

## Supplementary Issue: Disease Vectors

## Sexual Harassment and Feeding Inhibition Between Two Invasive Dengue Vectors

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**ABSTRACT:** Two invasive mosquitoes, *Aedes aegypti* and *Aedes albopictus*, have been interacting during the course of a rapid range expansion by *A. albopictus*. We investigated the potential for interspecific feeding interference by male mosquitoes interacting with females within and between these species. *A. aegypti* feeding on both sugar and blood was suppressed when females of this species were exposed to *A. albopictus* males, but no change was observed when exposed to conspecifics. *A. albopictus* feeding was not affected by males of either species. The potential consequences of these behaviors are discussed within the context of other known interspecific effects, all of which appear to favor the displacement of *A. aegypti* by *A. albopictus*.

**KEYWORDS:** *Aedes aegypti*, *Aedes albopictus*, invasion ecology, satyrization, interference competition

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## Introduction

Increasing globalized trade and travel have contributed to the introduction of non-native species to the United States. Rapid transportation of products from one region to another provides the opportunity for organisms to hitch a ride and survive long enough to inhabit new ecosystems.<sup>1</sup> Invasive species threaten the sustainability of indigenous biota and can influence human health. Historical records demonstrate that disease outbreaks in North America have coincided with the introduction of invasive mosquito species, such as *Aedes aegypti*.<sup>2</sup> To anticipate potential disease outbreaks, it is important to understand the community composition of mosquito species and changes in community structure, such as the potential replacement of one species by another. These dynamics may also change the abundance of disease vectors, the diseases that can be transmitted locally, as well as the intensity of disease transmission.

The introduction of *A. aegypti* to the United States from Africa in the 16th century led to epidemics of yellow fever throughout much of North America,<sup>2–4</sup> and the now-globally invasive *A. aegypti* and *Aedes albopictus* are responsible for arbovirus disease outbreaks across most continents today.<sup>5,6</sup> Dengue virus, transmitted by both aforementioned vectors, is a major global concern, with an estimated total of 390 million cases worldwide, and 96 million symptomatic cases.<sup>7</sup> *A. albopictus* is purportedly responsible for outbreaks of Chikungunya in countries along the Indian Ocean<sup>8</sup> and southern Europe.<sup>9</sup>

*A. albopictus*, first seen in the USA in 1983<sup>10</sup> and rediscovered in 1985,<sup>11</sup> was introduced from Japan.<sup>12</sup> These species have coexisted in some areas,<sup>13,14</sup> perhaps due to microclimate factors favoring egg survival by *A. aegypti*<sup>15</sup>; however, *A. albopictus* has quickly replaced *A. aegypti* through most of the southeastern United States and Bermuda.<sup>14,16–19</sup> Both



*A. albopictus* and *A. aegypti* develop within natural and artificial containers. Larvae and pupae develop in small natural water bodies such as rock pools and tree holes.<sup>2,17,20–24</sup> In human populated areas, the mosquitoes also reproduce in man-made containers such as tires, bird baths, and other containers.<sup>5,14</sup> In these habitats, both invasive species may compete with native mosquitoes and with one another for resources.<sup>24–26</sup>

