Mixed Swarms of the Molecular M and S Forms of *Anopheles gambiae* (Diptera: Culicidae) in Sympatric Area from Burkina Faso

ABDOULAYE DIABATÉ,[1,2] ROCH K. DABIRE,[1] PIERRE KENGNE,[3] CECILE BRENGUES,[3] THIERRY BALDET,[4] ALI OUARI,[1] FREDERIC SIMARD,[5] AND TOVI LEHMANN[2]

ABSTRACT The M and S molecular forms of *Anopheles gambiae* sensu stricto Giles are thought to be reproductively isolated through premating barriers. However, the exact mechanisms of recognition of conspecific partners are unknown. Because mating in *An. gambiae* occurs in swarms, one might expect swarming behavior between the M and S forms to be different and that this probably reduces the risk of contact between males and females of the different forms in areas where they are sympatric. We report the occurrence of four mixed swarms, containing males of M and S forms, out of a total of 26 swarms sampled in Soumousso, a typical savannah village of Burkina Faso, West Africa. However, the frequency of mixed swarms was lower than that expected by chance. This observation suggests partial segregation between the swarms of the molecular forms, which may contribute to their isolation. Because the frequency of mixed swarms seems too high to explain the low frequency of cross-mating and hybrids, we suggest that mate recognition in a swarm is more important than swarm segregation.

KEY WORDS mixed swarms, molecular M and S forms, *Anopheles gambiae*, mating behavior

The issue of reproductive isolation within *Anopheles gambiae* sensu stricto Giles has generated much debate. This species is highly polymorphic and is further subdivided into five forms that differ in their chromosomal inversion arrangements (Coluzzi et al. 1985, Touré et al. 1998, Coluzzi et al. 2002). These chromosomal forms seem more or less genetically isolated in the field, presumably through prezygotic barriers because viable and fertile hybrids have been obtained in the laboratory (Di Deco et al. 1980, Persiani et al. 1986, Touré et al. 1998). Cytogenetic analysis, however, is not a precise way to evaluate the degree of hybridization between forms because of the presence of cryptic "heterokaryotypes" that are difficult to identify and the adaptive nature of inversions strongly exposed to selection (della Torre et al. 2001, Coluzzi et al. 2002, Wondji et al. 2002). Recent studies using molecular markers such as X-linked ribosomal DNA suggested the existence of only two entities within *An. gambiae* that are referred to as M and S molecular forms (Favia et al. 2001). A deficit of hybrid M/S individuals has been observed in the field (della Torre et al. 2001). Although postmating barriers between the chromosomal forms have not been found (Di Deco et al. 1980, Persiani et al. 1986, Touré et al. 1998), no studies have evaluated postmating between the molecular forms. There are data that are consistent with the hypothesis of premating reproductive isolation between M and S (Tripet et al. 2001), but little is known on the structure of mating between the forms in the field.

Mating systems based on aerial male aggregations that function as encounter sites for mate-searching females have evolved repeatedly in various groups of insects (Sullivan 1981, Cooter 1989). In most swarming species, the swarms are composed of males. Females typically approach a swarm, acquire a mate, and leave in copula. The way the sexes are attracted to each other may contribute to specific mate recognition systems, which may prevent hybridization. The mechanisms that result in assortative mating in *An. gambiae* are still unknown. The hypothesis that flight-tone is used for mate recognition by sympatric *An. gambiae* and *Anopheles arabiensis* Patton (Brogdon 1998) was not confirmed by field studies (Wekesa et al. 1998). Recently, Tripet et al. (2004, 2005) did not find a difference in wingbeat frequency and sex proteins between the molecular M and S forms of *An. gambiae*. Studies on the swarming and mating behavior in the field suggest that males of *An. gambiae* avoid contact with interspecific partners mainly by swarming at different heights above markers such as those

[1] Institut de Recherche en Sciences de la Santé/Centre Muraz BP 390 Bobo-Dioulasso, Burkina Faso.

[2] Laboratory of Malaria and Vector Research, NIAID, National Institute of Health, 12735 Twinbrook Parkway, Room 2W13A, Rockville, MD 20852.

[3] Laboratoire de lutte contre les Insectes Nuisibles/Institut de Recherche pour le Développement Montpellier, 911 Avenue Agropolis, BP 5045, 34032 Montpellier Cedex 1, France.

[4] CIRAD-EMVT, UPR 16, Campus international de Baillarguet 34398 Montpellier Cedex 5, France.

[5] Organisation de Coordination pour la lutte contre les Endémies en Afrique Centrale/Institut de Recherche pour le Développement BP 288 Yaoundé, Cameroun.

