Assessment of Alternative Mating Strategies in *Anopheles gambiae*: Does Mating Occur Indoors?

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ABSTRACT Mating in *Anopheles gambiae* has been observed only in outdoor swarms. Here we evaluate whether mating also occurs indoors. Mark-release-recapture of virgin males and females in natural houses showed that mating occurred over a single day even when mosquitoes can leave the house through exit traps and without adaptation to laboratory conditions. In these experiments, insemination rate in the M molecular form of *An. gambiae* (and *An. arabiensis*) was higher than that of the S form (15 versus 6%). Under these conditions, smaller females of the M form mated more frequently than larger females of that form. Sampling mosquitoes throughout the day showed that both sexes enter houses around sunrise and leave around sunset, staying indoors together from dawn to dusk. In an area dominated by the M form, the daily rate of insemination in samples from exit traps was ≈5% higher than in those from entry traps, implying that mating occurred indoors. Importantly, frequency of cross mating between the molecular forms was as high as that between members of the same form, indicating that, indoors, assortative mating breaks down. Altogether, these results suggest that indoor mating is an alternative mating strategy of the M molecular form of *An. gambiae*. Because naturally occurring mating couples have not yet been observed indoors, this conclusion awaits validation.

KEY WORDS *Anopheles gambiae*, alternative mating strategies, molecular forms

Mosquito mating behavior is a poorly understood facet of their life, partly because direct observations are hampered by their small size, speed, and crepuscular or nocturnal activity. Hence, only a few studies address mating behavior of the principal African malaria vector, *Anopheles gambiae* under natural conditions (Charlwood and Jones 1980, Marchand 1984, Yuval et al. 1992, Charlwood et al. 2002b, Diabate et al. 2003, 2006). The *Anopheles gambiae* complex consists of seven sibling species (Coluzzi et al. 1979, 1985, Hunt et al. 1998, Toure et al. 1998). The M and S molecular forms of *An. gambiae* s.s. represent incipient species (Coluzzi et al. 1985, Toure et al. 1998, della Torre et al. 2001, Favia et al. 2001, Gentile et al. 2001, Mukabayire et al. 2001, Lehmann et al. 2003, Turner et al. 2005, but see Lanzaro et al. 1998, Yawson et al. 2007).

Many mosquito species initiate mating in swarms that typically consist of a group of males flying in zigzag pattern 1–3 m above ground (often over a distinct marker) and forming a distinct "cloud," into which females fly and depart paired with a male. However, swarms are not involved in mating of many cu-

licine and anopheline species (Nielsen and Haeger 1960, McIver et al. 1980, Lounibos et al. 1998) where mating occurs near the vertebrate host or near larval sites (Yuval 2006). As noted by Downes (1969), swarming in different "arenas" can facilitate premating isolation. The mating behavior of *An. gambiae* is key to understand the mechanisms of reproductive isolation of the partly sympatric molecular forms and sibling species. Observing only five couples in 34 swarms (Marchand 1984) casts doubt if mating occurs only in swarms. Although more couples were observed in *An. gambiae* swarms elsewhere (Charlwood et al. 2002, Diabate et al. 2003, 2006), this issue remains relevant because mating can be initiated in different ways by different individuals within species (Emlen and Oring 1977, Thornhill and Alcock 1983, Yuval 2006). Alternative mating strategies are often used by males that have low prospects of mating under "standard" conditions because of competitive disadvantage, such as smaller body size (Thornhill and Alcock 1983, Yuval et al. 1993, Charlwood et al. 2002a, Okanda et al. 2002). For example, *An. labranchiae atroparvus* swarms outdoors and indoors, yet most females are mated in sheltered "resting" sites (Cambounac and Hill 1940).

Although mating in *An. gambiae* is initiated in outdoor swarms, the possibility that mating also occurs independent of swarms has not been addressed. To evaluate if *An. gambiae* mates indoors, we (1) conducted experiments using progeny of wild females in

natural settings; (2) determined whether males and females naturally co-occur in houses, (3) assessed whether insemination rate is higher in females sampled from exit versus entry traps as expected if mating indoors occurred naturally; and (4) evaluated the specificity in indoor mating between the molecular forms.