The bulk of research on the displacement of *A. aegypti* has focused on larval competition, wherein a majority of studies show that *A. albopictus* is a superior larval competitor to *A. aegypti*.<sup>24–28</sup> However, the speed of displacement of *A. aegypti* in areas such as Bermuda<sup>19</sup> has raised questions about other factors that could be involved in the displacement. Nasci et al.<sup>29</sup> proposed that mating interference could be another explanation for population displacement. In a field study, they found that *A. aegypti* females were more likely to be inseminated by *A. albopictus* males than *A. albopictus* females were to be inseminated by *A. aegypti* males. Other studies have shown that *A. aegypti* and *A. albopictus* males transfer male accessory gland (MAG) proteins to females during mating, which induces monogamy.<sup>30–33</sup> The efficient transfer of MAG proteins from *A. albopictus* males to *A. aegypti* females causes them to produce inviable eggs and to be refractory to future mating, but the same is not true in the reverse case, where *A. albopictus* females do not become refractory to future matings after exposure to *A. aegypti* MAG proteins.<sup>34</sup> Together with unequal levels of cross-insemination between the species, this transfer of proteins and resulting sterilization of *A. aegypti* females by *A. albopictus* males has been termed satyrization.<sup>34</sup> Triplet et al.<sup>34</sup> found evidence of potential satyrization when they observed cross-insemination in field populations of sympatric *A. aegypti* and *A. albopictus*, but the incidence of cross-insemination was quite low. Further research suggested that rapid selection on satyrization-resistant *A. aegypti* could result in populations of *A. aegypti* largely resistant to satyrization,<sup>35</sup> potentially explaining why current field populations of *A. aegypti* in areas of sympatry with *A. albopictus* have low rates of cross-insemination. Such a selective response to satyrization could result in rebounding *A. aegypti* populations, as has been observed recently in some areas.

Due to the close relationship between mating and blood-feeding, males can potentially obstruct females' feeding behavior as well as interfering with mating. Both *A. aegypti* and *A. albopictus* males swarm near blood hosts to increase the likelihood of finding a female mate.<sup>36–38</sup> Hartberg<sup>36</sup> reported that a majority of biting females in the field were inseminated on or near the host. As females approach the host, males intervene by grasping them, proceeding to mate in the vicinity. Incidentally, males may increase the risk of mortality by agitating defensive blood hosts while females attempt to blood-feed, or just deter female feeding entirely through harassment. If *A. albopictus* males are not only more likely to inseminate *A. aegypti* females but are also more likely to harass them, then

*A. aegypti* population displacement could be influenced not only by larval competition and satyrization, but also by sexual harassment. We have observed *A. albopictus* males approaching and attempting to mate with blood-seeking *A. aegypti* females as well as other species in field and laboratory situations. These observations, and the possibility that such behavior might deter feeding, prompted the investigations reported here.

We test the hypothesis that *A. albopictus* males have a greater influence on feeding behaviors of *A. aegypti* females than conspecific males using two separate experiments. These experiments test whether male harassment interferes with sugar feeding and blood feeding, and we compare the rates of interference for conspecific and heterospecific combinations.

## Method

**Mosquito rearing.** *A. albopictus* used in these studies originated from a stock provided by the Connecticut Agricultural Experiment Station, where they had been maintained in colony since 2006, for approximately 20 generations. *A. aegypti* stocks originated from field collections of *A. aegypti* from Edinburg, Texas, in 2011 and held in colony at Clark University since then, for approximately 10 generations. Mosquitoes were kept in an insectary at 24 °C, 70% relative humidity (RH), and a 16:8 (L:D) photoperiod throughout each morphological stage. Eggs were stimulated to hatch in a nutrient broth solution (Difco Laboratories®) containing 1 g nutrient broth/L distilled water. Larvae were transferred into plastic containers filled with distilled water and fed a diet of ground mouse chow and powdered yeast (Kal®). Pupae were removed every other day and placed into vials where emerged adults could be collected and sorted by sex. Larval density conditions varied, resulting in size variation within and between species. Once sorted, each sex and species was placed in a different 20 × 20 × 20 cm cage. Adults were provided with a 10% sucrose solution to feed *ad libitum* until used in an experiment.

**Experimental procedures.** *Sugar feeding in the presence of males.* To determine whether the presence of males had any effect on sugarfeeding of females, the females of *A. aegypti* or *A. albopictus* were placed with conspecific or heterospecific males, or no males at all, in cages containing a 10% sucrose solution, in a total of six experimental cages (45 × 45 × 45 cm). Because of a shortage in the *A. aegypti* female stock, experiments involving *A. aegypti* females were limited to 11 female and 11 male mosquitoes (regardless of species), while experiments involving *A. albopictus* females included 15 female and 15 male mosquitoes. All mosquitoes had emerged between 3 and 14 days prior to the start of the experiment. Females were deprived of sucrose solution for 24 hours before being placed in experimental cages. After 24 hours, female mosquitoes were removed from the cage, anaesthetized using CO<sub>2</sub>, and weighed in grams. The length of the left wing, from the alula to the end of the wing excluding the fringe scales, of each female was then measured in millimeters.



