LRH: STEPHEN L. DOBSON ET AL.

RRH: *WOLBACHIA* SUPERINFECTION IN *AEDES ALBOPICTUS*

TITLE: ORIGIN OF *WOLBACHIA* SUPERINFECTION IN *AEDES ALBOPICTUS* BY SEQUENTIAL POPULATION REPLACEMENT

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ABSTRACT

A reproductive advantage afforded to female hosts by obligate intracellular *Wolbachia* infections can result in the spread of the vertically-inherited bacteria into the host population, replacing the uninfected cytotype (population replacement). Here we examine *Wolbachia* infection dynamics relevant to the origin of *Wolbachia* superinfection (i.e., host individuals that are co-infected with two or more *Wolbachia* types). Possible routes of origin for *Wolbachia* superinfection are: 1) the simultaneous invasion of an uninfected host population by the superinfected cytotype, or 2) the sequential invasion of an uninfected host population by a single-infection followed by the subsequent superinfection invasion. Relative to uninfected females, superinfected *Aedes albopictus* females are at a reproductive advantage due to both cytoplasmic incompatibility (CI) and a female fitness increase associated with *Wolbachia* infection. A host fitness increase combined with CI is predicted to reduce the threshold *Wolbachia* infection frequency required for population replacement. However, this prediction applies only to the simultaneous invasion hypothesis. To better understand events associated with sequential invasion, we have examined cytoplasmic incompatibility levels, host longevity, egg hatch rates and fecundity in introgressed *A. albopictus* strains that are uninfected, single-infected, and superinfected with *Wolbachia*. Relative to uninfected females, infected females are at a reproductive advantage due to both cytoplasmic incompatibility and a fitness increase associated with *Wolbachia* infection. In contrast, no fitness advantage was observed in comparisons of single- and superinfected females. We discuss the observed results in regard to the proposed origin of *Wolbachia* superinfection in *A. albopictus* via sequential population replacement. The hypothesized evolution of *Wolbachia* superinfection in *A. albopictus* by mutation and horizontal transmission is also discussed. Comparisons of similarly-infected *A. albopictus* strains that differ in host genotype demonstrate host fitness varies with host genotpe, but that cytoplasmic incompatibility and *Wolbachia*-induced host fitness effects are consistent regardless of the host genotype.

KEY WORDS:

cytoplasmic incompatibility, cytoplasmic drive, population replacement, reproductive parasite

Two evolutionary trajectories are commonly recognized for obligate, intracellular bacterial symbionts that are vertically-inherited. As the success of vertically-inherited infections is dependent upon host fitness, there is a selective advantage for bacterial variants that have an increasingly benign or beneficial relationship with their host, resulting in a trend toward commensialism or mutualism respectively (Ewald 1987; Fine 1975). Alternatively, when the bacterial infection is inherited exclusively through a single sex of the host population, direct selective pressure on the symbiont occurs only through the sex that is responsible for transmission, and a form of symbiosis known as reproductive parasitism can evolve.

Multiple examples of reproductive parasitism can be observed in infections of obligate intracellular *Wolbachia* bacteria. As *Wolbachia* is maternally transmitted to embryos via female host cytoplasm, males are an evolutionary dead end for *Wolbachia* infections, and there is no direct selection for beneficial symbiosis in infected males. To the contrary, cytoplasmic incompatibility (Hoffmann and Turelli 1997), male killing (Hurst et al. 1999), parthenogenesis (Stouthamer et al. 1993), and feminization (Rousset et al. 1992) each provide examples of *Wolbachia* symbioses in which infected female reproductive success is increased at the expense of infected male hosts.

The form of reproductive parasitism known as cytoplasmic incompatibility (CI) results in karyogamy failure and arrested development of early embryos in diploid insects (Tram and Sullivan 2002). Unidirectional CI occurs in matings between males that are infected with *Wolbachia* and uninfected females (see Fig. 1). The reciprocal cross and matings between individuals harboring similar infections are compatible. In host populations that include infected and uninfected individuals, CI provides a reproductive advantage to infected females since they can mate successfully with all male types. In contrast, uninfected females are incompatible with infected males, reducing reproductive success. The advantage afforded to females by CI comes at the expense of infected males, which are incompatible with uninfected females. As observed in both laboratory and natural populations (reviewed in Hoffmann and Turelli 1997), the reproductive advantage afforded by CI to infected females can result in population replacement, with the infected cytotype driving into the host population and replacing the uninfected cytotype. In addition to unidirectional CI, bidirectional CI (see Fig. 1) can occur in populations that harbor different *Wolbachia* infection types (Bordenstein et al. 2001; Guillemaud et al. 1997; Rousset and de Stordeur 1994).

Although the mechanism responsible for CI has not yet been identified, a poison/antidote model has been used to explain the observed phenomena (Werren 1997). A modification (*mod*) occurs on the male pronucleus, before *Wolbachia* are shed from maturing sperm (Presgraves 2000). Embryos that are fertilized with modified sperm are arrested in early development unless the rescue (*resc*) function is expressed in eggs from *Wolbachia*-infected females. The observation of bidirectional CI demonstrates that *mod* and *resc* interact in a specific manner, such that different infection types do not necessarily rescue the modification of a differing *Wolbachia* type (see Fig. 1; Bourtzis et al. 1998; Merçot and Poinsot 1998). CI patterns associated with superinfection demonstrate that differing *mod*/*resc* mechanisms may act autonomously and additively, such that co-infections may result in novel patterns of additive unidirectional CI (see Fig. 1; Dobson et al. 2001; Merçot et al. 1995; Perrot-Minnot et al. 1996; Sinkins et al. 1995b).

