Evidence of Limited Polyandry in a Natural Population of *Aedes aegypti*

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Abstract. The mosquito *Aedes aegypti* is a vector of yellow fever, dengue, and chikungunya. Control of the insect is crucial to stop the spread of dengue and chikungunya, so it is critically important to understand its mating behavior. Primarily, based on laboratory behavior, it has long been assumed that *Ae. aegypti* females mate once in their lifetime. However, multiple inseminations have been observed in semi-field and laboratory settings, and in closely related species. Here, we report the first evidence of polyandry in a natural population of *Ae. aegypti*. Female *Ae. aegypti* were captured around the New Orleans, LA, metropolitan area. They were offered a blood meal and allowed to lay eggs, which were reared to the third-instar larval stage. A parentage analysis using four microsatellite loci was performed. Out of 48 families, 3 showed evidence of multiple paternity. An expanded analysis of these three families found that one family group included offspring contributed by three fathers, and the other two included offspring from two fathers. This result establishes that polyandry can occur in a small proportion of *Ae. aegypti* females in a natural setting. This could complicate future genetic control efforts and has implications for sampling for population genetics.

INTRODUCTION

The yellow fever mosquito *Aedes aegypti* is also a vector of the viruses causing dengue fever and chikungunya. There are currently no approved effective vaccines for these latter diseases, so control of the vector is critical to restrain their spread. Promising forms of genetic control of *Ae. aegypti* are proposed using sterilized or genetically modified males being released to mate with native females.^{1–3} Understanding the details of the mating behavior of this vector is critical, both in terms of understanding the population genetics of the insect and increasing the effectiveness of potential genetic control programs.

One aspect of *Ae. aegypti* mating that remains unclear is the occurrence of polyandry in wild populations. Polyandry is defined here as a female producing offspring from multiple males, and not simply the copulation or insemination of a single female with multiple males. Polyandry is a frequent feature of insect mating systems,⁴ but is much less commonly observed in mosquitoes that are generally viewed as monandrous.⁵ A meta-analysis of experimental studies on the fitness effects of multiple mating in insects found that female fitness is increased by polyandry, because of increased egg production and fertility.⁴ The lack of polyandry in mosquitoes is thus both rare and difficult to explain from an evolutionary perspective.

Monandry in *Ae. aegypti* was established by early laboratory studies of mating behavior. Evidence shows that mating renders the female refractory to further insemination, and triggers postmating behaviors, such as faster digestion of the blood meal⁶ and increased oviposition.⁷ Further couplings with an already mated female can occur, but tend not to result in insemination.⁸

This resistance to further insemination is due to a substance passed to the female in the seminal fluid during mating.⁹ Transplantation experiments showed that an unknown factor in the male accessory glands is responsible, as implanting male accessory glands, but not other tissues, caused

virgin females to resist insemination attempts.⁹ Although this substance, originally known as “matrone,” is believed to be a protein, it has yet to be fully characterized.^{10–12} More recent work has systematically identified proteins transferred to the female through insemination in *Ae. aegypti*¹³ and *Ae. albopictus*.¹⁴ One or, likely, several of these proteins act on the female to cause mating refractoriness and other postmating behaviors. Other mosquito species, such as *Anopheles gambiae* and *An. stephensi*, and the closely related Asian tiger mosquito *Ae. albopictus* are also rendered monogamous by transfer of a male accessory gland factor during mating.^{9,15} On the basis of these and other findings, it was assumed that this mating-triggered resistance to further insemination meant monandry was standard in wild mosquito populations.

Although monandry is considered the norm in wild populations, polyandry has been observed in laboratory settings but these reflect highly artificial circumstances that cannot be easily extrapolated to wild populations.^{16,17} *Ae. aegypti* lay eggs in semipermanent water sources and mate in proximity to human hosts.⁵ The laboratory environment places reproductive adults in much more dense conditions than would be observed in the wild. Studies of polyandry in natural populations are difficult because they require the capture of mated females, and the availability of genetic or other markers to distinguish offspring from multiple males. Still, some such studies have been attempted. *An. gambiae* and *An. freeborni* displayed evidence of multiple insemination of wild females, at low frequencies.^{18,19} A parentage analysis of wild-caught *Ae. albopictus* using microsatellites found evidence of polyandry in a population on the island of La Reunion in 26% of families examined.²⁰ Helinski and others released radiolabeled *Ae. aegypti* males alongside females in semi-field enclosures.²¹ They found approximately 14% of the females mated with multiple males, though this experiment did not determine how many females laid eggs fertilized by multiple males.²¹ These studies suggest polyandry is possible in the wild even if monandry is the dominant mating system found in mosquitoes.