**Blood feeding in the presence of males.** Twelve experimental cages (45 × 45 × 45 cm) were established to test for blood-feeding interference. Using a mechanical aspirator, 25 males and 25 females were removed from source cages and relocated into experimental cages. Females of each species were placed with conspecific or heterospecific males, or no males at all, with each treatment condition replicated twice. Each treatment contained a 10% sucrose solution in a flask with a paper towel wick and a mouse restricted within a mouse rearing cage. Mice were obtained from a colony that had been maintained according to a protocol approved by the Clark University Animal Care and Use Committee and euthanized according to the same protocols following the experiment (IACUC No. 0094). The cages were stored indoor at approximately 20 °C with an ambient humidity. After 24 hours, all mosquitoes, live and dead, were collected; live mosquitoes were captured with a mechanical aspirator. The numbers of surviving males and females were recorded, and females were frozen and stored until further analysis. Females were inspected with a dissecting microscope to detect the presence of a blood meal in the abdomen.

**Statistical analyses.** All analyses were performed in R statistical software<sup>39</sup> using the package Deducer<sup>40</sup> and its dependencies, unless otherwise noted. All figures were plotted using the package ggplot2.<sup>41</sup> Data met assumptions of normality and equal variances, unless otherwise noted.

To analyze the success of females in sugar feeding in the presence of males, ANCOVA was performed with log-transformed female mass as a dependent variable, species and treatment as explanatory variables, and log-transformed wing length as a covariate. The log transformation of both variables was chosen to linearize the relationship between mass and wing length. We initially used interaction terms between species and treatment as well as between the covariate in the model, but when interaction terms were found to be non-significant, they were removed from the model. Tukey's Honestly Significant Difference (HSD) test was used to identify significant differences between treatment and species combinations, and the lsmeans package<sup>42</sup> was used to generate covariate-corrected means of female mass for each treatment.

A nominal logistic regression analysis was used to analyze the success of female bloodfeeding. The probability of successful bloodfeeding was modeled as a function of the two experimental factors (female species, male species) and the interaction between those factors.

## Results

**Sugar feeding in the presence of males.** Female species, male species, and the interaction between female and male species significantly influenced female mass (Table 1). A Tukey's HSD test showed that *A. aegypti* females weighed less in the presence of *A. albopictus* males than the controls or under conspecific treatments (Fig. 1). There were no

significant differences in female mass among any of the other treatments.

**Blood feeding in the presence of males.** Female species and the interaction between female and male species significantly influenced whether a blood meal was obtained (Table 2). The logistic regression indicates that both variables affected feeding behavior; however, it does not reveal which species was most affected. The lack of overlap of their 95% confidence intervals (Fig. 2) demonstrates that significantly fewer *A. aegypti* females fed in the presence of *A. albopictus* males.

## Discussion

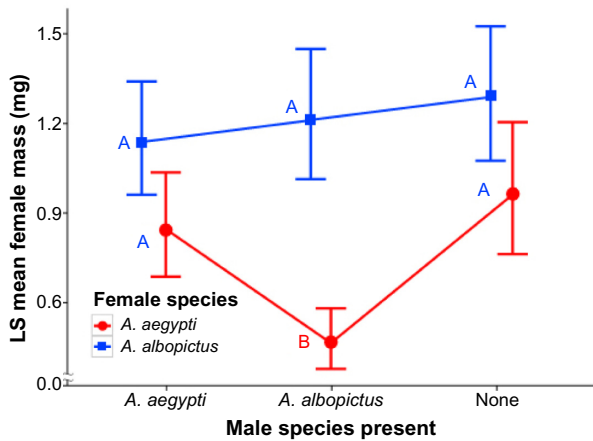
**Male interference with female sugar feeding.** After correcting for wing length differences, female *A. aegypti* weighed significantly less in the presence of *A. albopictus* than they did with no males or conspecific males, but *A. albopictus* females showed no difference in mass in any treatment. This difference indicates that *A. aegypti* feeding behavior was suppressed by males of *A. albopictus*, and that this feeding suppression is asymmetrical. Given that males and females require a carbohydrate source, it seems that nectar or other sugar sources could be the likely places for sexual interactions within and between species to occur. Our results demonstrate the possibility that *A. albopictus* males have a strong and negative effect on *A. aegypti* females when they encounter them in the field.

