OVIPOSITION BEHAVIOR OF FEMALE ANOPHELES GAMBIAE IN WESTERN KENYA INFERRED FROM MICROSATELLITE MARKERS

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Abstract. Anopheles gambiae females in a relatively isolated hut and all larvae from larval habitats within 100 m of the hut were collected in August 2001 in western Kenya. Among 42 aquatic habitats, 16 had A. gambiae larvae. Two hundred fifty larvae and 58 adults were genotyped using nine microsatellite markers to infer sibling relationship between the larvae and maternity between the females and larvae. The pairwise genetic relatedness of A. gambiae larvae per habitat ranged from –0.4112 to 0.9375, indicating that full siblings, half siblings, and genetically unrelated individuals were present at those habitats with multiple larvae. From a likelihood analysis, it was estimated that 56.6% of females had larvae in multiple habitats. These results substantiate that one A. gambiae female uses multiple breeding sites for oviposition, and thus, average genetic relatedness for breeding sites with high larval populations tends to be low.

INTRODUCTION

Anopheles gambiae Giles is the principal vector of human malaria across sub-Saharan Africa. Although many aspects of biology and behavior of the mosquito have been studied and well documented, as reviewed by Gillies and Coetzee, little is known about the oviposition behavior of A. gambiae under field conditions. When seeking novel avenues for ecological control of mosquitoes and mosquito-borne disease, mosquito breeding behavior should receive more attention.

The gravid females of A. gambiae sensu lato prefer black-bottomed water dishes to white-bottomed ones for oviposition, and most eggs are laid in pools with muddy and unvegetated edges. Other factors such as habitat size, substrate, semiochemicals, microbial fauna, predators, vegetation, and land cover types also affected the choice of aquatic breeding site by a female. Different volatiles and concentrations from potential habitats can either attract or repel anopheline females from laying eggs. The majority of A. gambiae s. l. adults invade huts within 300 m of their nearest larval habitats. Most larval habitats of A. gambiae s. l. are temporary aquatic habitats such as animal footprints and puddles in western Kenya, and the relative abundance of A. gambiae larvae is significantly associated with the distance between a larval habitat and the nearest hut. In nature, the abundance of anopheline larvae varies substantially among aquatic habitats. Some habitats contain high densities of anopheline larvae, whereas many others have none despite high densities of mosquito adults in surrounding huts. Such variation leads to the speculation that some aquatic habitats are more attractive or suitable for oviposition and larval development than others. If this is true, limited suitable aquatic habitats may lead to oviposition by multiple females. On the other hand, skip oviposition, laying a few eggs in several different sites, has been reported in the yellow fever mosquito, Aedes aegypti, from both laboratory studies and field evaluations. A. aegypti primarily develops in containers, and its larval density tends to be high (hundreds of larvae in a container). However, A. gambiae females generally use temporary aquatic habitats for oviposition, and their larval density in a habitat is generally lower (< 50 larvae).

Study area and mosquito collection. The study was conducted in Mbita, Suba District, western Kenya. A relatively isolated hut (S 00 26.021', E 34 12.765') and all aquatic habitats within a 100 m radius of the hut were surveyed for anopheline larvae from August 20 to 29, 2001 (Figure 1). Larval collection was carried out using the standard 350-mL dippers. At each breeding site, larvae were sampled on the oviposition behavior of anopheline mosquitoes in nature has been published.

Direct observation of mosquito oviposition in nature is not feasible because of the untraceable movement, nocturnal activity, and tiny size of mosquitoes. However, indirect methods such as genetic approaches can be useful tools for the study of mosquito oviposition behavior. Randomly amplified polymorphic DNA (RAPD) markers have been used to estimate the number of full-sibling families in A. aegypti. These studies have found that the average family size of mosquito larvae in a container is 11 and that family size distribution among the containers is skewed toward containers with one to two families. In A. gambiae, > 100 microsatellite markers have been isolated and genetically or cytogenetically mapped. The microsatellite markers are co-dominant, highly polymorphic, and usually neutral, and therefore more powerful in determining the genetic relatedness among mosquito individuals than dominant RAPD markers. Thus, these microsatellite markers represent excellent molecular tools for inferring genetic relationship among anopheline mosquitoes in a habitat and for studying their oviposition behaviors. Furthermore, the mother–larva inference made with microsatellite markers can be verified using the polymorphism in the mitochondrial NADH dehydrogenase subunit 5 (ND5) gene in A. gambiae.