Materials and Methods

Study Sites. Indoor mark, release, and recapture mating experiments and assessment of the presence of males with virgin females indoors were conducted in the villages of Banambani (8°3′ W, 12°48′ N) and Donéguébougou (7°59′5″W, 12°48′38″N), located 3–4 km apart and ≈15 km north of Bamako, Mali. These farming communities are situated among gently rolling hills covered with vegetation characteristics of wet savanna. Most houses have a single room, built of mud, shaped like a cylinder, and covered by a conic thatch roof.

Experiments comparing natural insemination rates in mosquitoes entering and exiting houses were carried out in the village Sokourani near Niono (14°17′ N, 8°5′ W). It is a rice cultivation area located 350 km northeast of Bamako in a habitat of dry savanna where, away from the river and the irrigation canals, vegetation is sparse. The inhabitants live in mud-walled houses, typically designed as a rectangular prism (box), with one door and two windows. Unlike in villages near Bamako, in Niono, many inhabitants use bednets regularly, but most bednets are not impregnated with insecticides.

Mosquitoes Used in Release Experiments. Females of *An. gambiae* s.l. were collected from houses in Banambani and Donéguébougou using aspirators during October–November 2004, July–August 2005, and July–August 2007. They were transported to the insectary at the Malaria Research and Training Center in the University of Bamako and maintained at 27°C and 75–85% RH. Blood-fed half-gravid and gravid females were placed in 1-gal cages and kept for 2 d before they were placed individually in a 50-ml Falcon tube containing 15 ml deionized water for oviposition. A strip of filter paper (2 cm wide) surrounded the water edge, providing a wet surface to collect the eggs. Females who laid eggs were identified to species and molecular form using molecular assays (Fanello et al. 2002). First-instar larvae were pooled by species and molecular form in groups of 200 per pan. Pans (25 cm width by 30 cm length by 6 cm depth) were filled with 400 ml of deionized water, and 0.1 g ground fish food (Tetramin Tetra, Inc., Melle, Germany) was provided daily. When most larvae reached the third instar, 400 ml of water was added. Pupae were collected daily and transferred into emergence cages. Emerging males and females were separated within 24 h to prevent mating. Virginity of females was confirmed by dissection and examination of spermatheca (below) from 8 to 20 females from each cage. All verifications (n = 339) were negative.

Indoor Release Experiments. Several one-room houses in Banambani and Donéguébougou were selected for the experiments. Before release, all openings of the house in the village were sealed by nets, and exit traps were mounted in the screen that covered the door opening (Fig. 1A). Five traps (30 by 30 by 30 cm) were mounted on the door, spaced more or less evenly from bottom to top. One trap (60 by 60 by 60 cm) was mounted on the window. To increase survival and recapture rate, spiders and cobwebs were removed, three to five wet towels were hung to increase air humidity, and three pads of cottons soaked in 5% sucrose solution were suspended by strings from the ceiling. A few hours before release, virgin females (2–5 d old) were dusted with a fluorescent dye (DayGlo; DayGlo Color, Cleveland, OH). Each species and molecular form was marked with a different color. Males were not marked. Males and females were released into the house together, 4–1 h before sunset. Mosquitoes were removed from exit traps continuously throughout the experiment to prevent mating in the exit traps, and males and females were placed in separate cages. The next morning, mosquitoes were collected indoors using a different aspirator and a different cage for each mosquito color. After the live collection, pyrethrum spray collection (PSC) was performed to collect all remaining mosquitoes.

Insemination status was determined by examination (×100 magnification or higher) of dissected spermatheca, pressed under a microscope coverslip, for the presence of sperm. All recaptured mosquitoes were preserved in 85% ethanol, and their species and molecular form were determined as described above. To determine whether smaller body size is a determinant of indoor mating, one wing of randomly selected females (n = 190) was removed, mounted on a slide, and measured to the nearest 0.1 mm using a dissecting scope equipped with micrometer ruler under ×10 magnification.