**Male interference with female blood feeding.** The feeding behavior of *A. aegypti* females was suppressed by *A. albopictus* males, but not by conspecific males, whereas the feeding behavior of *A. albopictus* females was not affected by the presence of males of either species. Perhaps, the insensitivity of *A. albopictus* to male activity is due to the more aggressive bloodfeeding behavior of *A. albopictus*.<sup>43</sup>

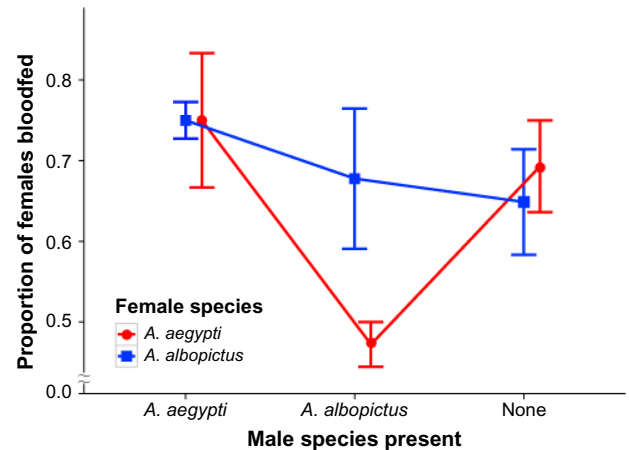
**Implications for population displacement.** One difficulty with the satyrization hypothesis for the displacement of *A. aegypti* by *A. albopictus* lies in the low rates of cross-insemination that have been reported for *A. aegypti* under field conditions. Tripet et al.<sup>34</sup> reported only a low frequency of this phenomenon (1.8%) in *A. aegypti* in Florida. However, the recent rate of cross-insemination may be a poor indicator of the rate experienced by *A. aegypti* early in the *A. albopictus* invasion. Bargielowski et al.<sup>35</sup> found that *A. aegypti*

**Table 1.** ANCOVA on effects of males on the mass of sugarfeeding females, accounting for wing length.

SOURCE	df	SUM OF SQUARES	F	P
Wing Length (Log)	1	0.82	42.11	<0.0001
Female Species	1	0.82	41.95	<0.0001
Male Species	2	0.28	7.29	<0.01
Female Species: Male Species	2	0.22	5.60	<0.01
Residuals	62	1.21		



**Figure 1.** Mean female mass after 24 hours in a cage with sugar solution and with no males, with males of one species, or the other. Means are those generated from the ANCOVA model of female mass by species and treatment, adjusting for female wing length, evaluated at the overall mean female wing length. Different letters indicate significant differences based on a Tukey’s HSD test. Confidence intervals are parametric 95% confidence intervals around the mean estimates from the ANCOVA model.



**Figure 2.** Mean proportions of females successfully attaining a blood meal after 24 hours in the presence of males or no males. Each point represents the mean of replicate cage proportions for each treatment combination. Bars represent bootstrapped mean 95% confidence intervals.

from populations in areas of sympatry with *A. albopictus* were less affected by cross-insemination than those from allopatric areas, suggesting that rapid recent selection for reduced susceptibility to the cross-insemination and the subsequent infertility has occurred. Although it may be true that satyrization was once much more important as it appears to be at present, this mechanism remains hypothetical in the absence of data on cross-insemination rates early in the invasion.

