Interspecific mating bias may drive *Aedes albopictus* displacement of *Aedes aegypti* during its range expansion

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**Abstract**

*Aedes albopictus* is the most invasive mosquito in the world and often displaces *Ae. aegypti* in regions where their populations overlap. Interspecific mating has been proposed as a possible cause for this displacement, but whether this applies across the range of their sympatry remains unclear. *Aedes albopictus* and *Ae. aegypti* collected from allopatric and sympatric areas in China were allowed to interact in cage experiments with different crosses and sex-choices. The results confirm that asymmetric interspecific mating occurs in these populations with matings between allopatric *Ae. albopictus* males and *Ae. aegypti* females being significantly higher (55.2%) than those between *Ae. aegypti* males and *Ae. albopictus* females (27.0%), and sympatric mosquitoes showed a similar but lower frequency bias, 25.7% versus 6.2%, respectively. The cross-mated females can mate second time (remate) with the respective conspecific males and the 66.7% remating success of female *Ae. albopictus* was significantly higher than the 9.3% of *Ae. aegypti* females. Furthermore, 17.8% of the matings of *Ae. albopictus* males exposed to mixed pools of *Ae. albopictus* and *Ae. aegypti* females and 9.3% of the matings of *Ae. aegypti* males with mixed *Ae. aegypti* and *Ae. albopictus* females were interspecific. The difference in the length of clasper between male *Ae. albopictus* (0.524 mm) and *Ae. aegypti* (0.409 mm) may be correlated with corresponding mates. We conclude that stronger *Ae. albopictus* male interspecific mating and more avid female intraspecific remating result in a satyr effect and contribute to competitive displacement of *Ae. aegypti* as allopatric *Ae. albopictus* invade during range expansion.

**Keywords:** *Aedes aegypti*, *Aedes albopictus*, interspecific mating, reproductive interference

**Significance Statement:**

*Aedes albopictus* and *Ae. aegypti* are highly invasive mosquitoes and important vectors of arboviruses that cause disease in humans, which make them major public health threats. Both species have sympatric and allopatric populations and appear to be in competition where they overlap with *Ae. albopictus* often displacing *Ae. aegypti*. We show that there is sex-based, asymmetric interspecific mating between the two species in China that is characterized by stronger interspecific mating of male *Ae. albopictus* and higher intraspecific remating by female *Ae. albopictus* that may drive *Ae. albopictus* displacement of *Ae. aegypti* during its range expansion. These findings confirm and extend previous studies on interspecific competition between the two species and could benefit novel mosquito control methods and prevent mosquito-borne diseases.

**Introduction**

*Aedes aegypti* is the primary vector of dengue, Zika, and yellow fever viruses and is distributed mainly in the global tropical regions (1–3). *Aedes albopictus* is the most important vector of chikungunya virus and also can transmit dengue viruses. Furthermore, it is the most invasive mosquito species worldwide and is distributed widely in tropical, subtropical, and temperate regions. It is now found in all continents except Antarctica (4–6). *Aedes* mosquitoes and their transmitted viral pathogens represent major threats to public health (7).

*Aedes aegypti* and *Ae. albopictus* share similar life cycles and ecological niches and often are found distributed sympatrically (8, 9). *Aedes aegypti* was found in the state of Florida in the United States, since 1880 (10). When *Ae. albopictus* invaded the state in the 1980s (11, 12), it gradually replaced *Ae. aegypti* as the local dominant species (13, 14), even causing total displacement in some regions (15–17). Similarly, *Ae. aegypti* used to be the predominant species during the 1980s in Hainan, China (18) and caused several dengue fever pandemics on the island and in the neighboring Leizhou peninsula, a joint area among Guangdong, Hainan, and...
Guangxi Provinces (19). At the time, Ae. albopictus was found rarely on the island. Several recent studies report that Ae. aegypti is now rare in the region and that Ae. albopictus is widespread (20). Both Ae. aegypti and Ae. albopictus have been on the Leizhou peninsula since the 1980s (21). Since then, the population and density of Ae. aegypti has been decreasing in this area and now only small numbers are found in the limited regions of Wushi town and Qishui town of Zhanjiang city, and there is a trend of Ae. albopictus completely replacing Ae. aegypti in the near future (22). Similar trends of species replacement also have been reported in the southeastern United States (23, 24) and Bermuda (25), where Ae. albopictus displaced Ae. aegypti with comparable rapidity. It is unclear how Ae. albopictus replaces Ae. aegypti although competition for breeding sites, and asymmetries in interspecific mating and the effects of male accessory gland proteins have been proposed (24, 26–28).

Satyrization is a form of mating interference in which males of one species mate with females of another species, produce no hybrid progeny, and significantly decrease the reproductive fitness of the species from which the females originate (27, 29, 30). Hybrids are asymmetric, with Ae. aegypti males being capable of satyrizing females of other species of the Stegomyia subgenus, potentially leading to competitive displacements, and possible extinctions, especially of endemic species on islands (27). However, the general dynamics of interspecific matings between Ae. albopictus and Ae. aegypti are not known.

Collections of Aedes albopictus and Ae. aegypti from allopatic and sympatric regions in China were used to investigate the dynamics of satyrization (Figure S1 and Table S1, Supplementary Material). Interspecific and intraspecific matings and rematings between Ae. albopictus and Ae. aegypti show a strong bias for favoring Ae. albopictus in regions where they might overlap with Ae. aegypti. These data confirm and extend what was seen with populations in Florida, USA (24, 31).