Abundance of Males and Females in Houses. Male and female mosquitoes were collected from five houses in Donéguébougou or Banambani by PSC at each of the following times: 0600, 0700, 1700, 1800, 1830, 1900, 2000, 2200, and 2400 hours. Weekly collections were performed from July to September 2005 alternately in the each village, allowing 2 wk before repeated sampling of the same house.

Natural Indoor Mating Assessment: Using Entry and Exit Traps. To ascertain if indoor mating occurs naturally, experiments were undertaken to compare female insemination rates in entry and exit traps mounted on houses during April–May 2006 in Sokourani (Niono), when mosquito density was high. If indoor mating occurs naturally, insemination of females from exit traps was expected to be higher than in females from entry traps as long as mosquitoes can also enter and exit the house through another window or door that was not fitted with traps. We assumed that indoor mortality was not higher in virgin females than in mated females. In the first experiment, funnel-based entry and exit traps were used. Each trap measured 30 by 30 by 30 cm. Two exit and two entry traps were

Fig. 1. Mounting exit traps in MRR experiments in Doneguebougou (top) and in experiments to assess natural insemination in Niono (bottom) using entry and exit traps (see text for details).

mounted on three different houses every other night for four nights. The traps were mounted in pairs on the door and on one window similar to those depicted in Fig. 1A. Mosquitoes freely entered and exited the house through the other window, which was left open. In the second experiment, larger traps (50 by 50 cm wide by 200 cm high) were used instead. One entry trap was mounted inside the house, and one exit trap was mounted outside (Fig. 1B). Funnels were not used in these traps, and mosquitoes flew into them in greater numbers. Mosquitoes entered and exited the house through the door opening, which was loosely covered by a cloth curtain, leaving space for flying mosquitoes. Such curtains were seen on many houses in that area (Fig. 1B). As customary in this region, three to four people slept in each house, and often a few people slept outside. The experiment did not change any of these conditions, but we confirmed that

no insecticide-impregnated bednets have been used in experimental houses. Traps were installed before 1400 hours for 24 h (first series) or 3–4 consecutive d (second series). To prevent mating inside traps, mosquitoes were continuously collected, and males and females were immediately separated in different cups and pooled based on the following time intervals: 1500–1700; 1700–1900; 1900–2000; 2000–2400; 2400–0300; 0300–0600; 0600–0900; 0900–1200; and 1200–1500 hours. During the experiments, sunset and sunrise times were close to 1900 and 0600 hours, respectively. The gonotrophic stage of females was recorded, and their insemination status was determined.

Specificity of Indoor Mating Between the Molecular Forms. The mating specificity of the molecular forms was assessed using indoor mark-release-recapture (MRR) experiments in Banambani and Donégué-

Table 1.   Assessment of indoor mating using mark, release, recapture experiments

Release date	Female recaptured/released			Insemination rate (%)			
	M	S	Ar	M	S	Ar	Mean
19 Nov. 2004	32/60	44/100	32/100	3.1	0	0	1.0
13 Jul. 2005	28/100	0/0	32/100	3.6	—	12.5	8.1
29 Jul. 2005	60/100	59/100	62/100	8.3	5.1	4.8	6.1
21 Aug. 2005	67/100	70/100	62/100	35.8	8.6	38.7	27.7
31 Aug. 2005	43/100	32/100	25/100	16.3	3.1	24	14.5
Overall	230/460	206/400	213/500	16.5	4.9	17.4	12.9

M, S, and Ar represent M form, S form, and *An. Arabiensis*, respectively. The no. of females and males released per species/forms was equal. The insemination rates are based on dissection of all recaptured females.

