infection. HTR individuals were sibling mated in the  $G_1$  and  $G_2$ . Beginning in  $G_3$ , 50 virgin HTR females were outcrossed with 50 HT1 males in every generation.

Crosses were conducted to characterize the pattern of CI and egg hatch rates resulting from crosses between HTR individuals and individuals with differing *Wolbachia* infection types. In all crosses, 10 virgin females were mated with 10 males. Three replicate cages were conducted for each cross type. All individuals were less than 5 days old when crossed. Subsequently, the female groups were blood fed and provided with oviposition cups.

### (d) PCR, fluorescent *in situ* hybridization staining and maternal transmission assay

For PCR assays, DNA was extracted from adult ovaries or testes via homogenization in 100  $\mu$ l STE with 0.4 mg ml<sup>-1</sup> proteinase K as described previously (O'Neill *et al.* 1992). Presence of *Wolbachia* was detected using general *Wolbachia* primers (81F, 691R) (Zhou *et al.* 1998). *Wolbachia* infection type was determined using primers specific for the *wAlbA* (328F, 691R), *wAlbB* (183F, 691R) and *wRi* (169F, 569R) infections (Zhou *et al.* 1998; Kang *et al.* 2003). As additional confirmation of infection type, primers specific for a prophage sequence (phgWOf, phgWOr) were used (Masui *et al.* 2000). The latter primers result in a PCR amplification product with *wRi* but not with the *wAlbA* or *wAlbB* infections in Hou. For mosquitoes failing to amplify with the above primers (e.g. HT1), template quality was confirmed using 12S mitochondrial primers as described previously (O'Neill *et al.* 1992). For Fluorescent *in situ* hybridization (FISH) staining, oocytes were dissected from females 4 days after blood feeding and were fixed for 15 min in freshly prepared 4% formaldehyde in PBS and then FISH stained as described previously (Xi *et al.* 2005). For the maternal transmission assay, 20 HTR  $G_5$  females and 11 HTR  $G_6$  females were randomly selected and PCR assayed. Progeny ( $G_7$ ) from one infected HTR  $G_6$  female were reared to adult and PCR tested. In each generation, the maternal transmission efficiency was estimated using the percentage of PCR positive individuals among those tested.

### (e) Population suppression

All mosquitoes used in suppression cage tests were isolated as pupae to assure virginity. Fifty Hou females and 10 Hou males were present in all of the cages. The number of HTR males ( $G_5$ ) was varied between cages. Male Hou : HTR ratios were: 10 : 500, 10 : 100, 10 : 20 and 10 : 0. All males were less than one week post-eclosion. One day after adding males

Table 1. Survival of microinjected *Ae. albopictus* embryos and the resulting *Wolbachia* infection status in the G<sub>0</sub> individuals surviving to adult.

experiment	per cent survival			G <sub>0</sub> infection status (%) (infected/total)	
	hatch (%) (larvae/ injected eggs)	pupation (%) (pupae/larvae)	eclosion (%) (adult/pupae)	female	male
1	3.4 (8/233)	75.0 (6/8)	83.3 (5/6)	40.0 (2/5)	(0/0)
2	1.9 (6/316)	50.0 (3/6)	100.0 (3/3)	0 (0/2)	0 (0/1)
3	15.8 (23/146)	78.3 (18/23)	66.7 (12/18)	37.5 (3/8)	50 (2/4)

to cages, 50 Hou females (less than one week post-eclosion) were added to each cage. Prior to adding females, cages were examined to assure that minimal male mortality had occurred. Mating was observed immediately upon addition of females to cages. Following blood feeding, females were allowed to oviposit for one week. Egg hatch rates were determined as described previously.

#### (f) Statistics

Statistical comparisons of egg hatch rate were conducted using chi-square. Kruskal-Wallis analysis was used in statistical comparisons of egg mortality (CI levels). All statistical comparisons were performed using SAS v. 8.0 (SAS Institute, Cary, NC).