**Results**

**Asymmetric interspecific matings occur at different frequencies in laboratory- and field-derived Ae. albopictus and Ae. aegypti in China**

A total of two interspecific mating groups for both laboratory- and field-collected mosquitoes were examined. Intermating I consists of male Ae. albopictus crossed with female Ae. aegypti and Intermating II has male Ae. aegypti crossed with female Ae. albopictus. In total, two control mating groups also were done: Control I had male Ae. albopictus crossed with female Ae. albopictus, and Control II had male Ae. aegypti crossed with female Ae. aegypti. Interspecific mating was observed in both groups (Fig. 1; Table S2, Supplementary Material). The interspecific mating rate for the laboratory mosquitoes was significantly higher in group I (55.2 ± 2.2%) compared to group II (27.0 ± 3.3%; t = 7.15, d.f. = 4, and P = 0.0020). This asymmetry also was observed and significant in the sympatric field-collected mosquitoes, 25.7 ± 1.0% and 6.2 ± 1.3% for Intermatings I and II, respectively. (t = 12.07, d.f. = 4, and P = 0.0003) but was less frequent. These data support the conclusion that interspecific matings between Ae. albopictus and Ae. aegypti are asymmetric, with Ae. albopictus males having a much higher rate of successful intermating than Ae. aegypti males. Furthermore, the higher interspecific mating rates between allopatic laboratory populations and sympatric Guangdong field-derived populations (Student t tests, all P < 0.01) support the hypotheses that prolonged sympathy may lead to selection for more robust premating barriers between the two species, especially for Ae. aegypti (32).

**Interspecific-mated female mosquitoes do not produce viable offspring**

The overall oviposition rates were high for females in all mating groups (χ² = 6.51, d.f. = 5, and P = 0.0852), regardless of being unmated or mated interspecifically or intraspecifically (Table S3, Supplementary Material). However, the number of eggs per female varied among mating groups, and the egg-hatching rates among all cross-species-mated were zero, and consequently, no progeny were produced. The control intraspecific egg-hatching rates were 74.1% to 79.4% and these combined data support and confirm previous reports that interspecies matings in either direction do not result in viable progeny (Fig. 1) (24).

**Remating between interspecific mated females and conspecific males**

Remating experiments between previously interspecific mated females and conspecific males were carried out only for the laboratory-reared mosquito colonies. As expected, remating rates were low, 9.3 ± 1.0%, for Ae. aegypti for which it is known that the first mating inhibits subsequent mating (33, 34) (Fig. 1; Table S4, Supplementary Material). Aedes albopictus rematings were significantly higher, 66.7 ± 2.4%, (t = 11.24, d.f. = 4, and P = 0.0004) and the odds ratio of remating was 25.6 (95% CI: 7.3 to 90.1) for Ae. albopictus over Ae. aegypti. Similarly, the hatching rate of eggs produced by those previously cross-species mated females was significantly highly for Ae. albopictus (35.8 ± 4.2%) compared to Ae. aegypti (4.9 ± 1.8%; t = 6.78, d.f. = 4, and P = 0.0025). These data support the conclusion that cross-mated female Ae. albopictus and Ae. aegypti can remate with their conspecific males with the former being more likely to contribute to the subsequent generation. However, a basal difference in remating rates between the species could explain these observed differences (35).

**Male Ae. albopictus and Ae. aegypti mate differentially with conspecific and interspecific females in a competition assay**

The results of a series of two mating experiments (“male-choice”), Male-alb, comprising Ae. albopictus males crossed with mixed Ae. albopictus and Ae. aegypti females, and Male-aeg. Ae. aegypti males crossed with Ae. aegypti and Ae. albopictus females, revealed interspecific mating rates of 17.8 ± 0.1% and 9.3 ± 1.3% (t = 5.25, d.f. = 3, and P = 0.0135), respectively, for laboratory populations and 11.8 ± 0.6% and 2.0 ± 0.4% (t = 13.77, d.f. = 4, and P = 0.0002), respectively, for Guangdong field-derived populations (Fig. 2; Table S5, Supplementary Material). Control intraspecific mating rates were 100%. Male Ae. albopictus had 2- to 5-fold higher proportion of interspecific matings compared to male Ae. aegypti, and the allopatic populations (laboratory colonies) were higher than the sympatric populations (Guangdong field-derived). These findings support the conclusion that male mosquito choice is a significant factor in interspecific matings and is consistent with prolonged sympathy selecting for more robust premating barriers (31, 32).
Fig. 1. Interspecific matings and outcomes between *Ae. albopictus* and *Ae. aegypti*. (A) Intermate I: male *Ae. albopictus* × female *Ae. aegypti*, Intermate II: male *Ae. aegypti* × female *Ae. albopictus*. A total of 120 males and 120 females were exposed in a cage for 7 days. (B) Female spermathecae were dissected to determine their mating status. (C) Eggs and hatched larvae per female mosquito were recorded after interspecific exposure. (D) Interspecific mating and hatching rates between *Ae. albopictus* and *Ae. aegypti*. Lab: laboratory strain; Fgd: field Guangdong strain. (E) Eggs per female mosquito were detected for male specific *Nix* gene by PCR to confirm whether the mosquito had been interspecific mated or not. (F) Remate I: intermated female *Ae. aegypti* × male *Ae. aegypti*, Remate II: intermated female *Ae. albopictus* × male *Ae. albopictus*. “△” represents females that had previously mated interspecifically. In total, one interspecific mated female was exposed to one conspecific male in a cup for 5 days, then laid eggs and hatching situation from every female mosquito were recorded. (G) Remating and hatching rate between intermated females and conspecific males. The black mosquito icons represent *Ae. albopictus* and brown mosquito icon represents *Ae. aegypti*. Gray columns represent intermating or remating rate; black columns represent hatching rate. Bars represent the standard error of mean. Statistics were performed using Student t test. **P < 0.01 and ***P < 0.001. These assays were repeated three times.