Here, we used nine microsatellite markers to estimate genetic relatedness among A. gambiae larvae from aquatic habitats around a hut and to infer their maternity from the female mosquitoes collected in the hut. This study addressed three questions related to mosquito oviposition behavior: 1) what was the genetic relatedness among the larvae from each habitat; 2) did a female mosquito deposit her eggs in more than one aquatic habitat; and 3) if a female laid her eggs in multiple aquatic habitats, what was the spatial distribution of the sibling larvae around the hut?

MATERIALS AND METHODS

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exhaustively. Female adults were collected using the indoor pyrethrum spray method. All anopheline larvae and adults were preserved in 95% ethanol right after collection. In total, 321 anopheline larvae and 77 adults were collected.

**DNA extraction and species identification.** Genomic DNA was extracted from individual mosquitoes and larvae. Because *A. gambiae* and *A. arabiensis* are morphologically indistinguishable, the rDNA–polymerase chain reaction (PCR) method was applied to identify specimen within the *A. gambiae* species complex. Up to three PCR reactions were conducted for identification of one specimen.

**Microsatellite loci and genotype scoring.** To individually genotype the *A. gambiae* larvae and adults, nine microsatellite markers were used: AGXH1D1, AGXH131, and AGXH503 on Chromosome X, AG2H46, AG2H79, and AG2H117 on Chromosome 2, and AG3H29C, AG3H33C, and AG3H158 on Chromosome 3.

**ND5 gene sequencing.** To verify the inferred relationship between female adults and larvae from the microsatellite markers, a segment of the *ND5* gene was amplified using primers 19CL and DMP3A and was double-strand sequenced with the ABI PRISM BigDye Sequencing Kit (Applied Biosystems, Foster City, CA) in 5 female mosquitoes and their 17 inferred larvae, with 2–5 larvae in each female–larvae group.

**Data analysis.** Pairwise relatedness (r) for larvae from each habitat was calculated using a regression measure available in Kinship 1.0. Genetic relationship between two individuals was categorized as unrelated (r ≤ 0.125), half sibling (0.125 < r ≤ 0.375), or full sibling (r > 0.375). The mean genetic relatedness of larvae per habitat was calculated using Mininab. Because of unequal variances and small sample sizes, the average relatedness was analyzed between habitats with three or more larvae and with two larvae by the nonparametric Mann-Whitney test. The average sibling frequency at the 12 *A. gambiae* habitats was 57.6 ± 6.3%. Furthermore, the median of average relatedness at the 12 habitats with three or more larvae (~0.0292) was significantly lower than at the three habitats with two larvae (0.0846, α = 0.05, Mann-Whitney test), indicating less sibling components at the habitats with three or more larvae.

Among the 250 *A. gambiae* larvae genotyped, 135 were assigned to 54 female mosquitoes at 95% confidence. According to the larval distribution among the habitats, there were from 1 to 22 females who chose the same habitat for their offspring, and one female had one to three larvae at a habitat (Table 1). In total, 115 larvae had no assigned mother, indicating their mothers were either not identified or not captured. For one habitat, there might be 1–22 larvae without any assigned mother. The habitat use by females is shown in Figure 1. There was no significant association between relative abundance of *A. gambiae* larvae (percentage of *A. gambiae* larvae in a habitat) and the distance from a habitat to the hut (r = −0.47, df = 15, P > 0.05).

**RESULTS AND DISCUSSION**

The rDNA PCR identified 250 larvae and 58 females as *A. gambiae*, 63 larvae and 5 females as *A. arabiensis*, 1 larva as a hybrid, and 7 larvae and 14 females as unknown in the larval and mosquito samples. *A. gambiae* was the predominant species in both larval and adult samples.

Among 42 aquatic habitats, 16 had *A. gambiae* larvae, with an average of 15.6 ± 4.3 (SE) larvae per habitat and a range of 1–53 larvae at each habitat. There were only six aquatic habitats with *A. arabiensis* larvae (10.5 ± 4.5, 1–28), all cohabitated with the *A. gambiae* larvae. The single hybrid larva was found to share a habitat with *A. gambiae* larvae. The average distance between the hut and *A. gambiae* habitats was 14.5 ± 1.4 m (Figure 1). There was no significant association between relative abundance of *A. gambiae* larvae (percentage of *A. gambiae* larvae in a habitat) and the distance from a habitat to the hut (r = −0.47, df = 15, P > 0.05).