Wolbachia infections in *Aedes albopictus* (Asian tiger mosquito) provide a useful system in which to study reproductive parasitism. Naturally occurring populations of *A. albopictus* can be single-infected with the *w*AlbA *Wolbachia* type or superinfected with *w*AlbA and *w*AlbB (Dobson et al. 2001; Kambhampati et al. 1993; O'Neill et al. 1997; Otsuka and Takaoka 1997; Sinkins et al. 1995b). Prior experiments demonstrate that the *w*AlbA infection is unidirectionally incompatible with uninfected hosts, and that the *w*AlbA/*w*AlbB superinfection has an additive effect, such that the superinfection is unidirectionally incompatible with both single- and uninfected hosts (see Fig. 1; Dobson et al. 2001). Based upon CI patterns, sequential population replacement has been hypothesized to explain the observed geographic distribution of single- and superinfections in naturally occurring *A. albopictus* populations (Perrot-Minnot et al. 1996; Sinkins et al. 1995b). Here we examine CI and *Wolbachia* infection dynamics in *A. albopictus* to better define conditions permitting the evolution of superinfection. As the interpretation of results can be complicated by differing host genetic backgrounds (Dobson et al. 2001), we compare the differing infection types within genetically homogeneous host strains generated via introgression.

A host fitness increase associated with *Wolbachia* superinfection in *A. albopictus* was observed in prior experiments (Dobson et al. 2002). As described in the previous report, mutualism associated with CI can facilitate population replacement by reducing the threshold infection frequency required for *Wolbachia* invasion and increasing cytoplasmic drive rates. However, the prior experiments compared superinfected hosts with uninfected hosts. The hypothesized origin of *A. albopictus* superinfection via sequential population replacement proposes that the superinfection replaced a population that was single-infected with the *w*AlbA cytotype (Perrot-Minnot et al. 1996; Sinkins et al. 1995b). To better understand conditions required for sequential population replacement events in *A. albopictus* we compare uninfected, single-infected, and superinfected hosts. As an additional examination of the level to which *Wolbachia* infection dynamics are influenced by host genotype, we also compare hosts that vary in their host genotype but that harbor similar infections.

MATERIALS AND METHODS

Mosquito Stocks, Culture, and Experimental Crosses

Mosquito strains and infection types are outlined in Table 1. Mosquitoes were maintained using standard conditions (Gerberg et al. 1994) at 28±2˚C and 75±10% RH with an 18 hour light cycle. Eggs were hatched in deoxygenated water and reared at low density in water augmented with liverpowder. For adult maintenance and experimental crosses, a constant supply of 10% sucrose was provided to adults. Females were provided weekly with a mouse for blood feeding and eggs were collected weekly.

Experimental crosses consisted of ten two-day-old virgin females and males (20 mosquitoes total) placed in cages. For all crosses, a constant supply of 10% sucrose was provided to adults. Females were provided a mouse weekly for blood feeding. An oviposition container was constantly available to females and changed weekly for egg collection. Eggs were dried over a 2 day period and then allowed to mature for five days at 28±2˚C 80±5% RH. Following drying and maturation, eggs were hatched by submerging in a deoxygenated water/liverpowder solution. Larvae used for maintenance

and crossing experiments were reared at low density in an excess of a liverpowder/yeast food suspension. Numbers of surviving males and females were recorded weekly. Egg papers were collected and counted until females in the cage were dead. For introgression crosses, four replicate cages were monitored for each of the crossing types.

Repeated measures ANOVA was used for comparisons of host fecundity and adult longevity (StatView 5.0.1; SAS Institute, Inc.). Due to unequal sample sizes, ANOVA and Bonferroni mean separation was used for comparisons of egg hatch rates (i.e., CI levels). For fecundity comparisons, per female oviposition rates were estimated as the number of eggs from a cage divided by the number of surviving females within the cage.

Introgressions

The *UjuT* nuclear genome was introgressed into the single- and superinfected cytoplasm types by repeated backcrossing. In brief, approximately 20 virgin *Hou* or *Koh* virgin females were mated with 20 *UjuT* males. In the next generation, the resulting hybrid daughters were mated with *UjuT* males. The introgression crosses were repeated for a total of seven generations.

In theory, each backcross generation will replace half of the maternal nuclear genome with the paternal nuclear genome. The percentage nuclear substitution in females is calculated as $[1-(1-0.5^n)]$, where n is the number of backcross generations. Thus, after seven generations, the amount of the original maternal genome remaining will be only 7.8×10^{-3} percent of the genotype. Although the original genotype is largely replaced by the *UjuT* genome in these introgressed lines, the maternally inherited *Wolbachia* infection should remain consistent during the introgressions. The resulting strains were designated *IK7* and *IH7* for the single-infected and superinfected introgressed lines, respectively (see Table 1).

PCR Assay

Infection type in mosquito strains initially was confirmed using diagnostic primers: *wAlbA* (primers 328F and 691R) and *wAlbB* (183F and 691R primers; Zhou et al. 1998). For samples failing to amplify using *Wolbachia*-specific primers (e.g., *UjuT* strain), 12S primers were used to amplify mitochondria DNA as a positive control for template DNA quality (O'Neill et al. 1992). For PCR amplifications, ovaries or testes from individual mosquitoes were isolated and homogenized in 100 µl STE [0.1 M NaCl, 10 mM Tris HCl, and 1 mM EDTA (pH 8.0)]. Proteinase K was added to a final concentration of 0.4 mg/ml, and this mixture was incubated at 56˚C for 1 hr. Following heat inactivation at 95˚C for 15 min, 1 µl of these samples were amplified in 50 mM KCl, 20 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 mM primers, and 1 U Taq DNA polymerase in a total volume of 20 µl. Samples were denatured for 3 min at 94˚C, cycled 35 times at 94˚C, 55˚C and 72˚C (1 min each), followed by a 10 min extension at 72˚C using a PTC-200 Thermal Cycler (MJ Research). 10 µl of each amplification was separated on 1% agarose gels, stained with ethidium bromide and visualized under ultraviolet illumination.

RESULTS

Molecular Confirmation of Wolbachia *Type in Introgressed Strains*

PCR amplification with primers diagnostic for the *w*AlbA and *w*AlbB infections (Dobson et al. 2001; Zhou et al. 1998) confirmed the *Wolbachia* infection type following introgression crosses. Both the *w*AlbA and *w*AlbB amplification products were observed in IH7 individuals, indicating superinfection. Only the *w*AlbA amplification product was observed in IK7 individuals. No amplification product was observed in UjuT individuals. This pattern of amplification products is similar to that observed in the original *A. albopictus* strains (Dobson et al. 2001) and demonstrates that the *Wolbachia* infection type remained constant throughout introgression crosses.