Table 1. Frequencies of the molecular forms of *An. gambiae* collected from 26 swarms

Swarm	Collection date (2004)	Location[a]	♂M_	♂S_	No. ♀
1	23 Aug.	1	0	20	0
2	23 Aug.	2	7	14	0
3	23 Aug.	3	0	34	1
4	23 Aug.	4	0	23	0
5	23 Aug	5	0	37	0
6	24 Aug.	1	0	12	0
7	24 Aug.	2	0	28	1
8	24 Aug.	3	0	46	2
9	24 Aug.	4	0	7	0
10	24 Aug.	5	5	23	0
11	24 Aug.	6	2	16	0
12	24 Aug.	7	0	17	0
13	25 Aug.	3	0	13	0
14	25 Aug.	5	0	21	1
15	25 Aug.	6	0	19	0
16	22 Sept.	1	0	17	0
17	22 Sept.	3	0	19	0
18	22 Sept.	5	0	16	0
19	22 Sept.	6	0	12	0
20	23 Sept.	1	0	9	0
21	23 Sept.	2	0	11	0
22	23 Sept.	3	0	49	1
23	23 Sept.	4	0	6	0
24	23 Sept.	5	0	11	0
25	23 Sept.	6	0	23	0
26	23 Sept.	7	3	8	0
Overall			17 (3.2%)	511 (96.8%)	6 (100%)

All six females collected were of the S form. Homogeneity of form composition across swarms was applied only to males. Homogeneity of form composition across swarms (males only) was rejected $P < 0.0001$ by using contingency table exact test.

[a] Location is a unique identifier of the swarm location defined by the projection of the center of a swarm onto the ground. If a location of a given swarm fell within 10 m from that of a previous swarm, the previous location identifier was used.

formed between grass areas and footpaths or bushes and secondarily by swarming at different times (Charlwood et al. 2002). Therefore, one might expect different swarming behavior between M and S forms that reduces contact between males and females of the different forms in areas where they are sympatric.

Materials and Methods

A survey of the swarming and mating behavior of *An. gambiae* was undertaken in Soumousso (11°00′ 46′ N, 4°02′ 45 W), a typical village in the savannah area of Burkina Faso, West Africa, where both M and S forms of *An. gambiae* coexist. *Anopheles funestus* Giles and *Anopheles nili* Theobald also are found there and contribute to malaria transmission. The highest density of *An. gambiae* occurs in September and is ≈30 bites per human per night. The relative frequencies of the two molecular forms of *An. gambiae* change over time. The M form is predominant from December to June and the S form from July to November. Swarms were sampled with an insect net. Mosquitoes were aspirated into cups, killed with chloroform, identified, counted, and placed on silica gel in tubes. The location of the swarm, the time of collection, and the height above the ground were recorded. Indoor resting mosquitoes also were collected using aspirators in this village during the same time and processed as described above. Genomic DNA was extracted from single mosquitoes and polymerase chain reaction (PCR) was used to amplify the intergenic spacer of the rDNA

to identify the molecular forms as described previously (Favia et al. 2001).

We tested whether the observed number of mixed swarms agreed with expectation based on random sampling from a binomial distribution with the observed form composition estimated from a sample of indoor resting females from the same time and area. We drew 5,000 random sets, each of 26 samples with the same sample sizes as in our actual samples from swarms. For each random set, the number of mixed swarms was recorded. The distribution of these 5,000 values represented the expected distribution of mixed swarms (out of 26 samples) given the form composition and actual sample sizes. If our observed count of mixed swarms fell outside the central 95% of this distribution, we would reject the hypothesis that the swarms represented a random collection of males from a population with these frequencies. Contingency table analysis was used to test homogeneity of form composition in males across swarms. Exact test was used because of the presence of cells with expected values lower than 5. Statistical analysis was performed using SAS (SAS Institute 1999)

