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Rapid evolution of reduced receptivity to interspecific mating in the dengue vector *Aedes aegypti* in response to satyrization by invasive *Aedes albopictus*

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Abstract

In this paper we examine the effect of reproductive interference on the dynamics of two mosquito vectors of public health concern and add to the growing literature on the strength and speed with which interspecific reproductive interference may drive evolution. Recent evidence supports a role for asymmetric reproductive interference, or satyrization, in competitive displacements of *Aedes aegypti* by *Aedes albopictus*. However, populations of *A. aegypti* sympatric with *A. albopictus* in nature evolve resistance to satyrization. Here we report that *A. aegypti* from Tucson, Arizona (USA), where *A. albopictus* are not known to occur, are satyrization-susceptible. Furthermore, in cage experiments we demonstrate rapid evolution in satyrization-susceptible lines. Exposing allopatric strains of *A. aegypti* to *A. albopictus* in cages led to significant reductions, within 1–3 generations, in the frequency of reproductive interference. We also demonstrate that satyrization-resistant *A. aegypti* females derived from selection experiments are significantly slower to mate with conspecific males, suggesting a cost for the evolution of satyrization-resistance. Results show how interspecific interactions between these vector species are rapidly evolving, with implications for the arboviral diseases, especially dengue and chikungunya, which they transmit.

Keywords

Aedes; satyrization; rapid evolution; interspecific mating

Introduction

The ecological importance of reproductive interference is becoming clear as growing attention is given to its role as a driving force in the evolution of behaviour and distribution patterns. Caused by incomplete mate recognition (Reitz and Trumble 2002; Dame and Petren 2006) and ranging from signal jamming to hybridization and satyrization, evidence of the dramatic consequences of these interactions is mounting. The most frequently cited consequences of reproductive interference include the displacement of native species by invasive congeners (e.g. refs in Noriyuki et al 2012). Recent work, however, highlights the possibility of more subtle outcomes of reproductive interference between species, ranging from niche differentiation in cryptic butterfly species (Friberg et al 2013) to prey specialization in ladybirds (Noriyuki et al 2012).

In this paper we examine the effect of reproductive interference on the dynamics of two mosquito vectors of public health concern and add to the growing literature indicating the strength and speed with which interspecific reproductive interference may drive evolution.

Following its broad diaspora during the last three decades, the Asian tiger mosquito *Aedes albopictus* has established in new continents and countries where its invaded distribution often overlaps that of the yellow fever mosquito *A. aegypti*, which was broadly dispersed from Africa centuries earlier. These two species are considered the most invasive mosquitoes in history (Juliano and Lounibos 2005) and are the most important vectors of the epidemic forms of dengue and chikungunya viruses to humans (Kyle and Harris 2008; Paupy et al 2010). Their distribution and abundance therefore have profound epidemiological implications, and understanding and predicting the competitive interactions that influence these patterns in nature is important. The spread of *A. albopictus*, for instance, into regions occupied by *A. aegypti* has coincided with rapid declines in resident *A. aegypti* populations. One example of such competitive displacement between these two vector species was the rapid reduction in range and abundance of *A. aegypti* (Hobbs et al 1991; O'Meara et al 1995) following the invasion and spread of *A. albopictus* throughout most of the southeastern USA in the 1980s (Hawley et al 1987, Craig 1993). Although various mechanisms, including larval resource competition (Juliano 1998; Juliano and Lounibos 2005), greater reproductive efficiency in *A. albopictus* (Klowden and Chambers 1992) and apparent competition mediated by the intestinal gregarine protozoan *Ascogregarina taiwanensis* (Juliano and Lounibos 2005) were suggested to contribute to the competitive success of *A. albopictus*, a more powerful mechanism may be asymmetric reproductive interference, or satyriization, between *A. aegypti* and *A. albopictus* (Nasci et al 1989; Lounibos 2007; Bargielowski et al 2013).