Wolbachia *Effects in Introgression Crosses*

As shown in Table 2, cytoplasmic incompatibility was observed in crosses of the introgressed strains only when the male harbored a *Wolbachia* infection type that was not present in the female mate. Superinfected IH7 females were compatible with all male infection types. Single-infected IK7 females were incompatible only with superinfected males. Uninfected UjuT females were compatible with uninfected males only. Thus, crosses of the introgressed strains demonstrated an additive pattern of unidirectional cytoplasmic incompatibility. As illustrated in Figure 2, the egg hatch rate remained relatively constant over time.

Comparisons within the six compatible cross types demonstrated significantly lower egg hatch resulting from the UjuT x UjuT cross relative to the remaining five compatible crossing types ($p<0.0001$; see Table 2). Egg hatch in compatible crosses of infected females did not differ significantly. In a comparison of the three incompatible cross types, egg hatch was higher in crosses of single-infected males relative to incompatible crosses of superinfected males ($p<0.0001$). No difference in egg hatch was observed between incompatible crosses of superinfected males.

Differences in host longevity were observed in comparisons of the different *Wolbachia* infection types. As shown in Figure 3A, superinfected females were longer lived relative to uninfected females ($p<0.0001$). Single-infected females were also significantly longer lived than uninfected females ($p<0.0001$). No difference was observed in comparisons of single- and superinfected female longevity. Mate infection type and cross type (i.e., compatible versus incompatible mates) were not observed to have a significant effect on female longevity. Wolbachia infection type in males was not observed to effect male longevity (see Fig. 3B).

Crosses were examined for an effect of infection type on fecundity (see Fig. 3C). Uninfected females produced fewer eggs than single-infected females ($p<0.054$) and superinfected females ($p<0.032$). No difference was observed in fecundity comparisons of single- and superinfected females ($p>0.809$). Mate infection type and cross type was not observed to significantly effect female fecundity ($p>0.566$).

Wolbachia *Infections Within Differing Host Genotypes*

To better define the effect of host genotype on egg hatch, longevity, and fecundity, introgression cross results ("U" genetic background) were compared with results obtained in "H" genotype crosses (see Table 1). As *Wolbachia* infection and compatibility type can affect egg hatch, longevity and fecundity, comparisons were made between individuals with identical infection types. To examine host genotype effect on compatible cross egg hatch rates, two cross pairs were compared. Comparison of a superinfected cross pair [Hou x Hou versus IH7 x IH7] demonstrated higher egg hatch ($p<0.0005$; see Fig. 4) in the "U" genotype relative to the "H" genotype. Higher egg hatch was again observed in the "U" genotype ($p<0.0092$; see Fig. 4) in comparisons of the uninfected [UjuT x UjuT ; HT1 x HT1] cross pair. In an examination of fecundity in cross pairs, "H" genotype females were observed to be more fecund than "U" genotype females in a comparison of both uninfected ($p<0.0004$; see Fig. 5A) and superinfected ($p<0.0724$; see Fig. 5B) crosses.

"U" genotype individuals were longer lived relative to "H" genotype individuals. To examine the effect of genotype on female longevity, a comparison was made between superinfected females that were of the H or U genotype (i.e., the Hou and IH7 strains, respectively). As shown in Figure 6A, superinfected females with the U genotype lived significantly longer ($p<0.001$). "U" genotype females were again observed to be longer lived than "H" genotype females in comparisons of uninfected females ($p<0.0413$; see Fig. 6B). Female longevity was not observed to be effected by the genotype of their mates (data not shown). "U" genotype males were longer lived relative to H-genotype males in comparisons of both superinfected males (see Fig. 6C; $p<0.0310$) and uninfected males (see Fig. 6D; $p<0.0001$).

DISCUSSION

The Evolution of Superinfections

Two routes are hypothesized for the evolution of *Wolbachia* superinfections. The first route proposes a "mixing" of two or more different *Wolbachia* types via horizontal transmission between infected hosts (Perrot-Minnot et al. 1996). Although transmission of *Wolbachia* infections occurs primarily via maternal transmission through the cytoplasm, rare horizontal transmission is suggested by phylogenetic analyses (O'Neill et al. 1992; Rousset et al. 1992; Vavre et al. 1999; Werren et al. 1995; Zhou et al. 1998) and has been observed to occur in egg parasitoids (Huigens et al. 2000).

Alternatively, superinfection may evolve from *Wolbachia* mutation that results in a variant that is incompatible with the original infection (see Fig. 7). This hypothesized route is similar to an elegant model described by Charlat et al (2001). Contrary to the prior model however, the route described here does not assume homogeneous infections within host individuals. Instead, we assume that *Wolbachia* variants arising by mutation become a proportion of the total infection that is maternally transmitted to progeny. Thus, a variant *Wolbachia* "S1" ($mod^{B}resc^{A}$) would co-occur in the host along with the original infection "S0" ($mod^{A}resc^{A}$). Females infected with both S1 and S0 infections ("S0+S1") show the same compatibility pattern as females infected with S0 only (see Fig. 7), and the relative proportion of the S0 and S0+S1 infections in the host population is expected to change through genetic drift only (Charlat et al. 2001). Subsequently, an

additional variant "S2" ($mod^{B}resc^{B}$) arising by mutation of S1 would then be selected. Unlike the example by Charlat et al (2001) in which the second variant is bidirectionally incompatible with the original infection, the S0+S1+S2 infection would be unidirectionally incompatible with both the S0 and S0+S1 infections and could invade the host population at frequencies less than 50%. In a host population with individuals infected with the S0+S1+S2 types, there would not be selection to maintain the S1 type. Thus, the S1 variant could be lost by drift, resulting in a host population that is superinfected with the S0 and S2 *Wolbachia* types. The resulting superinfection would be analogous to the *w*AlbA ($mod^{A}resc^{A}$) and *w*AlbB ($mod^{B}resc^{B}$) superinfection occurring in *A. albopictus*. With the exception noted above, the assumptions and notation of this model are as previously described (Charlat et al. 2001).