To test if polyandry occurs in natural populations of *Ae. aegypti*, we performed a parentage analysis of the offspring of 48 female *Ae. aegypti*. Female mosquitoes were captured

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in the New Orleans, LA, area, blood fed and allowed to lay eggs. Eggs were hatched and offspring reared to the third-instar larval stage. The mother and twelve offspring were then genotyped at four to twelve microsatellite loci, and the minimum number of fathers required to explain the offspring's genotype was determined. Because the females were captured after mating, these results reflect the prevalence of polyandry in a natural population.

MATERIALS AND METHODS

Mosquito collections and rearing of larvae. Female *Ae. aegypti* were collected from sites in the New Orleans metropolitan area, chosen based on the historic abundance of *Ae. aegypti* relative to other mosquito species in the area. Mosquitoes were collected using human landing collections. Collections were performed during daylight hours, mainly at dawn and dusk. Adult female *Ae. aegypti* were collected using inhaling-type aspirators fitted with high-efficiency particulate arrestance filters. The sex of each mosquito was visually confirmed before being placed in a temporary field cage. Male mosquitoes were immediately killed or dispersed away from the collection site to avoid mating in the confined cages. Individual female mosquitoes were aspirated into 8 × 8 × 8 in. collapsible metal cages (Bioquip, Rancho Dominguez, CA) after being identified as *Ae. aegypti*.²² Mosquitoes were collected, in 2012 to establish baseline genotypic frequencies, and in 2014 to perform the parentage analysis.

Females were provided with blood and those successfully fed were given a small, black plastic cup lined with seed germination paper (an ovipositing substrate) and partially filled with distilled water. Collected eggs were allowed to dry in a humidified chamber for 1 week. The eggs were then hatched and larvae were reared using standardized laboratory protocols. Larvae were grown to the third or fourth instar, dipped briefly in boiling water, and stored in 70% ethanol.

Microsatellite analysis. DNA was extracted from adults and larval offspring using the Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany). The 12 microsatellite loci used in this study are the same as those used by Brown and others.²³ These microsatellites were designed to be multiplexed in three sets of four. In each set, two polymerase chain reaction (PCR) reactions, each amplifying two nonoverlapping markers are pooled together for fragment analysis. The multiplex PCR protocol, including thermocycling conditions was performed as described by Brown and others.²³ Microsatellite alleles were measured by a 3730xl DNA Analyzer, and data scored using GeneMapper software (Applied Biosystems, Waltham, MA). χ^2 tests were used to compare genotypic frequencies between the population collected in 2012 and 2014. These tests were performed in R, using the `chisq.test` function, with the simulate *P* value set to TRUE.²⁴ This setting used Monte Carlo simulations to estimate *P* values. Tests for Hardy–Weinberg equilibrium were performed using Genepop on the web, version 4.2, with *P* values estimated by a Markov chain algorithm with the following parameters: dememorization 10,000; batches 200; and number of iterations per batch 10,000.^{25,26} The significance levels were adjusted for multiple comparisons using the sequential Bonferroni correction method.²⁷

Parentage analysis. Adult female mosquitoes were initially genotyped at all 12 microsatellite loci. Exclusion probabilities for each multiplexed set of four microsatellite loci were cal-

culated using GERRUD.^{28–30} The set of four multiplexed loci that yielded the highest joint exclusion probability was chosen for the initial parental analysis. GERRUDSim2 was used to simulate families with multiple fathers, and perform a simulated parentage analysis using the GERRUD algorithm.²⁸ This analysis estimated the number of fathers that contributed to the offspring, given only the knowledge of the maternal and offspring genotypes. These simulations only considered the four loci that yielded the highest joint exclusion probability, and the simulated parental genotypes had the same frequencies as the female adults used in this study. GERRUD was also used to perform the actual parentage analysis, determining both the minimum number of fathers given the maternal and offspring genotypes, and the most probable paternal genotypes.

Forty-eight families with a minimum of 12 offspring were selected for parental analysis. In six families, the maternal genotypes could not be accurately determined due to quality of the genetic material or the presence of null alleles. These families were maintained in the analysis because the knowledge of the maternal genotype does not greatly increase the chances of reconstructing the paternal genotype by GERRUD.²⁸ In one family, only 11 larvae could be genotyped, and in 2 other cases only 10 larvae were successfully genotyped.