Though reproductive barriers between *A. aegypti* and *A. albopictus* have long been recognized (Leahy and Craig 1965), interspecific mating nonetheless occurs in the field (Tripet et al 2011). The similar life histories and mating behaviour of these two species may increase the likelihood of interspecific mating when they first come in contact. Both species of the subgenus *Stegomyia* aggregate at vertebrate hosts during similar diurnal peak activity periods (Hartberg 1971; Gubler and Bhattachaya 1972) and initiate mating in flight by following visual and auditory cues (Roth 1948; Cator et al 2009). Interspecific mating in this species pair does not lead to viable offspring production or hybridization and is less deleterious to *A. albopictus* as heterospecific male accessory gland products render *A. aegypti* but not *A. albopictus* females refractory to further insemination by conspecific males (Tripet et al 2011). The mistake of mating with an *A. albopictus* male is, therefore, extremely costly for *A. aegypti* females and has been shown to lead to reproductive character displacement in affected populations (Bargielowski et al 2013). Female *A. aegypti* from populations that have been exposed to the selective pressure of satyriization in the field in central Florida show a lower frequency of interspecific matings than do females from allopatric populations (Bargielowski et al 2013) from Miami and the Florida Keys. This suggests that selection to avoid heterospecific mating is high enough for the evolution of prezygotic mating avoidance in *A. aegypti* females. It is conceivable that the evolution of resistance to satyriization may allow *A. aegypti* to re-invade areas from which they had been excluded by *A. albopictus*, in particular, if climate change favors the more desiccation-tolerant *A. aegypti* (Juliano et al 2002; Lounibos et al 2010).

In this paper we demonstrate that *A. aegypti* allopatric to *A. albopictus* in Tucson, Arizona (USA) are satyriization-susceptible and we further investigate two aspects of satyriization between *A. albopictus* and *A. aegypti* in more detail. Firstly, we look at the speed with which satyriization pressure (i.e. the presence of *A. albopictus* males) can alter female (*A. aegypti*) mating behaviour in selection experiments and secondly, we provide evidence that the

changes in female mating behaviour induced by heterospecific exposure affect intraspecific mating behaviour in *A. aegypti*. In particular, we show that a reduced propensity to engage in interspecific mating is linked with reduced intraspecific mating rates and thus, that the evolution of mechanisms in female *A. aegypti* to counteract satyriation may have a cost for mating behaviour towards conspecifics. The success of an *A. aegypti* resurgence may therefore hinge, in part, on the relative cost of resistance.

Methods

Laboratory and rearing conditions

Three lines of *A. aegypti* from populations allopatric to *A. albopictus* in the USA (inner city Miami (FL), Key West (FL) and Tucson (AZ)) and one line of *A. albopictus* from a population allopatric to *A. aegypti* in the USA (St. Louis (IL)) were established in the laboratory following collections of aquatic immatures from artificial containers such as cemetery vases and discarded tires. Adults used in the experiments were second generation (F₂), except for the allopatric strain of *A. albopictus* which was F₅. Second generation mosquitoes were used to minimize non-genetic maternal effects (Mousseau 2000). Experiments were carried out in screened, plastic Bug Dorm cages (30cm × 30cm × 30cm) in an insectary maintained at 27 (± 0.62)°C and 89 (± 5.28)% RH under a 14L:10D photoperiod. Larvae were reared from hatch to pupation in pans containing 1l of tap water (100 larvae per pan) and provided 0.6g of a 1:1 brewer's yeast / lactalbumin mix on day one. Pupae were sexed according to morphological differences in their external genitalia and segregated by species and sex in small containers (10–20 per container) for emergence. If a mistake during sexing was detected after emergence, the container was discarded. All adult mosquitoes were provided continuous access to 10% sugar solution and were 3–4 days old when used in experiments.

Interspecific mating assays for Tucson line

To assess the susceptibility of female *A. aegypti* of the Tucson strain to satyriation by *A. albopictus*, three cages containing 150 virgin females and 150 virgin males were established. Following three weeks of exposure the surviving females were removed, dissected and their spermathecae examined for the presence of sperm. The reciprocal crosses between *A. albopictus* females and *A. aegypti* males were also performed. This experiment is analogous to experiments carried out to assess the susceptibility of female *A. aegypti* from Key West (KW) and Miami in our previous report (Bargielowski et al 2013).