It is interesting to note, that the above model could be modified in that a variant "S3" ($mod^{A}resc^{B}$) arises instead of S2. The dynamics of the latter evolutionary route would be identical to the above. In a host population with individuals infected with S0+S1+S3, there would not be selection to maintain the S0 type. Thus, the original S0 infection could be lost by drift, resulting in a host population that is superinfected with S1+S3. The latter would provide strong selection for high maternal transmission rates of both infections and the maintenance of superinfection, since the loss of either infection from the host population would result in suicidal infections (i.e., females single-infected with either S1 or S3 would be incompatible with all males in the population, including males infected with the same infection type). Although an example of the latter has not been described, this may simply reflect the crude tools that have been used to recognize *Wolbachia* infections. A host population infected with S1+S3 would show a unidirectional compatibility pattern that is identical to a single-infection. The co-infecting S1 and S3 infections would be closely related and would likely escape differentiation in comparisons of only a few loci. The recently initiated *Wolbachia* genomics initiative (Slatko et al. 1999) may aid in recognizing examples of the latter evolutionary trajectory. Recombination between co-infecting *Wolbachia* variants (Jiggins et al. 2001; Werren and Bartos 2001) could result in the consolidation of multiple rescue loci on the genome of a *Wolbachia* variant ("S4"; $resc^{AB}$) and a generalization of the rescue mechanism. Subsequently, selection to maintain the S1 and S3 infections would be relaxed.

Origin of Superinfection in A. albopictus

Given the high level of genetic divergence between *w*AlbA and *w*AlbB infections in *A. albopictus*, the origin of the *w*AlbA+*w*AlbB superinfection via a horizontal transmission event provides a more parsimonious explanation than the alternate evolutionary route by mutation. Based upon independent phylogenetic analyses of the ftsZ and wsp loci, the *w*AlbA and *w*AlbB infections have been placed in the A and B clades, respectively (Werren et al. 1995; Zhou et al. 1998). However, the two evolutionary routes described above are not exclusive, and with more elegant molecular tools, the infections that are currently defined as *w*AlbA and *w*AlbB may be subsequently recognized to include additional *Wolbachia* variants.

The previously described geographic distribution of infection types in naturally-occurring *A. albopictus* populations combined with an additive pattern of unidirectional

incompatibility suggests that the *w*AlbA infection is the more primitive infection that was subsequently replaced by the *w*AlbA+*w*AlbB superinfection (sequential population replacement hypothesis; Perrot-Minnot et al. 1996; Sinkins et al. 1995b). Superinfection has been described in naturally occurring *A. albopictus* populations from Japan, India, America, Malaysia, and Thailand (Kambhampati et al. 1993; Kittayapong et al. 2000; Otsuka and Takaoka 1997). In contrast, naturally occurring *w*AlbA single-infections have only been described in island populations (Koh Samui and Mauritius; Dobson et al. 2001; Kambhampati et al. 1993; Kittayapong et al. 2000; Sinkins et al. 1995b) that are geographically isolated from superinfected populations. Additional hypotheses are: 1) that a primitive *w*AlbB single-infection was subsequently replaced by the superinfection or 2) that the superinfection replaced an uninfected cytotype (simultaneous population replacement hypothesis). The observation of naturally occurring *w*AlbA populations and the failure to observe the *w*AlbB single-infection in natural or manipulated laboratory populations (Dobson and Rattanadechakul 2001; Otsuka and Takaoka 1997) suggests that, if a *w*AlbB population was replaced by the superinfection, then this occurred in addition to the superinfection replacement of the *w*AlbA infection. The observed distribution of *w*AlbA and *w*AlbA+*w*AlbB populations could also be explained by the replacement of an uninfected population by the *w*AlbA+*w*AlbB superinfection. Island populations single-infected with *w*AlbA could then result from maternal transmission failure (Perrot-Minnot et al. 1996) and founder effects. However, maternal transmission failure in superinfected *A. albopictus* has not been observed, and laboratory manipulations (Dobson and Rattanadechakul 2001; Otsuka and Takaoka 1997) removing infection from superinfected females do not result in single-infected progeny. Thus, the sequential invasion hypothesis provides a more parsimonious explanation for the observed distribution of infections.

To better understand events associated with the evolution of the *Wolbachia*/*A. albopictus* symbiosis, *Wolbachia* infection dynamics important in sequential population replacement and *Wolbachia* maintenance within *A. albopictus* populations, we have examined *Wolbachia* single- and superinfections within a uniform host genetic background. Previous models demonstrate that cytoplasmic incompatibility levels and host fitness effects determine the initial *Wolbachia* infection frequency required for invasion and the rate at which the infection invades (reviewed in Hoffmann and Turelli 1997). Therefore cytoplasmic incompatibility levels, *Wolbachia* effects on host longevity and fecundity were examined. To examine host genotype effects on *Wolbachia* infection dynamics, we also compared cross results of *Wolbachia* infections that occur within different host genotypes.

In crosses of introgressed strains, an additive pattern of unidirectional cytoplasmic incompatibility was observed (see Table 2). Superinfected females are compatible with all of the male infection types and therefore are at a reproductive advantage relative to single-infected females, which are incompatible with superinfected males. Cytoplasmic incompatibility affords single-infected females a reproductive advantage relative to uninfected females, which are incompatible with both single- and superinfected males. This pattern of additive unidirectional cytoplasmic incompatibility is consistent with previous reports (Dobson et al. 2001; Dobson et al. 2002; Kambhampati et al. 1993; Otsuka and Takaoka 1997; Sinkins et al. 1995b).

Crossing results also demonstrate an increased host fitness associated with *Wolbachia* infection in *A. albopictus*. Based upon model predictions (Dobson et al.