### 3. RESULTS

Cytoplasm from *wRi*-infected DSR embryos was microinjected into aposymbiotic HT1 *Ae. albopictus* embryos. A total of 695 HT1 eggs were injected in three experiments, resulting in 15 G<sub>0</sub> females that survived to adult (table 1). PCR assays were conducted to diagnose *Wolbachia* infection in G<sub>0</sub> adults. As shown in table 1, *Wolbachia* infection was detected in 33% of surviving G<sub>0</sub> females and 40% of surviving G<sub>0</sub> males.

Of the five PCR positive G<sub>0</sub> females, only one female produced hatching eggs. The remaining G<sub>0</sub> females either failed to oviposit or their eggs failed to hatch. Three daughters (G<sub>1</sub>) resulting from the PCR positive G<sub>0</sub> female were sib-mated, blood fed, isolated and allowed to oviposit. Following oviposition, the G<sub>1</sub> females were PCR assayed for *Wolbachia* infection similar to G<sub>0</sub> females. Each of the three G<sub>1</sub> females tested positive for *Wolbachia* infection. One of the latter isofemale lines was randomly selected for subsequent experiments and designated as the 'HTR' strain. Subsequently, PCR assays of HTR individuals have consistently detected *Wolbachia* infection to G<sub>11</sub>, immediately prior to the submission of this report.

As an initial characterization of infection frequency, HTR females were randomly selected (G<sub>5</sub> and G<sub>6</sub>) and PCR assayed for *Wolbachia* infection. *Wolbachia* amplification products were observed in 29/31 (93.5%) females. As a direct test of maternal inheritance rates, the progeny (G<sub>7</sub>) from an infected G<sub>6</sub> female were reared to adult and PCR tested. In the latter test, PCR amplification products were observed in 18/20 (90%) of the assayed G<sub>7</sub> females.

FISH was used to examine the distribution of *Wolbachia* in transfected HTR oocytes. *Wolbachia* localization in HTR oocytes was observed to differ from that observed in naturally superinfected Hou oocytes (figure 1). *Wolbachia* was observed to be concentrated towards the centre of HTR oocytes. In contrast, the anterior and posterior are the focus of infection in naturally infected

Hou oocytes and the transfected HTB strain (Xi *et al.* 2005).

To determine the type of *Wolbachia* infection present in HTR, diagnostic PCR primers were used (figure 2). Consistent with expectations for host insects infected with the *wRi* infection only, PCR assays of HTR and DSR individuals result in amplification products of the expected size using the 169F/569R primer set, but not with the 328F/691R or 183F/691R primer sets. The 169F/569R primer set did not amplify any of the other *Ae. albopictus* strains. As an additional confirmation of *Wolbachia* type, DNA from each of the *Ae. albopictus* strains and DSR flies was amplified using the phgWof/phgWOr primer set. The latter amplification was similar to the 169F/569R primer set, in that amplification products were only obtained from HTR and DSR (data not shown). The Koh and HTB strains amplify with the 328F/691R or 183F/691R primer sets, respectively (figure 2). The Hou strain amplifies with both the 328F/691R and 183F/691R primer sets. The HT1 strain does not amplify with the *Wolbachia*-specific primers.

Given that *wRi* causes CI in its natural *D. simulans* host, crosses between HTR and HT1 were conducted to determine whether the *wRi* infection causes CI in *Ae. albopictus*. Additional crosses were conducted to determine the CI pattern relative to the other *Ae. albopictus* infections (i.e. the *wAlbA* single infection, *wAlbB* single infection and the superinfection). As shown in table 2, crosses of HTR individuals with the other infection types resulted in a unique pattern of CI. Specifically, a typical pattern of unidirectional CI was observed in crosses between HTR with uninfected HT1 individuals. Bidirectional CI was observed in crosses of HTR with single-infected (Koh, HTB) and superinfected (Hou) individuals.

The ability of the *wRi* infection in HTR to induce CI was measured as the level of egg hatch resulting from crosses of HTR males with females differing in their infection type. The strongest CI (less than 4% egg hatch) was observed in crosses with uninfected HT1 females. A similar CI level (ca 6% hatch) was observed in crosses with *wAlbA*-infected Koh females. In contrast, higher egg hatch levels were observed in crosses with *wAlbB*-infected HTB females and superinfected Hou females (9.9 and 14.4% hatch rate, respectively). Reciprocal crosses were conducted to examine the ability of *wRi* to rescue CI caused by the other infection types. Low egg hatch (less than 3%) was observed in crosses of HTR females with males harbouring different *Wolbachia* types. Consistent with expectations for *Wolbachia*-induced CI, HTR females are compatible with both HTR males and uninfected males.