Our results suggest that the aggressive and non-selective behavior of *A. albopictus* males may have implications beyond satyrization. Sexual harassment of *A. aegypti* females during feeding, either for sugar or blood, can have consequences that could ultimately lead to reduced fecundity and population decline. Sugar feeding, although not essential to female reproduction, can greatly extend the life span of *A. albopictus* females.<sup>44</sup> *A. aegypti* appears similar to *A. albopictus* in this regard; females do not require sugar meals for survival, as long as they have access to blood meals, and sugar feeding can delay oviposition but prolong survival.<sup>45</sup> Styer et al showed that fitness is improved for females having access to sugar as well as blood meals.<sup>46</sup> Females denied access to a sugar meal may suffer mortality before they find another opportunity. A similar

risk lies in the quest for blood meal, which is essential for egg production in *A. aegypti*. Interruption in access to a blood meal may result in either delayed reproduction or female mortality. Either of these forms of harassment could outweigh the effects of satyrization, but in concert with satyrization, the influence of *A. albopictus* male aggression may account for much of the collapse of *A. aegypti*.

**A web of potential interactions.** By showing the potential impact of sexual harassment by *A. albopictus* males on the feeding in *A. aegypti* females, our results add to an increasingly complex set of potential interactions between *A. aegypti* and *A. albopictus*. This topic has received considerable attention, with several potential mechanisms that may have contributed to the outcome. Each of these proposed mechanisms appears to work in favor of *A. albopictus*, each is difficult to test under natural conditions, and difficulties are associated with each mechanism when taken separately.

**Competition.** Interspecific competition for resources is most likely to occur between mosquito species at the larval stage. Experiments have addressed this mechanism for displacement, with results that lack clear consistency. Juliano<sup>27</sup> conducted experiments within enclosures in tire water and was able to detect competitive effects between species, suggesting that *A. albopictus* had an advantage. Braks et al.<sup>28</sup> conducted a comparable experiment in Brazil, with similar results. However, neither of these experiments could be used to calculate competition coefficients needed to conclusively demonstrate competitive exclusion potential by *A. albopictus*. Results of another field experiment in tire habitats<sup>19</sup> were amenable to competition coefficient calculation, but no significant competitive effects were found between species. Additional skepticism about competition stems from the unexpected speed with which displacement of *A. aegypti* occurred: in Bermuda, the displacement was complete within only 5 years.

**Table 2.** Nominal logistic regression summary for the likelihood of female bloodfeeding as a function of female species and the interaction between female and male species.

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SOURCE	df	L-R Chi-SQUARE	P
Female Species	1	11.74	<0.001
Female Species*Male species	2	8.33	<0.05
Male species	2	3.30	0.19



While competition may have been a contributing factor to displacement, we suspect that additional interactions are necessary for a full understanding.

**Egg hatch inhibition.** The suppression of egg hatch by larvae has been demonstrated in field experiments using *Ochlerotatus triseriatus* in tree holes.<sup>22</sup> Laboratory studies have been extended to the community level by examining interspecific effects among *A. albopictus*, *A. aegypti*, and *O. triseriatus*.<sup>23</sup> These results also appear to favor *A. albopictus*, which showed an ability to hatch in the presence of high densities of larvae, regardless of the larval species present, whereas egg hatching in *A. aegypti* was completely suppressed at high larval density. Although it is possible to show that this mechanism can occur under field conditions, its importance remains untested at the population level, and larval densities may not typically reach the levels necessary to confer a consistent advantage to *A. albopictus*.

**Disease introduction.** A parasite could have been introduced with *A. albopictus*, which could have had more adverse effects on *A. aegypti* than on its native host species.<sup>47</sup> This idea has not been tested extensively, although Juliano<sup>27</sup> did examine larvae of both species and did not find evidence for infection of *A. aegypti* by the gregarine parasite of *A. albopictus*, *Ascogregarina taiwanensis*.

**Higher order effects.** We expect that two or more of the factors described above could interact to produce effects stronger than the additive effects of each operating in isolation. We also anticipate that any of these factors could interact with climate to produce more severe effects. Where the displacement has been particularly pronounced and rapid, eg, in the southeastern USA and Bermuda, *A. aegypti* was able to succeed in the absence of other domestic container *Aedes* species, but those latitudes are on the edge of the ecological range for this primarily tropical species. *A. albopictus*, which was introduced from temperate Asia, may be better adapted for success in subtropical to temperate climates.