Results

A survey of swarms in Soumousso was conducted in August and September 2004, when the composition of the S and M forms based on indoor females collection ($n = 71$) was 82 and 18%, respectively. In total, 26 swarms of *An. gambiae* were sampled, yielding 528
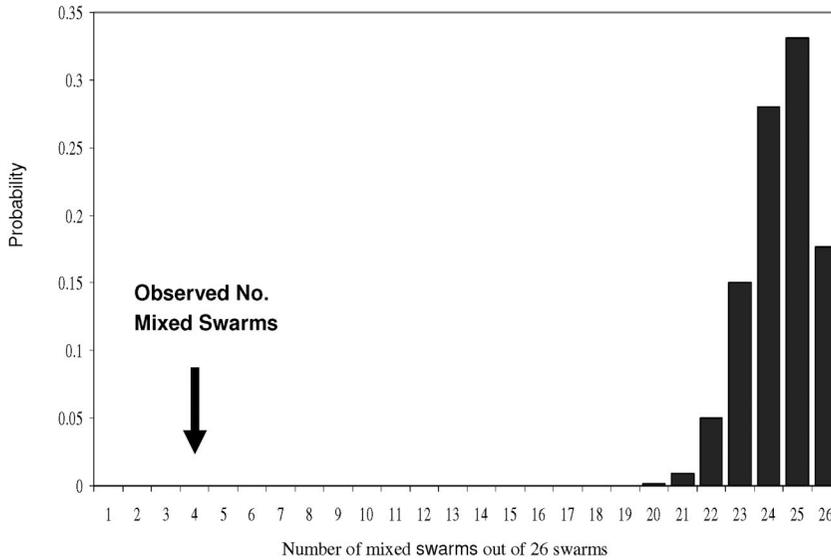
Fig. 1. Distribution of the number of mixed swarms expected out of 26 samples based on 5,000 sets of random samples drawn from a binomial distribution. The probability of success in the binomial distribution was 18.2%, based on the form composition in indoor resting female collection in the same time and area where swarms were collected. Observed number of mixed swarms is shown by the arrow. See text for details.

male and six female *An. gambiae* (Table 1). Of these 26 swarms, four were composed of M and S males (Table 1). The other 22 swarms collected were exclusively of the S form. Only six females of the S form were collected from the latter 22 swarms, and no females were collected from the mixed swarms. All the specimens collected from swarms were PCR identified. *An. arabiensis* was not collected in these swarms in accordance with its low frequency in this area (Diabaté et al. 2004). Based on the composition of the two molecular forms with indoor females collection, the expected number of mixed swarms out of 26 swarms was calculated and was found to be significantly higher than the number of mixed swarms observed, suggesting partial segregation in the swarming behavior of the two forms (Fig. 1). Even considering swarming males alone, homogeneity of form composition across swarms was rejected by contingency table analysis (Table 1; $P < 0.0001$ exact test). However, we could not find any difference between mixed and nonmixed swarms' general characteristics, such as the height above the ground, place of collection, time of collection, and movement. They were collected from 1 to 3 m above the ground; however, they could fly up to 4 m above the ground. Swarming always began 5 to 10 min after sunset by one or two males who were progressively joined by other males. After the swarm had formed, mosquitoes flew around a pivotal position occasionally moving forward, backward, or up and down. We could not associate any physical marker, such as a tree or a fence, with the sites where swarms were found (Diabaté et al. 2003). In the seven swarming sites, three were in open flat areas, one at the edge of a cornfield, one near a shed, one close to cow herd, and one above grasses. There was no exclusive spot for mixed swarms (Table 1).

Discussion

Most investigators have found swarms of mosquitoes to be composed of males of a single species, even when several species were swarming in the same area (Clements 1999). However, mixed swarms of *An. gambiae* and *An. arabiensis* have occasionally been reported, suggesting that segregation of swarms in time and space was not the key to the reproductive isolation of these sibling species (Marchand 1984). The lower than expected frequency of mixed swarms reported here revealed differences in swarming behavior between the molecular forms that may contribute to reduced cross-mating between them. However, if these four mixed swarms out of 26 would succeed in mating regardless of the molecular form, this would result in a high estimate of cross-mating. Previous results from Soumousso did not find any hybrid between M and S (Diabaté et al. 2004). Indeed, low frequency of cross-mating, 1.4% (Tripet et al. 2001) or hybrids 0.0026% (della Torre et al. 2001) has been reported from the field. Together with these previous results, our data suggest that mate recognition in a swarm is more important than swarm segregation to explain assortative mating. The occurrence of mixed swarms does not imply that cross-mating and hybridization will occur in such swarms. We hypothesize that *An. gambiae* females are important in mate recognition because mated females are able to evade copulation attempts by males, thus preventing further inseminations (Charlwood and Jones 1979). We did not find swarms composed mostly or exclusively of the M form, but the relatively low proportion of the M form in the swarms (Table 1) compared with that we observed in indoor females collection (18%) suggests that such swarms do exist.

This is the first report of mixed swarms of M and S forms of *An. gambiae*. Further studies of mate recognition and swarming behavior of this mosquito species will be valuable to understand speciation in *An. gambiae* and gene flow between them that determines the spread of traits such as insecticides resistance and transgenes (della Torre et al. 2002).

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