The egg hatch resulting from the HTR×HTR cross at G<sub>3</sub> was significantly lower than other compatible crosses,



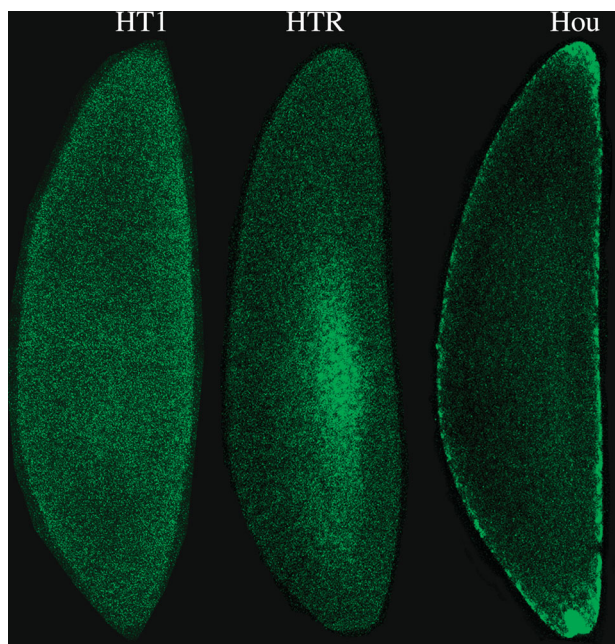


Figure 1. *Wolbachia* distribution in oocytes of naturally superinfected (Hou), aposymbiotic (HT1) and the *wRi* transfected HTR line. In HTR oocytes, *Wolbachia* is concentrated in the centre of oocytes. In contrast, *Wolbachia* is focused in the anterior and posterior of naturally infected Hou oocytes.

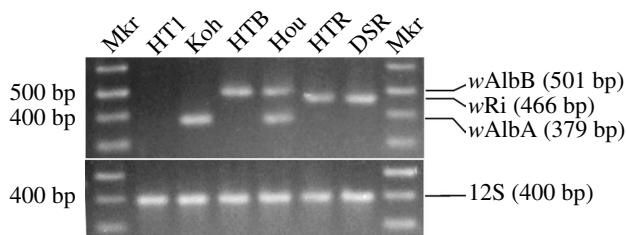


Figure 2. Diagnostic pattern of amplification products resulting with *Wolbachia*-specific PCR primers. The gel illustrates all of the amplification products that result when each *Ae. albopictus* strain is amplified with all four primer sets. The mitochondrial 12S primers are used to confirm template DNA quality. For the electrophoresis gel shown in the figure, amplicons resulting from separate PCR amplifications were combined.

including the HTR×HT1 (female×male; chi-square test,  $p < 0.0001$ ) and compatible crosses of Koh, HT1 and Hou (chi-square test,  $p < 0.05$ ). To reduce possible inbreeding depression effects associated with the use of isofemale lines in generating the HTR strain, HTR females were repeatedly outcrossed to HT1 males beginning in  $G_3$ . The HTR egg hatch was observed to increase from 58% hatch in  $G_3$  to 77% in  $G_4$ , 85% in  $G_6$  and 92% in  $G_8$ . While the increase in hatch rates is consistent with the hypothesized inbreeding effect, the results do not exclude an alternate hypothesis that the low egg hatch resulting in compatible crosses between HTR individuals in early generations was due to variable *Wolbachia* levels and maternal infection loss.

The bidirectional CI observed between HTR and the naturally superinfected Hou strain suggests a strategy for suppressing *Ae. albopictus* populations via releases of HTR males. As an initial test of this strategy, cages were established with varying ratios of HTR : Hou males. As shown in figure 3, the egg hatch was observed to decrease with an increasing ratio of HTR males in the population.