2002), increased host fitness associated with *Wolbachia* infection would facilitate population replacement by decreasing the initial infection frequency threshold required for *Wolbachia* to invade the *A. albopictus* population and increasing the rate at which population replacement occurs. Increased fitness associated with superinfection in *A. albopictus* was previously reported in studies comparing an aposymbiotic strain with the superinfected strain from which it was derived (Dobson et al. 2002). Important for understanding events associated with sequential population replacement however, the prior study was unable to examine genetically similar single-infected mosquitoes. Here, we observed that single- and superinfected females are longer lived than uninfected females (see Fig. 3A). A comparison of compatible crosses demonstrates that egg hatch in crosses of single- and superinfected females are significantly higher than that of uninfected females (see Table 2). Greater fecundity was also observed in single- and superinfected females relative to uninfected females (see Fig. 3C).

In contrast with comparisons of infected and uninfected hosts, no difference was observed in host fitness comparison of the single- and superinfected individuals. Thus, the reproductive advantage of superinfected females relative to single-infected females is due to cytoplasmic incompatibility only. Based upon this observation, different dynamics would be predicted for sequential population replacement events. As discussed above, cytoplasmic incompatibility in combination with increased host fitness is predicted to reduce the initial *Wolbachia* infection frequency threshold and increase the population replacement rates for superinfection invasion into an uninfected population (Dobson et al. 2002). However, the absence of observed fitness differences between single- and superinfected individuals indicates that the hypothesized subsequent invasion of the *w*AlbA+*w*AlbB superinfection into a host population that is single-infected with *w*AlbA would be driven primarily by unidirectional cytoplasmic incompatibility and not host fitness differences. Thus, the threshold infection frequency required to initiate the replacement of a single-infected *w*AlbA population with the *w*AlbA+*w*AlbB superinfection would be relatively higher, and replacement would proceed at a slower rate relative to the replacement of an uninfected population with the *w*AlbA single-infection. It is important to note that the comparisons described here are limited and do not exclude the possibility of additional fitness effects induced by *Wolbachia* infections (e.g., *Wolbachia* effects in immature hosts and other life stages, male mating competitiveness).

Similar to a previous report (Dobson et al. 2001), egg hatch rates in incompatible crosses of single-infected males were significantly higher than incompatible crosses of superinfected males. As the female and male in the incompatible UjuTxIK7 and IK7xIH7 crosses both differ by one infection, this suggests that cytoplasmic incompatibility is not simply additive. However, a currently unavailable strain of *A. albopictus* that is single-infected with *w*AlbB would be required to clarify the level to which the lower hatch rate is due to differences in the two *Wolbachia* types or an interaction between the *w*AlbA and *w*AlbB infections in the male.

Wolbachia infection was not observed to affect male longevity. As males are an evolutionary dead end for *Wolbachia* infections, there is no direct selection on *Wolbachia* to affect male fitness. Indirectly, *Wolbachia* will be selected to affect male fitness if the male effect corresponds with a benefit to infections in females (reproductive parasitism; Bandi et al. 2001). Comparisons of introgression crosses also

demonstrate that male infection type does not significantly effect egg hatch in compatible crosses, female mate longevity or fecundity.

Although the overall pattern of compatibility observed in crosses of the introgressed lines is similar to previous reports, there are differences in the egg hatch, longevity, and fecundity (Dobson et al. 2001; Dobson et al. 2002). The observed differences could be explained as a complication caused by variable genotypes. To examine host genotype effects on *Wolbachia* infections, we compared crossing results of similar *Wolbachia* infection types within differing genotypes. Significant differences were observed between the U and H genotypes in egg hatch rates, longevity and fecundity. Comparing superinfected strains, egg hatch was higher in crosses of U-genotype strains relative to crosses of H-genotype strains (see Fig. 4). Higher egg hatch in U-genotype crosses was again observed in a comparison of uninfected strains. In similar comparisons examining fecundity, egg production was higher in the H-genotype, relative to the U-genotype (see Fig. 5). U-genotype females and males were longer lived relative to H-genotype individuals (see Fig. 6). The observed differences demonstrate the importance of uniform host genotype in comparisons of *Wolbachia* infection effects. However, *Wolbachia* effects on cytoplasmic incompatibility and host fitness are consistent regardless of host genotype.

The results presented here are important to the hypothesized origin of superinfection in *A. albopictus* via horizontal transmission. As described above, based on the observed fitness effects and CI pattern, a higher threshold infection frequency is predicted for the invasion of a *w*AlbA-infected population by the superinfected cytotype. Therefore, a horizontal transmission event that results in superinfection via the "mixing" of the *w*AlbB infection with the more primitive *w*AlbA infection (sequential population replacement hypothesis) must occur at a higher rate to establish the localized threshold infection frequency required for population replacement (Dobson et al. 2002; Frank 1998). Therefore, future experiments should include an examination of potential routes for horizontal transmission of *Wolbachia* infections in *A. albopictus*.

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FIGURE 1. Generalized crossing pattern illustrating unidirectional and bidirectional cytoplasmic incompatibility (-) and compatibility (+) that results in crosses of uninfected (O), single-infected (A or B), and superinfected (AB) individuals. Note that incompatibility is observed when the male host harbors an infection type that is not present in the host female mate.

FIGURE 2. Mean proportion egg hatch rate (± standard error) over time. Crosses are shown as female x male. Week number indicates time post eclosion. In A and B, the symbols are offset for clarity.

FIGURE 3. Comparison of (A) female longevity, (B) male longevity and (C) fecundity by *Wolbachia* infection type. Week number indicates time post eclosion.

FIGURE 4. Comparison of host genotype effects on mean proportion egg hatch rate (± standard error), comparing cross pairs of similar infection type. See Table 1 for genotype designation.

FIGURE 5. Fecundity comparison of cross pairs in which the host strains differ in genotype but are similarly (A) uninfected or (B) superinfected. Bars indicate standard error. Week number indicates time post eclosion.

FIGURE 6. Comparison of female and male longevity using cross pairs in which the host strains differ in genotype but are similarlysuperinfected or uninfected. Bars indicate standard error. Week number indicates time post eclosion.