bougou from July to August 2007. The same procedures described above were used to produce virgin offspring of wild females, conduct the MRR experiments, and process the adults, but no exit traps were used. Two experimental designs were used, both with males of one molecular form. In the single female design, the females were all of a single form, either the same or the other form of the males (i.e., $\male^M\female^M$, $\male^M\female^S$, $\male^S\female^S$, and $\male^S\female^M$). In mix female design, females from the two molecular forms were released together in equal numbers (i.e., $\male^M\female^{M\&S}$, $\male^S\female^{M\&S}$). Every combination in the single female design was replicated three times, and those of the mix female design were replicated four times. The first two replications were carried out with 200 mosquitoes of each form and sex. In subsequent experiments, 150 males and a total of 150 females were used in both experimental designs (i.e., 75:75 M:S females in the mix female design). The fluorescent dyes were used alternately; females of each form were marked with the same color combinations across replicates and color was not confounded in molecular form.

In addition to analysis of female insemination status using logistic regression, we calculated a specificity index for each form in each experimental design in every replication as the difference between insemination rates of the pure versus cross form standardized by the average insemination rate across forms: $[Ip - Ic]/[(Ip + Ic)/2]$, where Ip and Ic represent the insemination rate of the females of the same and the other form as that of the males, respectively. The index varied from two (highest specificity) to -2 (complete preference of the other form), and 0 indicated no specificity.

Results

Insemination Rate in Indoor Mark, Release, and Recaptured Mosquitoes. To test whether *An. gambiae* might mate in natural houses, virgin fluorescently marked females and unmarked males (2–5 d old, F_1 offspring of wild caught females) were released into houses, and insemination status was determined the next morning. Exit traps (Fig. 1A) allowed mosquitoes to leave the house. Overall across species and molecular forms, the insemination rate was 13% (range, 1–27%; Table 1). The fraction of females and males collected in exit traps varied considerably between experiments (7–83% for females and 31–99% for males), possibly because of the relative humidity indoor or outdoor, moonlight intensity, etc. Overall, 62% of the females and 80% of the males were collected from exit traps. Insemination rates in females collected in houses (14%) and in the exit traps (13%) did not differ ($P > 0.28$, G test; df = 1). Mosquitoes could not mate in exit traps because they were removed as soon as they entered.

To compare the insemination rate of the species and forms, we used logistic regression accommodating variation among experiments (excluding the experiment without the S form; Table 1). Differences between species and molecular forms were found ($P < 0.0001$; Table 2), although insemination rates varied among experiments ($P < 0.0001$; Table 2). The interaction between experiment and species/form was not significant ($P > 0.56$; Table 2) and was removed. Insemination rate of the S form (6%) was lower ($P < 0.001$) than that of the M form (15%) and *An. arabiensis* (18%), whereas the difference between the M

Table 2.   Body size (wing length, mm) difference between inseminated and uninseminated females

Source	ANOVA results			Means (mm) and significance[a] of difference			
	df	F/MS	P	SpForm	Inseminated	Unnseminated	P
Model	8	15.0/0.293	0.0001				
Error	219	ND/0.020	ND	Ar	3.20	3.18	0.6
Experiment	3	15.9/0.311	0.0001	M	2.97	3.08	0.0012
Species/form	2	29.6/0.581	0.0009	S	3.15	3.20	0.3
Insemination	1	3.3/0.064	0.073				
SpFo × insemination[b]	2	4.1/0.080	0.018	All	3.11	3.16	0.073