#### 4. DISCUSSION

Here, we have demonstrated that embryonic microinjection can be used for interspecific transfer of *Wolbachia* from *D. simulans* into *Ae. albopictus* to generate the HTR strain. FISH staining shows that the *wRi* infection in *Ae. albopictus* females is transmitted to offspring during oogenesis. PCR assays demonstrate the HTR infection to be stable, with more than 90% infection frequency throughout the 11 mosquito generations encompassed by this report. The *wRi* infection is able to induce CI in the transfected HTR strain and displays a unique pattern of CI relative to other infections in *Ae. albopictus*.

Prior *wRi* transfection research has yielded a range of CI levels and maternal transmission rates, suggesting that host type can affect *Wolbachia* dynamics. Specifically, high CI levels are observed in *Drosophila mauritiana*, *Drosophila serrata*, the *Drosophila yakuba* complex and *Laodelphax striatellus*, similar to that observed in the native *D. simulans* host (Hoffmann *et al.* 1986; Giordano *et al.* 1995; Clancy & Hoffmann 1997; Kang *et al.* 2003; Zabalou *et al.* 2004). In contrast, a relatively low CI level (*ca* 75% hatch) was

Table 2. Egg hatch resulting from crosses of the transfected HTR line ( $G_3$ ).

expected CI type	cross <sup>a</sup>	infection type		per cent egg hatch (%) <sup>b</sup>	eggs scored
		female	male		
bidirectional CI	Koh×HTR	<i>wAlbA</i>	<i>wRi</i>	5.9±4.8	2119
	HTR×Koh	<i>wRi</i>	<i>wAlbA</i>	2.8±1.5	2040
	HTB×HTR	<i>wAlbB</i>	<i>wRi</i>	9.9±5.4	1434
	HTR×HTB	<i>wRi</i>	<i>wAlbB</i>	0.3±0.5	2152
	Hou×HTR	<i>wAlbA</i> , <i>wAlbB</i>	<i>wRi</i>	14.2±6.6	2972
	HTR×Hou	<i>wRi</i>	<i>wAlbA</i> , <i>wAlbB</i>	0.4±0.4	3216
unidirectional CI	HT1×HTR	—	<i>wRi</i>	3.8±3.3	1916
	HTR×HT1	<i>wRi</i>	—	75.5±9.7	2157
compatible	HTR×HTR	<i>wRi</i>	<i>wRi</i>	57.1±10.5	2136
	Koh×Koh	<i>wAlbA</i>	<i>wAlbA</i>	81.0±4.6	635
	HT1×HT1	—	—	80.8±0.3	1159
	Hou×Hou	<i>wAlbA</i> , <i>wAlbB</i>	<i>wAlbA</i> , <i>wAlbB</i>	82.6±2.7	1883

<sup>a</sup> Female×male.

<sup>b</sup> Average±s.d.; three cages/cross type.

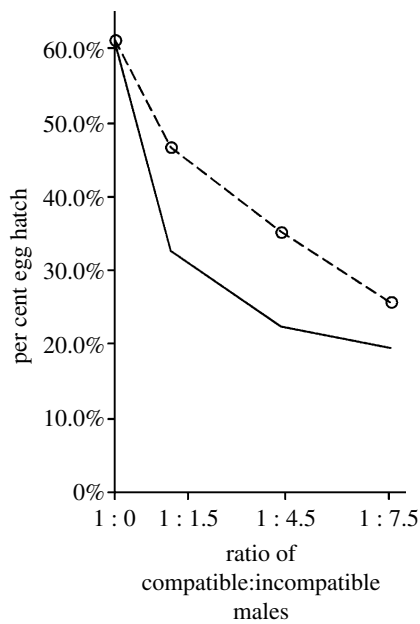


Figure 3. Suppression of egg hatch in populations of naturally superinfected *Ae. albopictus* (Hou) via releases of transfectured HTR males. The circles and dashed line illustrate the egg hatch observed in population cage tests. The solid line illustrates the expected egg hatch assuming equal competitiveness of HTR and Hou males (Arunachalam & Curtis 1985). Estimations of the ratio of compatible : incompatible males in the cages ( $x$ -axis) assume that 10% of HTR males that are released into cages are uninfected due to incomplete maternal transmission.