To make these studies logistically tractable, throughout this paper, the assessment of susceptibility of females to satyriation is measured by examining insemination rates in non-choice experiments, i.e. *A. aegypti* females are caged with only *A. albopictus* males (or vice versa). This experimental design does not allow for estimates of natural cross-insemination rates - in fact we predict these to be much lower in the field - rather it is the relative changes in insemination frequencies that are informative.

Development of satyriation resistant lines and interspecific mating assays

In each generation and for each line of *A. aegypti* (Miami, Key West, Tucson) three 'cross cages' were set up to assess the frequency of interspecific mating, while one 'stock cage' was set up to produce the next generation. Cross cages contained 120 virgin *A. aegypti* females and 120 virgin *A. albopictus* males. Following three weeks of exposure the females were removed, dissected and their spermathecae examined for the presence of sperm. The stock cages contained 200 *A. aegypti* females and 100 *A. aegypti* males (from the same line) as well as 100 *A. albopictus* males. Females were provided heated (37°C) bovine blood in a sausage membrane casing one week after cage exposures and suitable egg-laying substrate

two days following their blood meal. The eggs were removed and stored in humid containers for at least a week prior to hatching the next generation. Following this procedure, crosses were carried out and stock cages maintained through 5–6 generations. To ensure that any changes in the frequency of interspecific mating detected in the two *A. aegypti* lines were not merely artifacts of colonization, control cages containing only *A. aegypti* (200 females $\times$ 100 males) were cycled through a further four generations (F_6) before cross cages were set up to assess the frequency of interspecific mating as described above.

Intraspecific mating assays

To examine whether the evolution of resistance to satyriation affects intraspecific mating behaviour we compared the receptivity of females from two of the laboratory selected satyriation resistant lines (above), KW and Tucson, with their allopatric counterparts (original lines). Cages were set up containing either 15 male and 15 female *A. aegypti* from the original lines (KW or Tucson) or 15 males and females each from the selected lines, following 6 generations of exposure to *A. albopictus* (KWG6, TucsonG6). Following 15 minutes of exposure, the females were removed from the cages, dissected and examined for the presence of sperm. Seven repeats of each treatment were conducted in the KW line and six in the Tucson line.

Statistical analysis

Data were analysed with JMP (version 8) (<http://www.jmpdiscovery.com>). To compare insemination frequencies between *A. aegypti* (Tucson, AZ) and *A. albopictus* females, we arcsine transformed the proportions of females inseminated and performed Student's t-test. The transformed interspecific insemination rates of the three allopatric lines of *A. aegypti* (Tucson, KW and Miami) were compared by ANOVA. In our data set describing the development of satyriation resistance in the various lines, comparisons between lines and generations were assessed with a nominal logistic model, with line, generation and line $\times$ generation as factors. Heteroscedasticity was assessed using Bartlett's test for homogeneity of variances. In our data set comparing intra- and interspecific mate acceptance, we arcsine transformed the proportions of females inseminated before performing Student's t-test.

Results

Interspecific mating assays for Tucson line

A significantly higher mean proportion ($35.97 \pm 1.82\%$) of *A. aegypti* females was inseminated by *A. albopictus* males than in the reverse cross (*A. albopictus* females $\times$ *A. aegypti* males) ($8.60 \pm 2.12\%$) ($t_{(4)} = -9.75$, $p < 0.001$). Mean interspecific insemination rates of the three allopatric lines of *A. aegypti*, Tucson ($35.97 \pm 1.82\%$), KW ($54.13 \pm 7.56\%$) and Miami ($35.63 \pm 6.81\%$), were not significantly different ($F = 3.22$, $df = 2$, $p = 0.11$). Three-week survivorship of females was generally high (Tucson: $73.56 \pm 4.94\%$, KW: $76.17 \pm 2.20\%$, Miami: $77.47 \pm 3.49\%$, St. Louis: $73.78 \pm 4.9\%$) and did not vary significantly among the lines ($F = 0.40$, $df = 3$, $p = 0.75$). The data describing insemination rates of the KW and Miami lines are taken from our earlier report (Bargielowski et al 2013). Though not conducted simultaneously, the experiments had analogous design.