[a] Statistical significance of the contrast between inseminated and uninseminated females of *An. arabiensis* (Ar) and the M and S molecular forms of *An. gambiae*.
[b] Interaction between species/form and insemination.
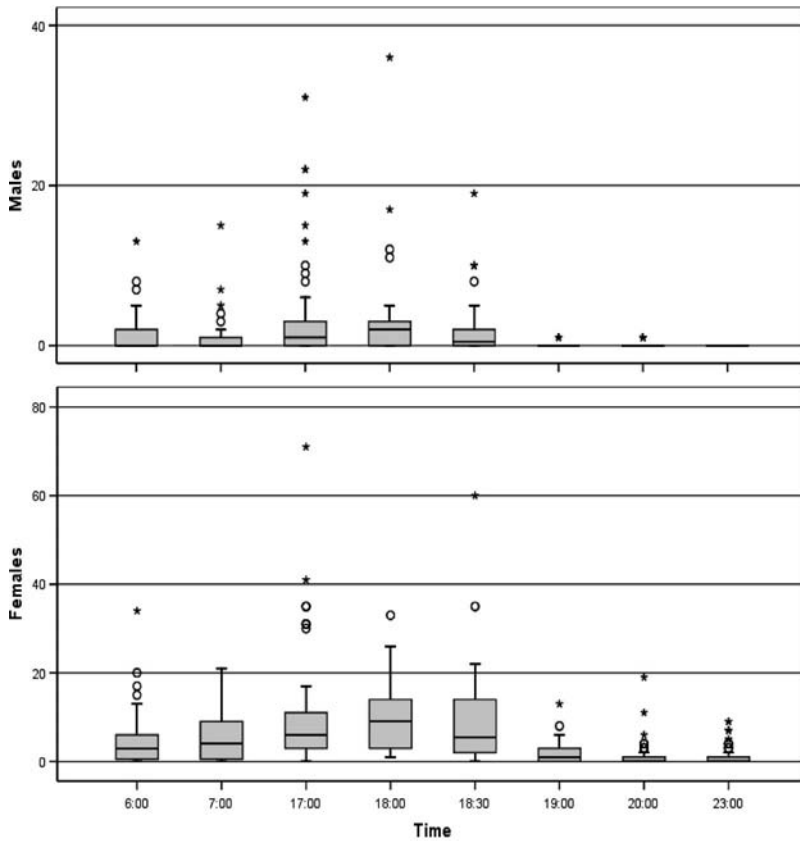ND, not determined.

Fig. 2. Mosquito density in houses estimated using pyrethrum spray catch in Doneguebougou and Banambani (pooled) over time showing variation among houses. In box-whisker plots, the box extends between the 25th and the 75th percentile, i.e., across one interquartile range (IQR), and the whiskers extend up to the most extreme value but not beyond 1.5 times the IQR. o, outlier values located 1.5–3 IQR from the median; *extreme outliers located over three IQR.

form and *An. arabiensis* were not significant ($P > 0.8$; Table 2). These results show that both species and molecular forms can mate indoors without adaptation to laboratory conditions even if they can leave the house through exit traps. The results suggest that indoor conditions are more suitable for *An. arabiensis* and the M form of *An. gambiae* than for the S form. Notably, nearly 90% of the females remained virgins under these conditions despite access to males and the advantage of early reproduction, suggesting that they preferred mating in outdoor swarms. Nevertheless, these experimental results did not show that this occurs naturally.

In experimental houses, flight activity peaked ~5–10 min after sunset and remained so for up to an hour. During this time, a humming sound, produced by flying mosquitoes was heard. Swarms, defined as groups of males flying together and forming a stationary "cloud," were not observed indoors. Instead, many males and females concentrated on the bottom half of the screen covering the door and on the adjacent walls and engaged in short (≈20 cm) flights. Small clusters of mosquitoes (6–20) were also observed near the eaves in houses with thatch roofs. Couples mating were observed flying and falling to ground near the

better illuminated door area by twilight (this was especially noticeable if white sheets were spread on the floor). However, the events leading to couple formation were not observed.

To assess if female body size affected her probability of mating indoors, we compared wing length of all inseminated females and that of a random sample of uninseminated females from each replicate experiment. Overall, inseminated females tend to be smaller than uniseminated females ($P = 0.073$), but only in the M form was this difference significant ($P < 0.0012$; Table 2).