Female-choice does not play a major role in interspecific matings between *Ae. albopictus* and *Ae. aegypti*

Similar experiments with females (“female choice”) Female-alb, in which *Ae. albopictus* females were crossed with *Ae. albopictus* and *Ae. aegypti* males, and Female-aeg, crosses of *Ae. aegypti* females with *Ae. aegypti* and *Ae. albopictus* males, showed similar intraspecific mating rates, ranging from 83.2 ± 5.2% to 93.9 ± 3.9% (Fig. 2; Table S6, Supplementary Material). Furthermore, the interspecific mating rates also were similar, ranging from 3.5 ± 2.5% to 8.5 ± 4.3% (Student t test, all P > 0.05). These results support the conclusion that female choice of either *Ae. albopictus* or *Ae. aegypti* does not play a major role as a premating barrier for subsequent interspecific mating.
Fig. 2. Choice-mating among *Ae. albopictus* and *Ae. aegypti*. (A) Male-choice I: 100 male *Ae. albopictus* × (100 female *Ae. albopictus* + 100 female *Ae. aegypti*), Male-choice II: 100 male *Ae. aegypti* × (100 female *Ae. aegypti* + 100 female *Ae. albopictus*). Those males and females were exposed in a cage for 7 days, then female spermathecae were dissected to determine their mating status. (B) Female-choice I: 100 female *Ae. albopictus* × (100 male *Ae. albopictus* + 100 male *Ae. aegypti*), Female-choice II: 100 female *Ae. aegypti* × (100 male *Ae. aegypti* + 100 male *Ae. albopictus*). Those females and males were exposed in a cage for 7 days, then female mosquitoes were used to detect male specific Nix gene by Nest PCR to identify whether the female had been intraspecific or interspecific mated. (C) Choice mating in field mosquitoes. Both *Ae. albopictus* and *Ae. aegypti* mosquitoes collected in the field were used to detect male specific Nix gene by Nest PCR to identify whether the female had been intraspecific or interspecific mated. (D) Male-choice mating rates between *Ae. albopictus* and *Ae. aegypti*. (E) Female-choice mating rates between *Ae. albopictus* and *Ae. aegypti*. (F) Intraspecific and interspecific mating rates of *Ae. albopictus* and *Ae. aegypti* collected from the fields. The black mosquito icons represent *Ae. albopictus* and brown mosquito icon represents *Ae. aegypti*. Lab: laboratory stain (green columns). Fgd: field Guangdong strain (Zhanjiang, red columns). Fyn: field Yunnan strain (Jinghong, blue columns). (D)–(E) Bars represent standard error of mean, and statistics were performed using Student t test. (F) Bars represent 95% CI, and statistics were performed using χ²-test or Fisher exact test. *P* < 0.05, **P < 0.01, ***P < 0.001, and ns = not significant. Male- and female-choice mating assays were repeated three times. The field mosquitoes were collected five times in Zhanjiang, Guangdong and Jinghong, Yunnan, respectively.

**Evidence of interspecific mating between *Ae. albopictus* and *Ae. aegypti* in field-derived mosquitoes**

Examination of field-collected adult females showed intraspecific mating rates ranging from 46.9 ± 3.6% to 57.5 ± 7.2% for *Ae. albopictus* and *Ae. aegypti*, respectively (χ² tests, all P > 0.05; Fig. 2; Table S7, Supplementary Material). In contrast, the interspecific mating rates between male *Ae. albopictus* and female *Ae. aegypti* or male *Ae. aegypti* and female *Ae. albopictus* were 4.3 ± 2.9% and 1.6 ± 0.9%, respectively, in Zhanjiang samples, and 9.1 ± 3.1% and 5.5 ± 1.6%, respectively, in Jinghong samples. Only the matings biases of *Ae. aegypti* males with *Ae. albopictus* females between Zhanjiang and Jinghong were significant (P < 0.05). Interestingly, *Ae. albopictus* and *Ae. aegypti* have been sympatric in Zhanjiang for more than 40 years (21) but less than 10 years in Jinghong (36), and these data are consistent with the hypothesis that prolonged sympatry selects for premating barriers between these two species (32, 37).