FIGURE 7. Pattern of cytoplasmic incompatibility when co-infecting *Wolbachia* variants affect *mod* and *resc*. Incompatible crosses are illustrated as an "X".

TABLE 1. *Aedes albopictus* strains used in introgressions and experiments.

Strain Designation	Genotype	*Wolbachia* Type	Notes	Reference(s)
UjuT	U	uninfected	Artificially generated; aposymbiotic	(Otsuka and Takaoka 1997)
Koh	K	*w*AlbA	Naturally occurring; Field Collected	(Kambhampati et al. 1993; Sinkins et al. 1995a; Sinkins et al. 1995b)
Hou	H	*w*AlbA+ *w*AlbB	Naturally occurring; Field Collected	(Kambhampati et al. 1993; Sinkins et al. 1995a; Sinkins et al. 1995b)
HT1	H	uninfected	Artificially generated; aposymbiotic	(Dobson et al. 2002; Dobson and Rattanadechakul 2001)
IK7	U	*w*AlbA	Artificially generated; introgressed	this paper
IH7	U	*w*AlbA+ *w*AlbB	Artificially generated; introgressed	this paper

TABLE 2. Percent egg hatch resulting from crosses of introgressed strains (mean ± standard error). Superscripted letters indicate significant differences ($p<0.0001$)

	Male Type		
Female Type	IH7	IK7	UjuT
IH7	90.2±1.0^{A}	87.7±2.0^{A}	89.7±1.1^{A}
IK7	0.0±0.0^{C}	86.1±1.2^{A}	88.9±1.9^{A}
UjuT	0.0±0.0^{C}	0.2±0.04^{D}	73.6±3.9^{B}

Figure 1

	Male			
Female	O	A	B	AB
O	+	-	-	-
A	+	+	-	-
B	+	-	+	-
AB	+	+	+	+

Figure 2

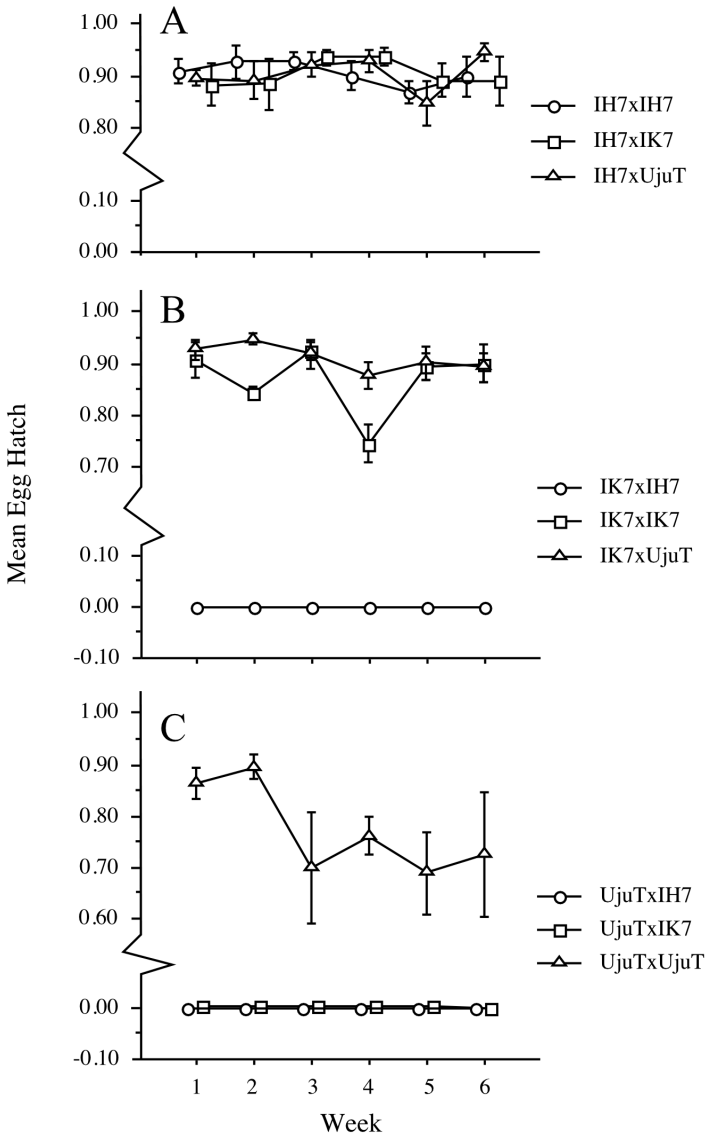

A
1.00
0.90
0.80
0.10
0.00
IH7xIH7
IH7xIK7
IH7xUjuT
B
1.00
0.90
0.80
0.70
0.10
0.00
-0.10
IK7xIH7
IK7xIK7
IK7xUjuT
C
1.00
0.90
0.80
0.70
0.60
0.00
-0.10
UjuTxIH7
UjuTxIK7
UjuTxUjuT
1
2
3
4
5
6
Week
Mean Egg Hatch