Abundance of Males and Females in Houses. Collections of males and females in houses were conducted throughout the day in Banambani and Donéguébougou by PSC. Male density indoors increased rapidly at sunrise (0615 hours) and remained stable (average = 4 males per house, max = 36) until sunset (1845 hours, sunset time varied between 1830 and 1900 hours during the experiment). Male density declined sharply to near zero by 1900 hours (Fig. 2), precluding indoor mating during the rest of the night when male density was effectively zero. Female density indoors followed this pattern, but female density remained above zero throughout the night (Fig. 2).
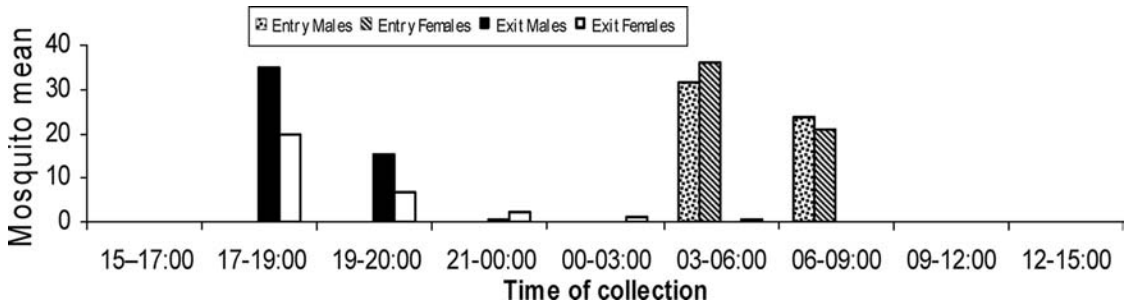
Fig. 3. Mean number of males and females collected in entry and exit traps mounted on houses in Niono over time.

Notably, the number of males (and females) was considerably higher in certain houses. Males and females represented both molecular forms (M: 38%, S: 42%) and *An. arabiensis* (20%). Indoors, 30% of females were virgin. Although they were present in houses throughout the day, relative abundance of virgin females varied among different times ($P < 0.0074$, $\chi^2 = 17.6$, df = 6), reaching peak abundance at 1830 hours (37%) and dropping to 17% at 1900 hours (data not shown). Departure of virgin females from houses, mating indoors, and entry of inseminated females indoors could explain the rapid decline in virgin females over this short period. Cohabitation satisfies a fundamental requirement for indoor mating and increased presence of virgin females indoors before and near sunset could provide an opportunity for mating. Samples from exit and entry traps mounted on houses in Niono (see Materials and Methods) similarly showed that males and females enter houses around sunrise and exit during and after sunset (Fig. 3). Altogether, these results suggest that both sexes rest indoors during the day, exit at dusk to swarm and sugar feed, and enter houses mostly during dawn. Accordingly, the main window of time available for indoor mating is during the day and around sunset.

Natural Indoor Mating. To determine whether indoor mating in *An. gambiae* occurs naturally, we compared insemination rates in samples of females from entry and exit traps mounted on a house for 24 h (or longer) of continuous collection. To allow free entry and exit of mosquitoes, traps were mounted on one window and the door, leaving another window open (first experiment) or on both windows, leaving the door open (second experiment, see Materials and Methods). If mating does not occur indoors, insemination rate of females exiting and entering the house over 24 h is expected to be equal, but if mating occurs indoors, the insemination rate in females exiting the house is expected to be higher than that in females entering the house. Experiments were conducted in the rice cultivation area near Niono, where the M form of *An. gambiae* exclusively abounds (Dolo et al. 2004). During our experiments, the M form was the only member of *An. gambiae* identified (experiment 1: 309 M form and 10 unidentified; experiment 2: 260 M form and 25 unidentified).