**Aedes albopictus** males have longer claspers than *Ae. aegypti* males

Morphological characteristics may be among the factors contributing to the observed differentials in interspecific matings between *Ae. albopictus* and *Ae. aegypti*. While female mosquitoes are generally larger than their conspecific males, the sizes and coloration of both male and female *Ae. albopictus* and *Ae. aegypti* are similar (Fig. 3; Table S8, Supplementary Material). The average weight, body length, wing length, and leg length in samples of 50 each male and female mosquitoes also are similar. In contrast, the average length of the clasper in male *Ae. albopictus* is 0.524 ± 0.003 mm and 0.518 ± 0.003 mm in laboratory and Zhanjiang field-derived mosquitoes, respectively, and these are significantly
Fig. 3. Morphological comparison and physical measurements of *Ae. albopictus* and *Ae. aegypti*. (A) Morphology and male clasper of *Ae. albopictus* and *Ae. aegypti*. (B) Weight of adult mosquitoes (mg). (C) Body length of adult mosquitoes (mm). (D) Wing length of adult mosquitoes (mm). (E) Leg length of adult mosquitoes (mm). (F) Clasper length of male mosquitoes (mm). Lab: laboratory strain. Fgd: field Guangdong strain. alb ♂: *Ae. albopictus* male (light gray); aeg ♂: *Ae. aegypti* male (light brown); alb ♀: *Ae. albopictus* female (dark gray); aeg ♀: *Ae. aegypti* female (dark brown). Bars represent standard error of mean. Statistics were performed using Student t test. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.0001, and ns = not significant. A total of 10 mosquitoes were assigned to each group. The measurements were repeated five times.

**Aedes albopictus** copulation bouts are of longer duration than those of **Ae. aegypti**

Video imaging of interspecific mating bouts of Intermate I: *Ae. albopictus* males with *Ae. aegypti* females revealed that the grasping episodes (n = 261; males attempting to engage females) of *Ae. albopictus* males with *Ae. aegypti* females were significantly lower than *Ae. aegypti* males against *Ae. albopictus* females (n = 2650), indicating that *Ae. aegypti* males were more active in pursuit of heterospecific females than male *Ae. albopictus* (Fig. 4; Table S9, Supplementary Material). The copulating attempts, successful copula, and copulate-fail rates were 101, 49, and 51.49%, respectively, in Intermate I, and were 1,365, 687, and 49.67%, respectively, in Intermate II. These data support the conclusion that the acceptances of both female mosquito species to the interspecific males are similar and the females do not play the dominant role in mate choice. Interestingly, the copulating duration and insemination rate in Intermate I was 14.78 ± 2.12 s and 17.54 ± 0.47%, which are significantly longer and higher than 6.54 ± 0.19 s and 7.66 ± 0.34% in Intermate II (Student t tests, all P < 0.01). The copulating duration in control I (*Ae. albopictus* males with *Ae. albopictus* females) was 20.60 ± 0.95 s, which also significant longer than 7.55 ± 0.32 s (Student t tests, P < 0.0001) in control II (*Ae. aegypti* males with *Ae. aegypti* females; Figure S3 and Table S9, Supplementary Material). These results are consistent with interspecific mating rates and coincident with male clasper lengths between *Ae. albopictus* and *Ae. aegypti*.

**Discussion**

The results of these experiments demonstrate that asymmetric interspecific matings can occur between *Ae. albopictus* and *Ae. aegypti* mosquito samples collected in China, and these findings are...
consistent with studies in southeastern United States (24, 38, 39) and Bermuda (25). The interspecific matings between *Ae. albopictus* males and *Ae. aegypti* females were significantly more frequent than the reciprocal matings of *Ae. aegypti* males with *Ae. albopictus* females. Interspecific matings also were more frequent in allopatric strains than in sympatric strains. Furthermore, females that had mated to interspecific males produced no progeny. This combination of results provide a basis for the observed gradual displacement of *Ae. aegypti* by *Ae. albopictus* in Zhanjiang. From these results, we conclude that *Ae. albopictus* suppresses the reproduction of *Ae. aegypti* by interspecific mating and this is an example of satyrization in species competition. These observations extend the conclusions of the previous findings to include large-scale arenas of invasion and displacement in China.

The insemination rate is typically used to assess male performance in mosquitoes (40–42), but the role of female choice and the degree to which it influences mating outcomes is not known (43, 44). The interspecific mating frequencies in male choice groups observed in this study were significantly different and *Ae. albopictus* males mated more frequently with *Ae. aegypti* females, even in the presence of conspecific females, than *Ae. aegypti* males did with *Ae. albopictus* females under similar choice conditions. In contrast, no significant differences were observed in the female choice experiments. These findings support the conclusion that interspecific matings between *Ae. albopictus* and *Ae. aegypti* is dominated by male behavior, and male *Ae. albopictus* engage in interspecific mating more frequently than male *Ae. aegypti*. While the evidence for female choice in mating bias is not evident here, it may arise as a premating barrier as the species experience longer periods of sympatry (32, 37). This asymmetric mating coupled with the infertility of interspecific-mated females would have a significant impact on species displacement.

Male mating behavior and morphology may provide a partial explanation for the asymmetrical interspecific mating bias. One correlated factor is that the average length of the clasper in *Ae. albopictus* males is significantly longer than that of *Ae. aegypti* males. The claspers are used to hold the female tightly during copulation and prevent easy disengagement during sperm transfer, and therefore, result in a more successful mating. Longer copulation times and increased insemination rates of *Ae. albopictus* male compared with *Ae. aegypti* were observed and detected in video experiments. We do not propose that these morphological differences alone account for the male-driven asymmetry in mating success, but they may have an additive effect in combination with other factors including sex pheromones (45) and wing-beat frequency (46, 47). Further studies are needed to clarify these and other potential mechanism for the asymmetric interspecific matings.