Figure 3

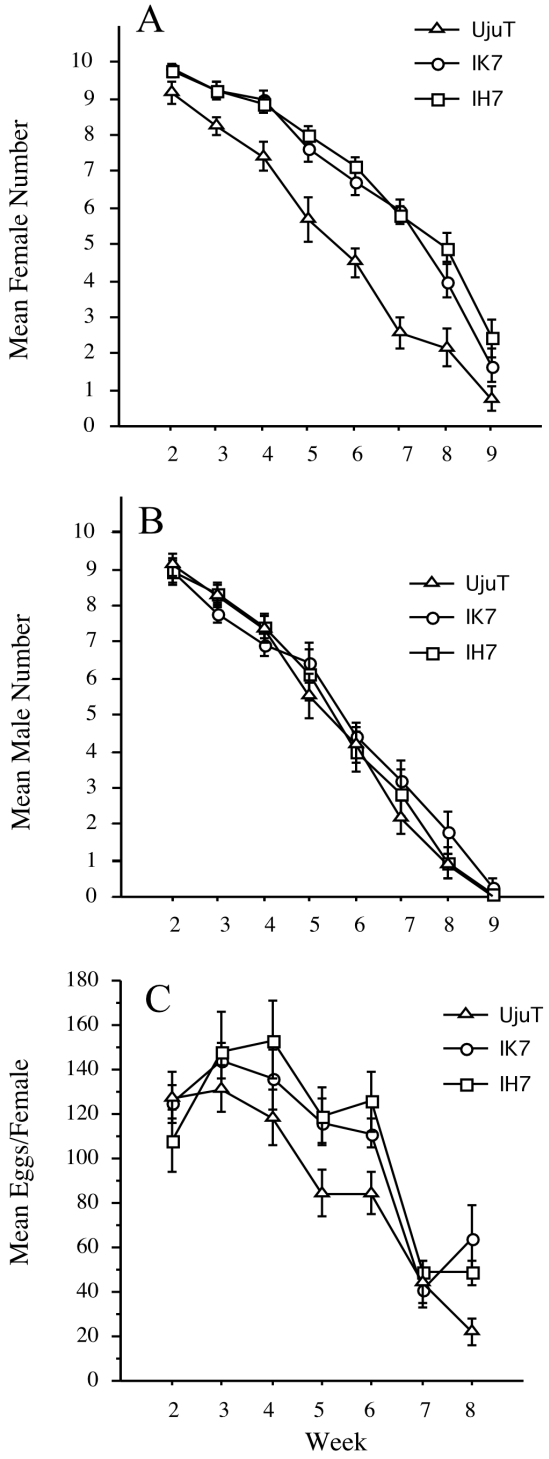

A
UjuT
IK7
IH7
Mean Female Number
B
Mean Male Number
C
Mean Eggs/Female
Week

Figure 4

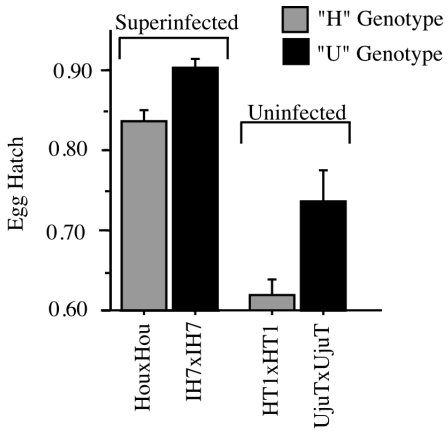

Superinfected
"H" Genotype
"U" Genotype
Uninfected
Egg Hatch
0.90
0.80
0.70
0.60
HouxHou
IH7xIH7
HT1xHT1
UjuTxUjuT

Figure 5

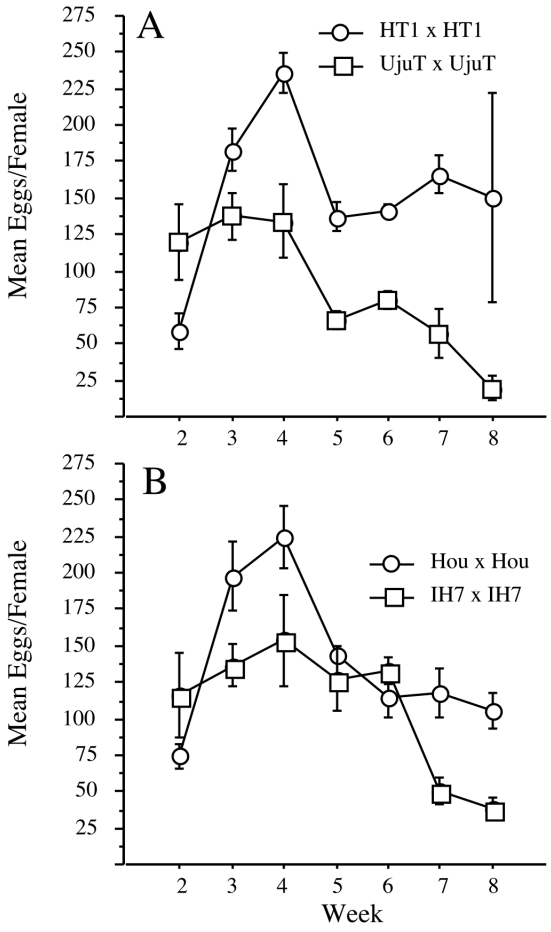

A
HT1 x HT1
UjuT x UjuT
Mean Eggs/Female
275
250
225
200
175
150
125
100
75
50
25
2
3
4
5
6
7
8
B
Hou x Hou
IH7 x IH7
Mean Eggs/Female
Week