Relatedness distributions of 12 *A. gambiae* habitats with three or more larvae are shown in Figure 2, and relatedness values of habitats with two *A. gambiae* larvae (habitats 3, 24, and 41) are listed in Table 1. The values of pairwise relatedness (Figure 2; Table 1) indicate that there were full-sibling (r > 0.375) and half-sibling (r > 0.125) larvae at the 15 habitats with two or more *A. gambiae* larvae. The average sibling frequency at the 16 *A. gambiae* habitats was 57.6 ± 6.3%. Furthermore, the median of average relatedness at the 12 habitats with three or more larvae (~0.0292) was significantly lower than at the three habitats with two larvae (0.0846, α = 0.05, Mann-Whitney test), indicating less sibling components at the habitats with three or more larvae.

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Each of the five *A. gambiae* females sequenced for the *ND5* gene segment (665 bp, positions 6,896–7,560, sequenced by Beard and others; GenBank no. L20934) had a unique haplotype. All of the larvae (two to five for each female) shared the same haplotype with their assigned mother (Table 2). This confirms that the maternal inference by microsatellite markers and likelihood method produces highly confident results.
The *A. gambiae* average sibling frequencies at the larval habitats in this study (57.6 ± 6.3%) were significantly higher than those in 13 adult populations of *A. gambiae* from Kenya, Nigeria, and Senegal (19.4 ± 0.6%); *t* = 6.02, df = 14, *P* < 0.01). Most measures of the mean relatedness for larval habitats in this study were also much higher than in the 13 adult populations reported by Lehmann and others, which ranged from –0.050 to 0.004. Adults in that study were collected from multiple huts at each location and should have shown lower proportions of siblings (16.5–24.1%).

The likelihood results above show that *A. gambiae* females used multiple habitats for oviposition and that some of the females shared one habitat. It is known that *A. gambiae* larvae mainly present at small, temporary habitats near huts, such as 10,11,21 that females have preferences for which habitats they select for oviposition, 24 and that the majority of adults are found a short distance (< 300 m) from their larval habitats. 21 These findings all indicate that preferred breeding habitats are limited for *A. gambiae* females in the field. Moreover, one *A. gambiae* female is capable of laying 50–200 eggs after a blood meal, 46 but < 50 eggs are found at most habitats, 26 indicating that one female needs more than one habitat to lay eggs. Such skip oviposition behavior is practiced by *A. gambiae* females.

The distribution of mosquito larvae is generally determined by the oviposition sites selected by females. 2 The local dispersal of *A. gambiae* could be driven by the search for oviposition sites. Increased adult dispersal caused by females searching for a suitable breeding site may facilitate the spread of malaria parasites. Control strategies against the malaria-vector mosquitoes at immature stages, including efforts to remove or destroy breeding sites, could be highly effective and could complement adult control interventions. They should be given priority for further development, evaluation and implementation as an integral part of the Rolling Back Malaria project. 47

It is well known that *A. gambiae* is highly anthropophilic. A higher proportion of *A. gambiae* larvae in habitats closer to huts was reported in the same area used for this study. 21 However, such a relationship was not found in this study because only one hut site was surveyed even though *A. gambiae* was predominant there.

Of the 16 polymorphic ND5 nucleotide sites, 4 were new and 3 were detected only in *A. arabiensis* by Besansky and others. 32 None of these nucleotide substitutions resulted in an amino acid replacement. With a high level of polymorphism,
this segment of the ND5 gene is an ideal genetic marker not only for maternal inference, but also for systems, evolution, and population genetics studies on anopheline mosquitoes.\footnote{2}\footnote{47} Although the female–larvae likelihood inferences were supported by the ND5 sequences, more microsatellite markers and genotyping data from candidate males may be used in the future to provide more power for distinguishing the female–larvae relationship and to verify the inferences in this study.

*Anopheles arabiensis* is also an important malaria vector in sub-Saharan Africa. The research methods used in this study can be applied equally to this sibling species to infer its oviposition behavior. Interspecific competition between the two sibling species has been shown to have a detrimental effect only on *A. arabiensis*.\footnote{38}\footnote{48} However, all 63 *A. arabiensis* larvae in this study shared habitats with the *A. gambiae* larvae. It is therefore unclear if a female mosquito will attempt to avoid a habitat occupied by its sibling species.

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