Female mosquitoes usually mate only once in their reproductive lifetime (48–50) because the sperm from the first mating can be stored in spermatheca and used throughout subsequent gonotrophic cycles (51). In addition, substances secreted by the accessory glands and passed to the female along with the sperm alter female mating behavior to prevent remating (33, 52). However, we showed here that *Ae. albopictus* or *Ae. aegypti* females previously experiencing an interspecific mating could remate with a conspecific male and produce viable offspring. It is notable that the remating and hatching rates of *Ae. albopictus* females were significantly higher than those of female *Ae. aegypti*. This is likely due to the differential effectiveness of male accessory gland protein suppression of remating in the interspecific crosses (24). The accessory protein HP-1 from the semen of *Ae. albopictus* could impose enforced monogamous paternity on *Ae. aegypti* females and inhibit secondary matings, but in contrast, secretions of *Ae. aegypti* males did not have the effect on female *Ae. albopictus* (53). Other factors correlated with the female activity in remating possibly exist in the semen of the male (33, 54) and need to be clarified with more study.

*Aedes albopictus* and *Ae. aegypti* share similar life cycle characteristics and ecological habits (8, 55, 56), as well as the similar size, body weight, and wing length. As the result, both mosquitoes also may have similar swarm behavior, wing beat frequencies, and other premating factors that make possible occasional interspecific mating. Because the reproductive cost of interspecific mating is high, no viable progeny, resistance to interspecific mating would be expected to be selected against during divergence of the
ancestral mosquitoes that give rise to these two species (32, 37). Consistent with this expectation, interspecific matings among field-derived Zhanjiang samples where Ae. albopictus and Ae. aegypti have been sympatric for at least 40 years were significantly lower than those samples where both mosquitoes have only been sympatric for less than 10 years. Similar observations have been reported for these two species in sympatric locales in Florida, USA (32, 37). It is possible that more prolonged close contact of both mosquitoes leads to stronger premating barriers, so that the errant interspecific matings could be avoided and decreased. This observed asymmetry also may account for the circumstances where Ae. albopictus as a species invading regions where Ae. aegypti is already extant leads to the suppression and elimination of the latter.

Conclusions
We can conclude from these studies that the asymmetric interspecific matings between Ae. albopictus and Ae. aegypti characterized by Ae. albopictus males exhibiting more frequent interspecific mating than Ae. aegypti males, and female Ae. albopictus having a greater frequency of conspecific remating is a general phenomenon in areas where these mosquitoes are sympatric. This results in a species competition known as satyrization in which Ae. albopictus can displace Ae. aegypti. This asymmetry is correlated with the length of clasper and female monogamy. These findings highlight some of the potential factors and mechanism of interspecific mating and species competition between Ae. albopictus and Ae. aegypti, two important vector and invasive species. Furthermore, the observed biases may complement and enhance the efficacy of sterile insect technologies (SIT) for impacting pathogen transmission dynamics in regions where the two species are sympatric and able to transmit the same pathogens.

Materials and Methods
Mosquito strains and rearing
Laboratory Ae. albopictus and Ae. aegypti strains have been colonized in our laboratory for many years. Field-derived colonies of Zhanjiang Ae. albopictus and Ae. aegypti were collected from artificial containers (discarded tires and buckets) in several places in Wushi Town, Zhanjiang City, Guangdong Province in 2019 to 2021, and these colonies used in the mating experiments were second to fourth generation (F2 to F4). All mosquitoes were reared at 27 ± 1°C, 70 ± 10% humidity and under 14 h light/10 h dark cycles. To obtain experimental mosquitoes, the larvae (200 larvae/1 water) were reared in stainless steel trays containing dechlorinated water and were provided daily with yeast and turtle food. Pupae of both species were collected individually and secured in 2 ml Eppendorf tubes with 1 ml water. When adults emerged, the species and sex of adults were determined by examination of the scutum and antennae, respectively. Adults of each sex and species were placed separately in paper bowls (9.5 × 6.7 × 6.2 cm³) with a mesh cover and offered a 10% sucrose solution on a cotton wick.

Noncompetitive (no-choice) interspecific mating experiments
Experiments were conducted in microcosms (20 × 20 × 30 cm³) using virgin males and females of each species, originating from the laboratory colonies and the field-derived colonies of Guangdong Province. All mosquitoes were between 2- and 3-old when used for mating experiments. A total of 120 males of one species was crossed with 120 unmated females of the other species in each enclosure (microcosm) using laboratory (lab) or field-derived mosquitoes. Mosquitoes were left to cohabit for 7 days. Conspecific microcosms containing males and females of either Ae. albopictus and Ae. aegypti were set up as normal mating controls. In addition, unmated females of each species were held alone in microcosms as egg-laying controls. All females were anesthetized with CO2 and spermathecae dissected to determine insemination status. The presence of sperm in spermathecae was recorded as an insemination event (Figure S2, Supplementary Material). Triplicate replicates were carried out for every cross combination.

Gene amplification (PCR) to detect the male-specific gene, Nix, in mated females
Nix is a male-specific gene present in both Ae. albopictus (57) and Ae. aegypti (58) and can be used to detect mated females. If female mosquitoes mated, the sperm of male mosquitoes would be transferred to the spermathecae of female mosquitoes. PCR assays were used to detect Nix in eggs to determine whether females had mated. Moreover, we established a sensitive and specific nested PCR assay to detect Nix in female mosquitoes to identify whether the female had been intraspecifically or interspecifically mated. The sequences of primers and program used in the study are given in Table S10 (Supplementary Material). AlbNix and AegNix were used to detect Nix in eggs as well as the first fragment in female mosquitoes, and Nest-AlbNix and Nest-AegNix were used to amplify the second fragment which is inside of the first fragment. For the first run, female genomic DNA was used for template. For the second run, the first run of PCR product was diluted 100 times as a template. Positive and negative controls were included in each first run experiment. Amplification products were observed under UV light after electrophoresis in 1% agarose gel containing and confirmed by DNA sequencing.