In the first experiment, two exit and two entry traps (see Materials and Methods) were mounted on three different houses every other day for 4 d (Fig. 4). Overall insemination rate in exit traps (44.0%) was greater than in entry traps (37.8%; $N = 1,166$; $P = 0.036$; one-sided Fisher exact test). The number of exiting females (870) was larger than those entering (296), indicating that mosquitoes avoid the entry traps. Although this did not necessarily bias the results, the traps were modified (see Materials and Methods) in
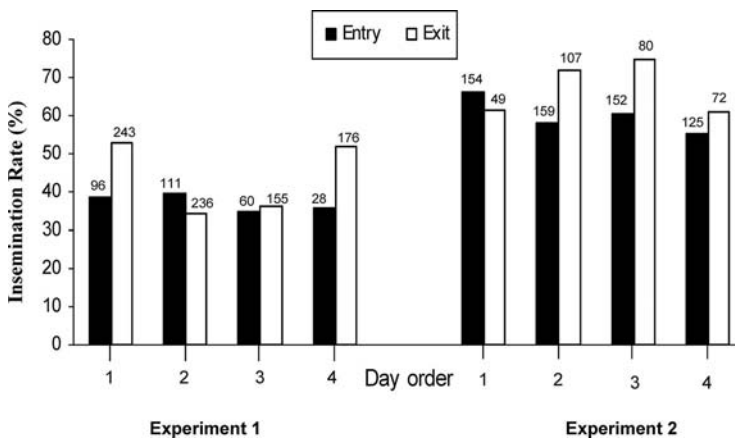

Fig. 4. Insemination rates in entry and exit traps over consecutive days. Bars represent a mean across houses pooled by day. Numbers above bars denote the number of total females sampled.
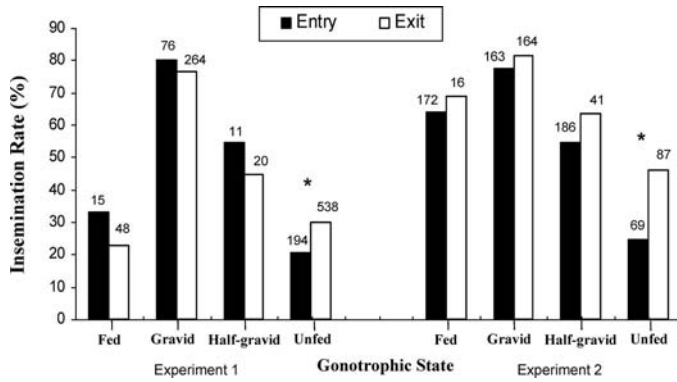
Fig. 5. Insemination rates of females in different gonotrophic states in entry and exit traps. Stars indicate significant difference between adjacent bars entry and exit traps (using one-side Fisher exact test). Numbers above bars denote the number of total females sampled.

the second experiment. The modified entry traps collected a total of 590 females versus 308 captured in the modified exit traps. The traps were used for 3–4 d consecutively on each house. As in the previous experiment, overall insemination rate in exit traps (68.5%) was greater than in entry traps (60.2%; N = 898; $P < 0.008$; one-sided Fisher exact test). When tested separately, significantly higher rate of insemination in exiting versus entering females was detected only in certain days (Fig. 4) and houses (data not shown), probably reflecting differences in power because of sample size and difference in conditions suitable for indoor mating, but no heterogeneity was detected among days (across houses) and among houses (Fig. 4; $P > 0.08$, Breslow-Day test for homogeneity) in both series of experiments. Corresponding to the movement pattern into and from houses (Fig. 3), a higher fraction of virgin females entered the house at dawn, whereas a higher fraction of inseminated females exited at dusk and throughout the night (data not shown).

Insemination rate varied with the gonotrophic state, being highest in gravid females and lowest in unfed females (Fig. 5; $P < 0.001$, likelihood ratio $\chi^2 > 90$, df = 3). Higher insemination rates in exit versus entry traps were detected in unfed females in both experiments (Fig. 5; $P < 0.013$, likelihood ratio $\chi^2 > 6$, df = 1) and was not significant in the other gonotrophic states (Fig. 5; $P > 0.4$, likelihood ratio test, df = 1), suggesting that some unfed virgin females entered to the house to mate. Importantly, higher insemination rate in females from exit traps was observed in the same physiological state (unfed females), indicating that the difference between exit and entry traps was not confounded by higher frequency of gravid and half-gravid females. These results suggest that the M molecular form of *An. gambiae* in Niono mates indoors naturally. Indoor mating may occur in certain houses and days in different intensity. Because conventional outdoor swarms were observed nearby and insemination rate in exit traps was near 5% higher than that in entry traps, we infer that only a fraction of mosquitoes mate indoors.