Figure 6

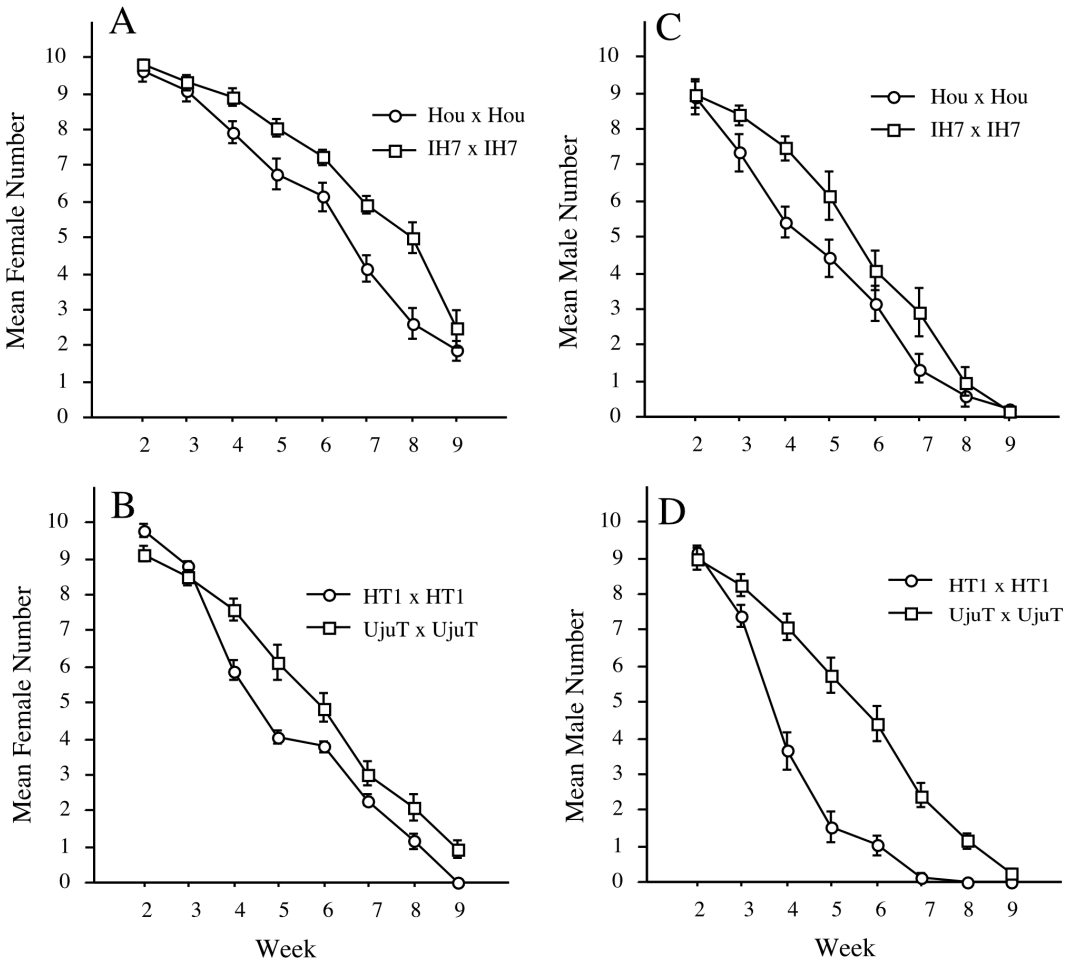
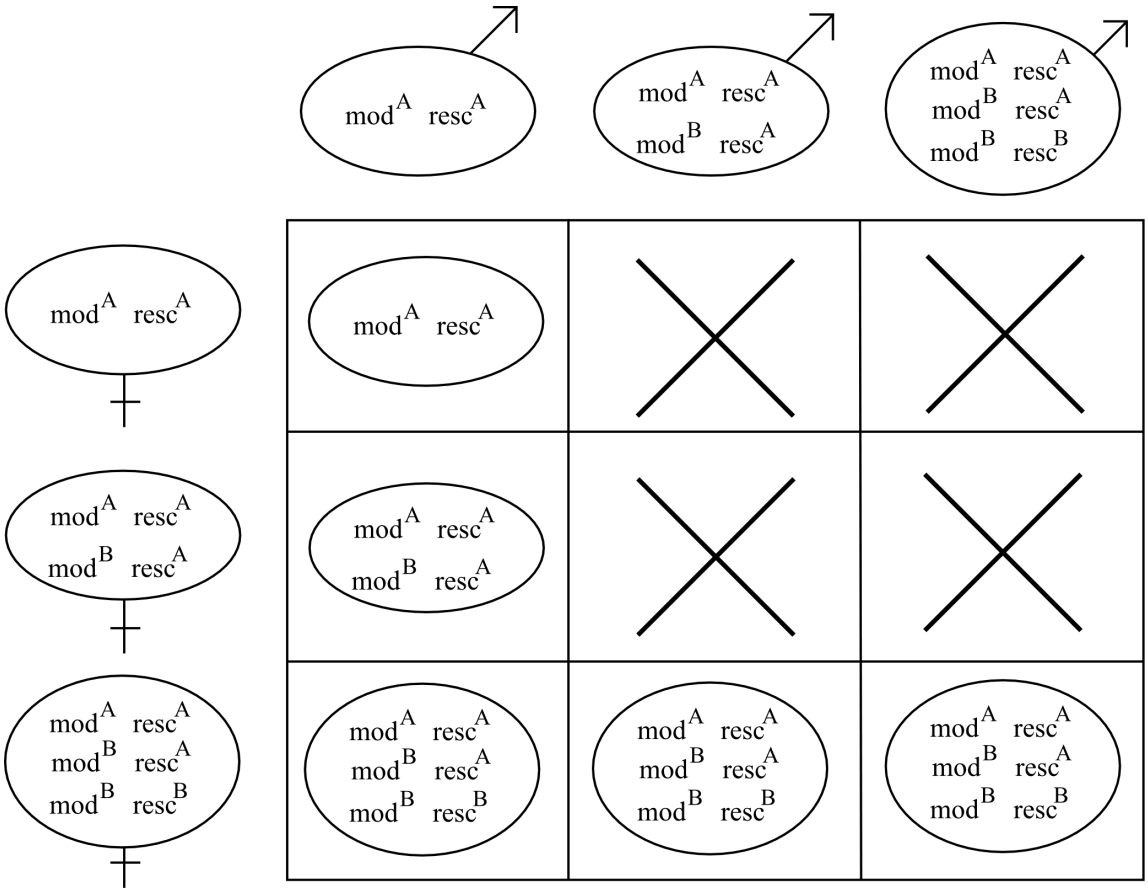

A
B
C
D
Mean Female Number
Mean Male Number
Week
Hou x Hou
IH7 x IH7
HT1 x HT1
UjuT x UjuT
0
1
2
3
4
5
6
7
8
9
10

Figure 7


mod^A resc^A
mod^A resc^A
mod^B resc^A
mod^A resc^A
mod^B resc^A
mod^B resc^B
mod^A resc^A
mod^A resc^A
mod^B resc^A
mod^A resc^A
mod^B resc^A
mod^A resc^A
mod^B resc^A
mod^B resc^B
mod^A resc^A
mod^B resc^A
mod^B resc^B
mod^A resc^A
mod^B resc^A
mod^B resc^B