Remating experiment
The remating assays recapitulated the noncompetitive (no-choice) experiment described above. Females from the intercross experiments were offered a blood meal after a week of exposure to the interspecific males. Restrained mice were put in the cages, in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. Individual engorged females were transferred to a 250-ml paper cup with filter paper. Females were given 3 days to lay eggs. The pools of eggs were counted and the DNA extracted used as a template for gene amplification to detect the presence of Nix gene. Only mosquitoes positive for Nix-eggs were used for remating to ensure female had indeed mated with an interspecific male. These females then were transferred to a paper cup and mated for 5 days with conspecific males at a ratio of 1:1. Blood fed again. Every blood-fed female was transferred to a new paper cup with filter paper. Eggs collected from each female were counted and hatched to determine if a female deposited viable or nonviable eggs. Production of viable eggs was used as a proxy for successful intraspecific insemination. In the controls, 15-d-old virgin females were exposed to 3-d-old conspecific males for 5 days and subsequently allowed to blood feed. Each blood-fed female was allowed to oviposit. There were 10 to 20 females per replicate and three repetitions were performed.

Male-choice and female-choice mating experiment
A total of 100 males were aspirated into microcosms (20 × 20 × 30 cm³) containing 100 conspecific and 100 heterospecific females.
in the male-choice experiment. They were left to cohabit for 7 days. After that, the females were removed and morphologically identified as *Ae. albopictus* and *Ae. aegypti*. The spermatheca of female mosquitoes then were dissected and examined the sperm microscopically. A total of 100 females were aspirated into microwells containing 100 conspecific and heterospecific males for the female-choice experiments. After 7 days, females were removed. Each female mosquito was transferred into a 1.5-ml Eppendorf tube containing 50 μl of lysis buffer from MiniBEST Universal Genomic DNA Extraction Kit (Takara-Bio, Shiga, Japan). The mosquito was digested by proteinase K and RNase A overnight at 56°C according to the manufacturer’s instructions. Following DNA extraction, samples were stored at −20°C until PCR analysis.

**Detection of cross-mating in natural populations**

Adult mosquitoes were collected from randomly selected locales in two regions in Southern and Southwestern China, where *Ae. albopictus* and *Ae. aegypti* coexist to determine potential interspecific matings in natural populations. Human landing catches were carried out using power aspirators at two different cities in China. Wushi town (109°86′E, 20°56′N), located in the southwest of Leizhou Peninsula, is one of the main fishing ports in Guangdong Province. The other city, Jinghong (101°31′E, 22°36′N), is located in the southern part of Yunnan Province and adjacent to Myanmar. Both cities have larval habitats for *Ae. albopictus* and *Ae. aegypti*. Wild-caught females were identified morphologically as *Ae. albopictus* and *Ae. aegypti* and were stored in ethanol. They were transferred to a plastic plate and washed three times in deionized water to remove ethanol. In order to further determine which male mosquitoes the females mated with, Nix (*Ae. albopictus*, AlbNix and *Ae. aegypti*, and AegNix) diagnostic fragments were amplified from their genomic DNA using the previously described nested PCR system.

**Measurement of adult mosquito**

The weight, body length, wing length, and leg length of adult male and female *Ae. albopictus* and *Ae. aegypti* (laboratory strains), as well as the length of the male mosquito clasper (laboratory and field-derived Guangdong strains) were measured. Mosquitoes were placed in an oven to dry for 1 h, and their weight was measured in groups of 10 mosquitoes (repeated five times). Forceps and dissecting scissors were used to separate the legs, wings, and body of mosquitoes and the male mosquito claspers. These were examined microscopically and images recorded using a computer and camera. Image-pro Plus software was used to measure the length of the organs. Measurements of 50 mosquitoes of each species and sex were taken.

**Video observation of interspecific mating interactions**

Newly emerged 2- to 3-d-old mosquitoes of laboratory strains were used for mating behavioral video recording. A total of 50 males and 50 females were transferred into a custom video cage (7 × 11 × 12 cm³) and a digital camera (Logitech Capture, 1080P, 60fps) was used to record the mating process between *Ae. albopictus* and *Ae. aegypti*. The videos were recorded at intervals from ZT0-3 (3 h after light on) and ZT11-14 (3 h before light off) for five consecutive days. The number of grasp (male grasps female), copulation attempt (male rolls its abdomen to try copulate), copulation (male copulates with female successfully), copulation fail rate, and mean of copulate duration and insemination rate were observed and counted.

**Statistical analysis**

All statistical analyses were performed using SPSS version 20.0 (IBM, Chicago, IL). Mating rates, remating rates, hatching rates, mosquito eggs, body size, and male clasper length were compared using the Student t test (significant level of α = 0.05). χ²-test or Fisher exact test (if any n < 5) (significant level of α = 0.05) was used to compare the mating rate in wild-caught female mosquitoes. The odds ratio of remating success (after interspecific mating) was calculated for *Ae. albopictus* against *Ae. aegypti*.

\[
\text{Odds ratio} = \frac{\text{Observed} - \text{expected}}{\text{expected}} \times 100.
\]

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**Supplementary Material**

Supplementary material is available at *PNAS Nexus* online.