Development of satyriation resistant lines and interspecific mating assays

Exposure to *A. albopictus* males over multiple generations significantly lowered the frequency of interspecific mating by *A. aegypti* females of all three lines ($\chi^2_{(4)} = 88.51$, $p < 0.001$) (Fig 1). The average cross-insemination rate dropped from 54.13% to 14.68% (over five generations) and to 10.68% (over six generations) in the KW line, from 35.63% to 11.81% (over five generations) in the Miami line and from 35.97% to 9.43% (over five

generations) in the Tucson line. There were no overall differences in cross-insemination rates among lines ('line effect': KW, Miami, Tucson) ($\chi^2_{(2)} = 4.34, p = 0.11$). However, the interaction between 'line' and 'generation' was significant ($\chi^2_{(10)} = 35.21, p < 0.001$). The decrease in interspecific mating frequency across six generations was accompanied by a decrease in variance of the trait in two of the lines (Bartlett's test KW: $F = 2.74, df = 5, p = 0.02$, Miami: $F = 3.19, df = 5, p < 0.01$). In the KW line the variance around the mean decreased from $\pm 13.09\%$ to $\pm 1.94\%$ over five generations and similarly in the Miami line from $\pm 11.80\%$ to $\pm 1.98\%$. The variance did not change significantly over time in the Tucson line ($F = 1.13, df = 5, p = 0.34$).

Intraspecific mating assays

Results showed a significant decrease in the proportion of females inseminated following 15 minutes of exposure to conspecific males in both the KWG6 line of *A. aegypti* ($32.38 \pm 5.33\%$) compared with the original allopatric KW line ($61.90 \pm 3.48\%$) ($t_{(12)} = 4.79, p < 0.001$) as well as in the TUCG6 line of *A. aegypti* ($81.11 \pm 3.18\%$) compared with the original allopatric Tucson line ($54.44 \pm 4.69\%$) ($t_{(10)} = -4.92, p < 0.001$).

Discussion

Evolution of satyrization resistance in *A. aegypti*

Recent work (Bargielowski et al 2013) provided evidence for the evolution of satyrization resistance in nature. Female *A. aegypti* from populations that have been in contact with *A. albopictus* showed decreased interspecific mating rates compared to females from populations allopatric to *A. albopictus* (Bargielowski et al 2013). Here we demonstrate that an additional allopatric line of *A. aegypti*, from Tucson, Arizona, is similarly susceptible to satyrization by *A. albopictus*, compared to previously tested lines (KW and Miami (Bargielowski et al 2013)). Furthermore, as in earlier work (Bargielowski et al 2013; Nasci et al 1989), we show that *A. aegypti* females are significantly more susceptible than *A. albopictus* females and confirm the asymmetric nature of bidirectional mating in this species pair.

As our measures of interspecific mating rates are derived from non-choice experiments, i.e. *A. aegypti* females were exposed to only *A. albopictus* males, we certainly expect cross insemination rates to be far lower in the field. In fact, a previous study (Tripet et al 2011) estimated around 1–3% heterospecific mating at two sites in Florida. However, the relative differences in insemination frequencies in cage tests are still informative and the fact that the sympatric populations, mentioned above (Bargielowski et al 2013), had been in contact for at most two decades, suggests satyrization to be a strong force for rapid evolutionary change in this system (Bargielowski et al 2013).

Here, our selection experiments, showing rapid decreases in the frequency of interspecific mating after only a few generations in cages, confirm these findings. Females of all three (KW, Miami and Tucson) lines of *A. aegypti* became increasingly less likely to engage in interspecific mating following several generations of exposure to satyrization. Furthermore, in two of the lines (KW and Miami) there was a significant decrease in the variance around the mean number of females inseminated over consecutive generations of exposure to *A. albopictus*. This rapid reduction in variance is further indicative of strong selection acting upon this trait. Though the three lines generally responded similarly (with a reduction in interspecific mating), the overall amplitude and pattern of their response differed. Prior to exposure to *A. albopictus*, female *A. aegypti* from Key West showed higher frequencies of interspecific mating and subsequently a greater decrease in interspecific mating than females from Miami and Tucson. In addition to this, in the KW line, the changes in mating

behaviour became apparent from the first generation following exposure, while in the Miami and Tucson lines we observed a lag of two generations.