reported in *Drosophila melanogaster* transfectured with *wRi* (Boyle *et al.* 1993). The same studies also demonstrate that maternal transmission of *wRi* can vary between hosts. High transmission (*ca* 90% fidelity) is observed in *D. simulans* and *D. serrata*, and lower transmission (*ca* 30% fidelity) is observed in *L. striatellus*. Thus, the CI levels and maternal transmission rate observed with HTR are equivalent to the highest reported for the *wRi* infection. A comparison with prior crossing results (Dobson *et al.* 2002b, 2004; Xi *et al.* 2005) demonstrates HTR females to be similar to HT1 females in their inability to rescue the *Wolbachia*-induced sperm modification in Koh, HTB and Hou males. Thus, the *wRi* infection is unable to rescue the modifications caused by either *wAlbA* or *wAlbB* in *Ae. albopictus*.

Relative to the other CI crosses of HTR males, higher egg hatch is observed when *wRi*-infected males are mated with females infected with *wAlbB* (chi-square test,  $p < 0.01$ ), including both HTB and Hou females. Interestingly, a similar pattern was observed in a prior transfection study examining the interaction between *Ae. albopictus* infections and *wRi* in *D. simulans* (Braig *et al.* 1994). In the prior study, egg hatch resulting from crosses of females infected with *Ae. albopictus* *Wolbachia* and *wRi* males was approximately 10% higher than the reciprocal cross. However, the prior study did not determine the specific infection type(s) transfectured from *Ae. albopictus* (Hou strain) into *D. simulans* (Braig *et al.* 1994). Thus, additional experiments are required to specifically test the hypothesis that the *wAlbB* infection is able to partially rescue the modification caused by *wRi*.

The observed pattern of *wRi* distribution in *Ae. albopictus* oocytes is distinct from that of natural infections

described in *Aedes* and *Culex*. The *wAlbA*, *wAlbB* and *wPip* infections are concentrated at the oocyte poles of *Ae. albopictus* and *Culex pipiens* (Rasgon & Scott 2003; Xi *et al.* 2005). In contrast, the *wRi* infection is concentrated in the oocyte centre (figure 1). The *wRi* distribution in HTR also differs from that reported in natural and transfectured *Drosophila* hosts, where it concentrates in the cortical region of oocytes (Veneti *et al.* 2004). The unusual distribution of *wRi* in HTR oocytes is consistent with a prior report showing that *Wolbachia* embryonic distribution can be affected by host type (Pintureau *et al.* 2000).

The bidirectional CI observed between HTR and the naturally superinfected Hou strain suggests a strategy for suppressing *Ae. albopictus* populations via releases of HTR males. As an initial test of this strategy, cages were established with varying ratios of HTR : Hou males. As shown in figure 3, egg hatch was observed to decrease with an increasing ratio of HTR males in the population.

Although cage experiments demonstrate that HTR male releases can suppress egg hatch in naturally infected *Ae. albopictus* populations, the results suggest that HTR is not suitable for an eradication strategy similar to prior *Culex* work (Laven 1967). Imperfect CI and maternal transmission failure is observed with HTR, resulting in *ca* 14% egg hatch in crosses of Hou females and HTR males. Thus, continued releases of HTR males into a naturally superinfected population would be expected to reduce, but not eliminate egg hatch. Furthermore, population cage tests suggest that HTR and Hou males are not equally competitive. As shown in figure 3, comparison of the observed egg hatch with theoretical expectations for equally competitive HTR and Hou males (Arunachalam & Curtis 1985) suggests that the HTR males suffer a reduced mating competitiveness relative to Hou males. Further studies are required to test the hypothesis that the observed difference results from persistent inbreeding effects that were not eliminated by two generations of outcrosses.

Demonstrating that microinjection can be used to successfully transfer *Wolbachia* across taxonomic families into a medically important mosquito emphasizes a need to repeat this work with additional disease vectors (e.g. *Anopheles*). Additional experiments should also include transfection of the *wRi* infection into superinfected *Ae. albopictus*, with the goal of generating a triple-infected strain capable of invading *Ae. albopictus* field populations. The latter could be used as a vehicle to drive desired transgenes (e.g. genes reducing vector competency) into naturally occurring *Ae. albopictus* populations.

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