RESULTS

Sixty-three adult mosquitoes collected from the New Orleans area were genotyped at 12 microsatellite loci to establish the baseline frequency of alleles in this population, and assess the suitability of using microsatellites to detect polyandry in this population. Using GERRUDSim2, we estimated the frequency of misclassifying a polyandrous family from this population as monandrous, assuming we genotyped 12 offspring, and that the second father contributed at least 8.3% of the offspring, as 0.149. This estimate is dependent only on the frequencies of the multiplexed set of alleles with the greatest exclusion probabilities. Screening a family from this population yields a relatively high frequency of 0.851 of correctly identifying polyandry. On the basis of this information, the probability of misclassifying a single polyandrous family (corresponding to a frequency of 0.021 in the population) is 0.0031. For the parentage analysis, we were able to screen 12 offspring from a population of 48 mothers, from the same area. The allelic frequencies from this sample of adults are similar to the frequencies found in the baseline population (Supplemental Table 1). Only one locus, A9, was found to be significantly different between the populations (*P* = 0.044), based on a χ^2 test. In addition, all loci except for A9 were found to be in Hardy–Weinberg equilibrium, using the exact test implemented in Genepop (Supplemental Table 2).²⁵

We established multiple paternity by using the GERRUD software program, which determines the likely number of fathers based on maternal and offspring genotypes, and reconstructs the most probable paternal genotypes.²⁸ Three out of 48 *Ae. aegypti* family groups (6.25%) showed evidence of multiple paternity after an initial screen of 12 offspring per family at four microsatellite loci (AC1, AC5, AG1, and AG5). To ensure this finding was not due to genotyping error or mutation, an expanded analysis was done on all offspring from each of the three families with evidence of polyandry. All offspring available for these families were

TABLE 1

Number of offspring sired by each father in families showing evidence of polyandry

Family	Number of offspring
Family 55	15
Father 1	1
Father 2	14
Family 48	23
Father 1	1
Father 2	10
Father 1 or 2	12
Family 33	44
Father 1	1
Father 2	7
Father 1 or 2	1
Father 3	35

For each polyandrous family, the number of offspring sired by each father was estimated by GERRUD. Offspring were assigned to a single father only if their genotype unambiguously supported that assignment. Otherwise, they were listed as belonging to either of the fathers.

genotyped at an additional eight microsatellite loci. This expanded analysis confirmed polyandry for the three families (Table 1).

In family no. 33, a total of 44 offspring were genotyped, and 5 loci have 3 or 4 alleles of paternal origin, indicating the presence of at least two fathers (Table 2). One locus, AG2, has five alleles of paternal origin, indicating three fathers. Each of the reconstructed fathers could unambiguously be assigned 1, 8, or 35 offspring, and one offspring could be assigned to either of two fathers (Table 2). Table 2 shows the genotypes of each offspring, along with the reconstructed paternal genotypes, and the father or fathers that are compatible with each offspring. Only the six loci that show evidence of multiple paternity are shown. Similar tables for the other polyandrous families are shown in Supplemental Table 3 (family no. 48) and Supplemental Table 4 (family no. 55).

In family no. 48, a total of 23 offspring were genotyped. The mother's genotype could not be ascertained at several loci. Still, genetic contributions from two males are required to explain the array of offspring genotypes. Although no loci have more than five alleles, definitively showing polyandry, locus AC5 has three alleles likely to be of paternal origin (Supplemental Table 3). Specifically, there are offspring homozygous for allele 157 and allele 156, meaning both the mother and one father are 156/157 heterozygotes. The presence of the 164 allele in other offspring must therefore be from a second father. Using GERRUD, one offspring could be unambiguously assigned to one father, 10 could be unambiguously assigned to a different father, and 12 could be assigned to either of these fathers.

In family no. 55, a total of 15 offspring were genotyped. There are three paternal alleles at the AG5 locus, one of which is only found in one offspring (Supplemental Table 4). This offspring also has an allele not shared by its siblings at locus AG1. This locus by itself does not technically exclude the possibility of a single father, since only two paternal alleles are present. However, it is extremely unlikely that a male would pass one of its alleles to only one out of 15 offspring, given that this locus was initially found to be in Hardy-Weinberg Equilibrium (Supplemental Table 2).³¹ Instead, it is more probable that two fathers inseminated the mother, with one contributing to 14 offspring, and the other contributing to a single offspring.