**Harassment and satyrization distinguished from competition, and evolutionary considerations.** Although it may seem that the distinction between the mating-related feeding inhibition and reproductive interference constitute only a pair of extended forms of competition, it is important to note that these interactions are likely to be frequency dependent. As the density of *A. albopictus* increased, the likelihood of encounters with *A. aegypti* should also have increased. The per capita likelihood of these encounters should also have increased as *A. aegypti* became more rare, so the two means of interference could have induced the extinction of *A. aegypti* more rapidly than one might expect from resource competition. Thus, satyrization and harassment could act in a manner similar to a sterile insect technique<sup>48</sup>, due to the increased intensity of these interactions as one species declines.

Ideally, tests for interspecific mating could be done as an invasion of *A. albopictus* spreads across an area. This may not be feasible within the USA, as the range of *A. albopictus* appears to have stabilized, although it could still be done in

some isolated localities occupied by *A. aegypti* that have not yet been reached by *A. albopictus*. A greater challenge lies in testing for the importance of sexual harassment by *A. albopictus* on the feeding behavior of *A. aegypti*.

Bargielowski et al.<sup>35</sup> reported differences in *A. aegypti* susceptibility to heterospecific mating that correspond to whether *A. aegypti* were drawn from populations sympatric or allopatric to *A. albopictus*, suggesting that *A. aegypti* has rapidly evolved resistance to satyrization. If such resistance is at the biochemical level associated with response to the male accessory gland protein, it may not confer resistance to sexual harassment in the form of feeding interference. On the other hand, if *A. aegypti* have evolved reduced attractiveness to *A. albopictus* males, perhaps through modified wing beat frequency, then such evolution could also reduce feeding interference. The comparisons of wing beat frequency of sympatric and allopatric populations of *A. aegypti* may therefore be of great interest. If such resistance to satyrization and/or feeding interference is possible, we may anticipate that *A. aegypti* populations could eventually rebound.

**Public health implications of the *A. albopictus* range expansion.** The global threat of introduction of *A. albopictus* to public health has been discussed extensively.<sup>6,7,9,49–52</sup> This is a highly successful species, with the ability to transmit viruses causing serious diseases including Dengue and Chikungunya. Because *A. albopictus* is not as efficient a vector as *A. aegypti* for the transmission of Dengue, and not yet known to transmit yellow fever virus,<sup>9,52</sup> the displacement of *A. aegypti* populations may be seen as a welcome change from a human disease perspective. However, this change is only of potential benefit in areas occupied by *A. aegypti*, and *A. albopictus* has occupied many localities with climates much colder than that *A. aegypti* can tolerate.<sup>49</sup> Chikungunya virus, formerly carried primarily by *A. aegypti*, has already adapted to capitalize on *A. albopictus* as a vector,<sup>53</sup> and recent epidemics (eg, 266,000 cases in Reunion) and spread of Chikungunya virus to new areas is traceable to *A. albopictus* invasions as well as human transport.<sup>8</sup> Since 2007, 231 cases of Chikungunya have been reported in 31 states of the USA, and three recent cases in Florida are not travel related and appear to have been transmitted by local mosquitoes.<sup>54</sup> Many domestic container habitats of these states are now occupied primarily by *A. albopictus* rather than *A. aegypti*, and this indicates the potential for the spread of exotic viruses via this introduced vector.

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## Author Contributions

Conceived and designed the experiments: JS, AS, KG, TL. Analyzed the data: JS, AS, KG, TL. Wrote the first draft of the manuscript: JS, AS, KG, RK, TL. Contributed to the



writing of the manuscript: JS, AS, KG, RK, TL. Agree with manuscript results and conclusions: JS, AS, KG, RK, TL. Jointly developed the structure and arguments for the paper: JS, TL. Made critical revisions and approved final version: JS, TL. All authors reviewed and approved of the final manuscript.

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