A possible explanation for these differences may be found in the histories of these three lines. While we can be relatively confident that the KW line was established from a truly allopatric population of *A. aegypti*, it is conceivable that the populations in Miami and Tucson may have had limited contact with *A. albopictus* in the past. Although no *A. albopictus* specimens have been collected from the Miami site over the last 16 years, collection records indicate *A. albopictus* was present at low numbers during several sampling visits in 1995–96 (Lounibos et al 2010). Therefore, the Miami line may already have evolved some resistance to satyriation in the field prior to sampling. The interspecific mating frequencies observed in the Tucson line of *A. aegypti* were very similar to initial insemination rates in the Miami line (35.97% vs. 35.63%), which may also be explained by possible prior exposure to *A. albopictus*. *A. aegypti* was first recorded in Tucson in 1946 (Bequeart 1946; Murphey 1953), but disappeared in 1969 (Engelthaler et al 1997; Merrill et al 2005). In 1994, *A. aegypti* reemerged (Engelthaler et al 1997; Merrill et al 2005) in Tucson and the following year in Nogales (Engelthaler et al 1997) and the southern Arizonan towns of Naco, Douglas and Benson (Fink et al 1998). Despite regular arbovirus surveillance, no records of *A. albopictus* exist for this region, so it is likely that the *A. aegypti* populations that have become established there have had little contact with *A. albopictus* since their reintroduction. However, genetic data suggest that the *A. aegypti* population that now exists in Tucson was most likely introduced from Mexico (Merrill et al 2005; Black et al 2002), where *A. albopictus* is indeed prevalent (Ibáñez-Bernal et al 1995; Villegas-Trejo et al 2010) and where satyriation resistance could have evolved in the field. Finally, it is conceivable that differences in the genetic backgrounds of the lines may play a role in their ability to evolve resistance to satyriation. In a recent paper, Damal et al (2013) show that even between our two Florida lines (KW and Miami) gene flow may be low.

Changes in intraspecific mating behaviour associated with satyriation resistance

Selecting, in the laboratory, for *A. aegypti* populations that are significantly less prone to satyriation by *A. albopictus* than when first invaded, allows the examination of questions regarding the evolution of behavioural changes associated with mate discrimination and acceptance.

Females in populations exposed to satyriation pressure face the added burden of species recognition when assessing potential mates. Theory predicts that selection should favor divergent phenotypes in allopatric and sympatric populations that minimize heterospecific mating (Brown and Wilson 1956; MacArthur and Levins 1964; Grant 1972; Slatkin 1980; Coyne and Orr 2004; Pfennig and Pfennig 2005). Furthermore, studies show that the evolution of character displacement in sympatric populations can lead to phenotypes that face fitness trade-offs. On the one hand the post-divergent phenotype will benefit from reduced interference for mates, but on the other, may suffer costs in other fitness components, such as, for example, settling for low quality males to ensure conspecific mating (Pfennig 2000). Our results show that *A. aegypti* females from populations that have been exposed to satyriation by *A. albopictus* for six consecutive generations, and have evolved notable resistance, are not only less likely to engage in interspecific mating, but are also slower to mate with conspecific males than their counterparts from allopatric populations. This increased latency in mate acceptance, seen in both the KW and Tucson line, is possibly the result of increased investment in mate assessment and is an indicator that the evolution of resistance to satyriation requires significant modification of mating behaviour.

Furthermore, as proposed by Carracado et al. (1989) for *Drosophila*, this relationship between interspecific mating (reluctance to accept heterospecific males) and intraspecific mating (slower to accept conspecific males), may suggest a common genetic basis for this behaviour. When reduced choosiness (for heterospecifics) was selected for in *Drosophila*, a reduced time to copulation with conspecifics was also found (Pineiro et al 1993). However, whether this modification is costly to *A. aegypti*, i.e. whether sympatric populations of *A. aegypti* carry a fitness burden compared with allopatric populations remains unknown. To answer this question we need to learn more about the mating cues that dictate female choice, which males females are ultimately selecting, and whether these choices have an impact on population growth. Questions as to whether resistance to satyriation would persist in the absence of satyriation pressure or whether this trait could spread into allopatric populations, for example, depend on the relative cost of expression.