The frequency of polyandry reported here, 6.25%, is likely an underestimate because males that inseminate the same female may have the same genotype at one or more loci, or one father may contribute such a small proportion of offspring that our assay fails to detect it. To estimate the frequency of polyandrous families not detected in our study (false negatives), we performed simulated experiments using GERUDSim2 software.²⁸ We simulated 1,000 families composed of one mother, two fathers, and twelve of their offspring under three scenarios. Simulated mothers and fathers were assigned genotypes for the four microsatellite loci used in the initial screen at the same frequencies as the real mothers used in this study. In one scenario, the fathers contributed equally (six offspring each), in another, one father contributed nine offspring, and in a final case one father contributed only one offspring. Table 3 shows the frequency of underestimating the number of fathers (the false negative rate). For example, if the case of one father siring one offspring is the most common scenario, then we would expect four family groups not to be detected in our experiment. Which scenario actually represents the reality of mating in *Ae. aegypti* is unclear, because only three families showed clear evidence of multiple paternity, and the frequency of offspring contributed by each father varied widely. Still, four seems to be a reasonable upper limit to the number of undetected instances of polyandry. This means the true frequency of polyandry in our study population could be up to 14.6% (7 out of 48 families).

DISCUSSION

Our results demonstrate for the first time the occurrence of polyandry in a natural population of *Ae. aegypti*. The frequency is low (6.25%), but also likely an underestimate, and is within the range of polyandry estimates in other mosquito species. The highest frequency of polyandry in a mosquito, 26%, was reported in a wild population of *Ae. albopictus*.²⁰ Much lower estimates of polyandry were reported in wild populations of *An. freeborni* and *An. gambiae* at 4.3% and 1.7%, respectively.^{18,19} Unlike Aedine males, males from these species insert a mating plug that can act as a physical barrier to further insemination attempts and make polyandry more difficult.^{5,32} The upper limit of our own polyandry estimate is 14.6%, which is similar to the study of *Ae. aegypti* females in semi-field studies that reported a multiple insemination rate of 14% (though this does not necessarily reflect the rate of polyandry).²¹ Taken together, these results paint a picture of mostly monandrous mosquito populations with a small but potentially consequential subset of polyandrous females. Although we demonstrate that polyandry does occur in natural populations, establishing the exact frequency will require further work. Our work is limited by the natural variability of the microsatellite loci we used. Genetic markers with greater diversity will lower false negative rates by reducing the probability that multiple fathers will share the same alleles by chance. Also, surveying additional families and additional populations will be necessary to establish exactly how prevalent polyandry is in *Ae. aegypti* species-wide.

The occurrence of polyandry in wild *Ae. aegypti* is important for several reasons. As a practical matter, wild-caught females cannot be assumed to give birth to offspring from