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**Authors’ Contributions**

X.-G.C., J.Z., and S.L. designed the research; J.Z., S.L., and H.L. performed the research; J.Z., S.L., and G.Z. analyzed the data; and X.-G.C., A.A.J., J.Z., and S.L. wrote the paper.

**Data Availability**

All relevant data are within the manuscript and its Supplementary Information files.

**References**

1. Kyle JL, Harris E. 2008. Global spread and persistence of dengue. Annu Rev Microbiol 62:71–92.
2. Marchette NJ, Garcia R, Rudnick A. 1969. Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. Am J Trop Med Hyg 183:411–415.
3. Auguste AJ, et al. 2010. Yellow fever virus maintenance in Trinidad and its dispersal throughout the Americas. J Virol 84:9967–9977.
4. Enserink M. 2008. Entomology: A mosquito goes global. Science 320:864–866.
5. Paupy C, et al. 2010. Comparative role of *Aedes* albopictus and *Aedes aegypti* in the emergence of Dengue and Chikungunya in central Africa. Vector Borne Zoonotic Dis 103:259–266.
6. Battaglia V, et al. 2016. The worldwide spread of the Tiger Mosquito as revealed by mitogenome haplogroup diversity. Front Genet 7:208.
7. Weetman D, et al. 2018. *Aedes* mosquitoes and *Aedes*-borne arboviruses in Africa: current and future threats. Int J Environ Res Public Health 15:220.
8. Hopperstad KA, Sallam MF, Reiskind MH. 2020. Estimations of fine-scale species distributions of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in eastern Florida. J Med Entomol 12:699–707.

9. Lounibos LP, Juliano SA. 2018. Where vectors collide: the importance of mechanisms shaping the realized niche for modeling ranges of invasive Aedes mosquitoes. Biol Invasions 20:1913–1929.

10. Tabachnick WJ. 1991. Evolutionary genetics and arthropod-borne disease: the yellow fever mosquito. Am Entomol 37:14–26.

11. Sprenger D, Wuithiranyagool T. 1986. The discovery and distribution of Aedes albopictus in Harris County, Texas. J Am Mosq Control Assoc. 2:217–219.

12. Lounibos LP, Bargielski I, Carrasquilla MC, Nishimura N. 2016. Coexistence of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in peninsular Florida two decades after competitive displacements. J Med Entomol 53:1385–1390.

13. Moore CG. 1999. Aedes albopictus in the United States: current status and prospects for further spread. J Am Mosq Control Assoc 15:221–227.

14. Fader JE. 2016. The importance of interspecific interactions on the present range of the invasive mosquito Aedes albopictus (Diptera: Culicidae) and persistence of resident container species in the United States. J Med Entomol 53:992–1001.

15. Hawley WA, Reiter P, Copeland RS, Pumpuni CB, Craig GB, Jr. 1987. Aedes albopictus in North America: probable introduction in used tires from northern Asia. Science 236:1114–1116.

16. O’Meara GF, Evans LF, Jr., Gettman AD, Cuda JP. 1995. Spread of Aedes albopictus and decline of Ae. aegypti (Diptera: Culicidae) in Florida. J Med Entomol 32:554–562.

17. Lounibos LP. 2007. Competitive displacement and reduction. J Am Mosq Control Assoc 23:276–282.

18. Ai C, et al. 1985. A longitudinal study of dengue virus infection in Ya county Hainan island, Guangdong province, during 1980-1983. Bull. Acad. Mil. Med. Sci.;(China) 5:503–508.

19. Xie H, Zhou H, Yang Y. 2011. Advances in the research on the primary dengue vector Aedes aegypti in China. Chin J Vector Biol Control 22:194–197.

20. Liu L, et al. 2004. Laboratory study on interspecific competition between Aedes albopictus and Aedes aegypti. Chin J Vector Biol Control 15:264–265.

21. Chen Z, et al. 2018. Investigation of distribution of Aedes aegypti and Aedes albopictus in Leizhou, Guangdong province. Chin J Vector Biol Control 29:46–49.

22. Chen Z, et al. 2020. An experimental study of interspecific competition between Aedes aegypti from Wushu town of Leizhou and Aedes albopictus from different places in Guangdong province, China. Chin J Vector Biol Control 31:486–489.

23. Nasci RS, Hare SG, Willis FS. 1989. Interspecific mating between Louisiana strains of Aedes albopictus and Aedes aegypti in the field and laboratory. J Am Mosq Control Assoc 5:416–421.

24. Tripet F, et al. 2011. Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. Am J Trop Med Hyg 85:265–270.

25. Kaplan L, Kendall D, Robertson D, Livdahl T, Khatchikian C. 2010. Aedes aegypti and Aedes albopictus in Bermuda: extinction, invasion, and extinction. Biol Invas 12:3277–3288.

26. Juliano SA. 1998. Species introduction and replacement among mosquitoes: interspecific resource competition or apparent competition? Ecology 79:255–268.

27. Bargielski IE, Lounibos LP. 2016. Satyrization and satyrization-resistance in competitive displacements of invasive mosquito species. Insect Science 23:162–174.

28. Groning J, Hochkirch A. 2008. Reproductive interference between animal species. Q Rev Biol 83, 257–282.

29. Ribeiro JMC, Spielman A. 1986. The satyr effect: a model predicting parapatry and species extinction. Am Nat 128:513–528.

30. Maiga H, Giles JRL, Lees R Susan, Yamada H, Bouyer J. 2020. Demonstration of resistance to satyrization behavior in Aedes aegypti from La Réunion island. Parasite. 27:22.