As discussed by Kokko and Mappes (2005), most experiments on female choice, our experiment being no exception, are conducted in a situation in which females have simultaneous choice over several males, as opposed to the situation in the wild, where mate choice is more commonly sequential. While it is conceivable that simultaneous mate assessment is a possibility in the field for this *Aedes* species pair owing to their mating habits (swarming at hosts), the numbers of individuals would certainly be far lower than in our caged design (during line selection / exposure to satyriation by *A. albopictus*). These conditions may therefore artificially foster the selection of choosiness amongst females. With the availability of suitable mates not being a limiting factor, reserving time to assess the “best” mate may be beneficial – a “luxury” not obviously afforded in the field. That being said, in this particular scenario, where females have the option of accidentally choosing a heterospecific mate (a reproductive dead end), the pressure for females to develop “wallflower-tendencies” (Kokko and Mappes 2005; deJong and Sabelis 1991), i.e. become increasingly choosy and reluctant to mate, is far greater than in a system where females need to distinguish only between low and high quality conspecifics.

Finally, though we mainly discuss female mate choice, the contribution of male selection cannot be distinguished in this experimental design. The reason we have chosen not to discuss it at length in this paper, is that our previous findings (Bargielowski et al 2013) as well as more recent work in our laboratory, indicate that female effects largely outweigh male effects in this system. This is most likely do to the asymmetric nature of mating interspecifically – males do not lose future mating opportunities, while females (*A. aegypti*) do. However, it has been shown that sperm production is limited in *A. aegypti* and that courting is costly to males (Bargielowski et al 2011). We, therefore, hope to identify the exact contribution of both sexes in more detail in the future.

Implications for species distributions and disease transmission

With the growing consensus that ecological and evolutionary changes can occur on the same time scale (reviewed in Pelletier et al 2009; Ezard et al 2009), recognizing the ways in which the two processes can feed into one another is the next step. Understanding such eco-evolutionary dynamics is becoming increasingly important in the face of changing environments (Burger and Lynch 1995; Gomulkiewicz and Holt 1995; Orr and Unckless 2008), particularly with regard to rapid human-driven change (e.g. species introductions). Here we show that the rapid directional evolution in the form of development of resistance to satyriation, has the potential to continually reshape community structure. As *A. aegypti* and *A. albopictus* are both vector species of significant public health concern, predicting outcomes of interactions between the two species that will affect their distribution and abundance, and by extension, the risk of arbovirus transmission, is important in its own right, however, it further provides a system in which evolutionary processes can feed back

into ecological, or more specifically, epidemiological outcomes. When populations first come in contact, satyrization may suppress *A. aegypti*, while the evolution of resistance to satyrization may allow for recovery. However, if the evolution of resistance to satyrization comes at a cost, resurgences in *A. aegypti* populations may not be stable, as the trait may decline or disappear if satyrization pressure eased. Resulting fluctuations in the relative abundances of the two species could on the one hand contribute to the patchy distribution patterns of these two species often observed where they co-occur around the world (Craig 1993; Benedict et al 2007; Higa et al 2010; Raharimalala et al 2012; Bargielowski et al. 2013). On the other hand fluctuations in vector prevalence could also negatively impact disease transmission. For example, with regard to dengue fever, *A. albopictus* is considered less of a public health threat than *A. aegypti* (Lambrechts et al 2010), so a predominance of *A. albopictus* may lower transmission rates, in turn lowering existing herd immunity. In this case, reinvasion by *A. aegypti*, considered the primary outbreak vector of epidemics of severe dengue fever (Lambrechts et al 2010), could easily cause a resurgence of disease (Bargielowski et al 2013). It may, therefore, be worthwhile for vector ecologists to consider the implications of emerging eco-evolutionary theory in the light of disease transmission cycles.

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Figure 1.

Evolution of resistance in *A. aegypti* females to satyrization by *A. albopictus* males. Solid lines show selected KW (a), Miami (b) and Tucson (c) strains, while dashed lines present controls from the same locations that were not exposed to *A. albopictus*.