TABLE 2
Genotypes of family 33

	AI	AG2	ACI	ACS	AGI	AG5	Compatible father
Mother	158/171	115/117	209/209	154/157	117/117	172/176	—
Father 1	162/158	115/119	197/209	156/159	117/121	170/178	—
Father 2	168/158	115/149	197/197	152/156	115/117	166/178	—
Father 3	162/168	141/145	195/197	152/158	115/115	170/166	—
NO33Off2	162/171	115/117	209/209	154/156	117/117	176/178	1
NO33Off14	158/158	115/115	197/209	154/159	117/121	172/178	1
NO33Off17	158/158	115/115	197/209	154/159	117/121	170/172	1
NO33Off20	162/171	115/119	209/209	154/156	117/117	172/178	1
NO33Off21	158/171	115/119	197/209	154/156	117/117	170/172	1
NO33Off22	158/158	117/119	197/209	157/159	117/121	170/172	1
NO33Off37	158/162	117/119	209/209	154/156	117/117	170/172	1
NO33Off16	158/158	115/117	197/209	156/157	117/117	176/178	1 or 2
NO33Off8	168/171	117/149	197/209	152/154	115/117	166/176	2
NO33Off1	158/162	117/141	195/209	152/157	115/117	170/172	3
NO33Off10	168/171	115/145	197/209	152/154	115/117	166/172	3
NO33Off11	168/171	117/145	197/209	152/154	115/117	170/176	3
NO33Off12	158/168	115/145	197/209	152/157	115/117	166/172	3
NO33Off3	162/171	117/145	195/209	154/158	115/117	166/172	3
NO33Off4	158/162	115/141	195/209	154/158	115/117	170/172	3
NO33Off5	168/171	115/145	197/209	152/157	115/117	170/172	3
NO33Off6	158/168	117/145	197/209	154/158	115/117	170/176	3
NO33Off7	168/171	117/145	197/209	152/154	115/117	166/176	3
NO33Off9	158/162	117/145	195/209	157/158	115/117	170/176	3
NO33Off13	168/171	117/141	197/209	152/154	115/117	170/176	3
NO33Off15	168/171	117/145	197/209	152/157	115/117	166/176	3
NO33Off18	162/171	115/141	197/209	152/154	115/117	170/172	3
NO33Off19	168/171	115/141	197/209	157/158	115/117	170/172	3
NO33Off23	158/168	115/145	197/209	154/158	115/117	166/176	3
NO33Off24	158/162	115/145	195/209	157/158	115/117	166/172	3
NO33Off25	158/162	117/141	195/209	157/158	115/117	170/176	3
NO33Off26	162/171	117/145	197/209	154/158	115/117	166/176	3
NO33Off27	158/168	117/141	197/209	152/157	115/117	170/176	3
NO33Off28	158/168	117/145	197/209	154/158	115/117	166/176	3
NO33Off29	158/168	117/141	197/209	154/158	115/117	166/176	3
NO33Off30	158/168	117/145	197/209	154/158	115/117	166/172	3
NO33Off31	168/171	115/145	197/209	154/158	115/117	166/176	3
NO33Off32	158/168	117/145	197/209	154/158	115/117	166/176	3
NO33Off33	158/168	115/141	197/209	152/157	115/117	166/172	3
NO33Off34	162/171	115/145	195/209	154/158	115/117	166/172	3
NO33Off35	168/171	117/141	197/209	157/158	115/117	166/176	3
NO33Off36	162/171	117/145	195/209	152/157	115/117	166/176	3
NO33Off38	168/171	117/141	197/209	152/157	115/117	170/176	3
NO33Off39	162/171	115/145	195/209	154/158	115/117	166/176	3
NO33Off40	168/171	117/145	197/209	152/154	115/117	166/176	3
NO33Off41	162/171	115/141	195/209	154/158	115/117	170/172	3
NO33Off42	162/171	117/145	195/209	154/158	115/117	170/176	3
NO33Off43	158/162	117/141	195/209	152/154	115/117	170/176	3
NO33Off44	158/168	117/145	197/209	154/158	115/117	166/176	3

Genotype of mother, reconstructed fathers, and offspring of family 33 at the six microsatellite loci showing evidence of polyandry. The paternal genotypes were reconstructed by GERRUD. The father(s) compatible with the offspring's genotype is listed in column 8.

a single father. This is important when sampling natural populations for genetics. Often females collected in nature are used to establish isofemale (single female) lines.³³ It had been assumed that the offspring represent four genomes sam-

pled from the natural population. This assumption needs to be reassessed.

Polyandry in *Ae. aegypti* could also affect the impact of genetic control programs. These programs depend on sterile males mating with females, effectively removing them from the population. Monandrous females would not contribute to the next generation after this mating, whereas polyandrous females could potentially remate with a nonsterile male. More males may ultimately be necessary in these programs to achieve similar results. The presence of polyandrous females also highlights the need to study aspects of postcopulatory biology in *Ae. aegypti*, such as sperm utilization and sperm competition, which have not received much attention. Given the wide variance in number of offspring per father that we observed, it will also be of interest to determine what factors affect reproductive success among males that mate with polyandrous females.

TABLE 3

Estimated frequency of misclassifying polyandrous families as monandrous

Scenario conditions	Frequency of simulated families where number of reconstructed fathers is incorrect
Father 1, 6 offspring	0.005
Father 2, 6 offspring	
Father 1, 9 offspring	0.014
Father 2, 3 offspring	
Father 1, 11 offspring	0.086
Father 2, 1 offspring	

Families consisting of a mother and two fathers contributing a variable number of offspring were simulated using GERRUDSim2. A total of 1,000 families were simulated for each scenario, and a parentage analysis was performed on each family. The frequency of families identified with an incorrect number of fathers was determined by the algorithm used by GERRUD.

Received November 15, 2014. Accepted for publication March 5, 2015.

Published online April 13, 2015.

Note: Supplemental tables appear at www.ajtmh.org.

Acknowledgments: Joshua B. Richardson was supported by a Parasitology and Vector Biology Training Grant (NIH: 5T32AI007404-23), and the research was supported by grant NIH RO1 AI101112 awarded to him.

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