31. Irka B, Erik B, Lounibos LP. 2015. The effects of interspecific courtship on the mating success of Aedes aegypti and Aedes albopictus (Diptera: Culicidae) males. Ann Entomol Soc Am 108:513–518.

32. Bargielski IE, Lounibos LP, Carrasquilla MC. 2013. Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors. Proc Natl Acad Sci USA 110:2888–2892.

33. Helinski ME, Deewatthanawong P, Sirot LK, Wolfner MF, Harrington LC. 2012. Duration and dose-dependency of female sexual receptivity responses to seminal fluid proteins in Aedes albopictus and Ae. aegypti mosquitoes. J Insect Physiol 58:1307–1313.

34. Craig GB, Jr. 1967. Mosquitoes: female monogamy induced by male accessory gland substance. Science 156:1499–1501.

35. Boyer S, Toty C, Jacquet M, Lempérière G, Fontenille D. 2012. Evidence of multiple inseminations in the field in Aedes albopictus. PLoS ONE 7:e42040.

36. Liu QY, Guo YH. 2016. Coexistence of Aedes aegypti and Aedes albopictus in Jinghong City, Yunnan Province: a survey of Aedes aegypti invasion. J Trop Dis 04:1000227.

37. Bargielski I, Lounibos LP. 2014. Rapid evolution of reduced receptivity to interspecific mating in the dengue vector Aedes aegypti in response to satyrization by invasive Aedes albopictus. Evol Ecol 28:193–203.

38. Carrasquilla MC, Lounibos LP. 2015. Satyrization without evidence of successful insemination from interspecific mating between invasive mosquitoes. Biol Lett 11:20150527.

39. Bargielski IE, et al. 2015. Widespread evidence for interspecific mating between Aedes aegypti and Aedes albopictus (Diptera: Culicidae) in nature. Infect Genet Evol 36:456–461.

40. Ng’habi KR, et al. 2008. Sexual selection in mosquito swarms: may the best man lose?. Anim Behav 76:105–112.

41. Bellini R, et al. 2013. Mating competitiveness of Aedes albopictus radio-sterilized males in large enclosures exposed to natural conditions. J Med Entomol 50:94–102.

42. Lang BJ, et al. 2018. The effect of larval diet on adult survival, swarming activity and copulation success in male Aedes aegypti (Diptera: Culicidae). J Med Entomol 55:29–35.

43. Aldersley A, Cator LJ. 2019. Femaleresistanceand harmonicconvergence influence male mating success in Aedes aegypti. Sci Rep 9:2145.

44. Diabate A, et al. 2011. Spatial distribution and male mating success of Anopheles gambiae swarms. BMC Evol Biol 11:184.

45. Wang G, et al. 2021. Clock genes and environmental cues coordinate Anopheles pheromone synthesis, swarming, and mating. Science 371:411–415.

46. Cator LJ, Arthur BJ, Harrington LC, Hoy RR. 2009. Harmonic convergence in the love songs of the dengue vector mosquito. Science 323:1077–1079.

47. Cator LJ, Harrington LC. 2011. The harmonic convergence of fathers predicts the mating success of sons in Aedes aegypti. Anim Behav 82:627–633.
48. Tripet F, Toure YT, Dolo G, Lanzaro GC. 2003. Frequency of multiple inseminations in field-collected Anopheles gambiae females revealed by DNA analysis of transferred sperm. Am J Trop Med Hyg 68:1–5.

49. Thanh-Xuan-Nguyen J L Cabes. 1977. Sexual receptivity in female and insemination power in male of Culex pipiens autogenicus (Diptera, Culicidae): rigorous monogamy case and possible polygamy case. C R Acad Hebd Seances Acad Sci D 284:929–931.

50. Helinski ME, et al. 2012. Evidence of polyandry for Aedes aegypti in semifield enclosures. Am J Trop Med Hyg 86:635–641.

51. Ramírez-Sánchez LF, Camargo C, Avila FW. 2020. Male sexual history influences female fertility and re-mating incidence in the mosquito vector Aedes aegypti (Diptera: Culicidae). J Insect Physiol 121:104019.

52. Gwadz RW, Craig GB, Hickey WA. 1971. Female sexual behavior as the mechanism rendering Aedes aegypti refractory to insemination. Biol Bull 140:201–214.

53. Duvall LB, Basrur NS, Molina H, McMeniman CJ, Vossshall LB. 2017. A peptide signaling system that rapidly enforces paternity in the Aedes aegypti mosquito. Curr Biol 27:3734–3742 e3735.

54. Baldini F, Gabrieli P, Rogers DW, Catteruccia F. 2012. Function and composition of male accessory gland secretions in Anopheles gambiae: a comparison with other insect vectors of infectious diseases. Pathog Glob Health 106:82–93.

55. Gubler DJ, Bhattacharya NC. 1972. Swarming and mating of Aedes (S.) albopictus in nature. Mosq News 32:219–223.

56. Hartberg WK. 1971. Observations on the mating behaviour of Aedes aegypti in nature. Bull World Health Organ 45:847–850.

57. Liu P, et al. 2020. Nix is a male-determining factor in the Asian tiger mosquito Aedes albopictus. Insect Biochem Mol Biol 118:103311.

58. Hall AB, et al. 2015. SEX DETERMINATION. A male-determining factor in the mosquito Aedes aegypti. Science 348:1268–1270.