Specificity of Indoor Mating. The specificity of mating indoors was evaluated using MRR experiments in which virgin males of one molecular form were released into experimental houses with virgin females of both forms (mix female design) or of one form (single female design). Logistic regression of female insemination status showed strong male form effect ($P < 0.001$; Table 3) ascribed to higher insemination of females of both forms by males of the M form (33%) compared with males of the S (9%), whereas insemination rate in the females of the S molecular form (19.5%) was slightly higher than that of the M form females (15.1%, $P < 0.007$; Table 3). Importantly, cross-mating (16.1%) was as common as mating between members of the same form (18.9%), providing no evidence for indoor mating specificity as reflected by the insignificant interaction between male and female forms ($P < 0.34$). Likewise, specificity index, calculated in each experiment as the difference in insemination rate between the pure and cross forms, standardized by the average mating rate (see Materials and Methods) depicted slightly higher specificity (positive values) involving S males (Fig. 6), but the difference between forms was not significant ($P > 0.06$), and even specificity of the S form was minimal. Because the molecular forms mate assortatively (Tripet et al. 2001) and M/S hybrids are rare (della

Table 3. **Mating specificity indoors measured as frequency of insemination in experiments with different form combinations of males and females using a logistic regression**

Source	Estimate	df	χ^2	$Pr > \chi^2$
Intercept	1.50	1	656.30	<0.0001
Experimental design (single/mix)	−0.13	1	6.22	0.0126
Male form	−0.77	1	175.51	<0.0001
Female form	0.16	1	7.33	0.0068
Male form × female form	0.06	1	0.88	0.3482
Likelihood ratio	—	3	5.31	0.1504

In single female design, females were of the same or other form, whereas in mixed female design, females of both forms were present. In both designs, males were exclusively of one form.
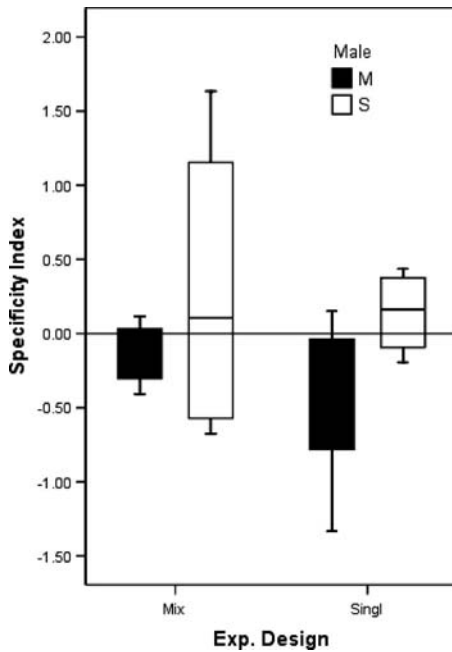
Fig. 6. Mating specificity between the molecular forms of *An. gambiae* in experiments with males of one form in the single female design (females of the same or other form than the male) or mixed female design (females of both forms with males of one form). Specificity index is calculated as the difference in insemination rate between pure and cross forms standardized by the average insemination rate in each experiment replicate. The index ranges from 2 (highest specificity) to −2 (complete preference of the other form); 0 indicates no specificity.

Torre et al. 2005), indoor mating cannot occur frequently, unless it involves a single form.

Discussion

As in many dipterans, mating in *An. gambiae* is initiated in swarms (Charlwood et al. 1980, 2002a, b, Marchand 1984, Diabate et al. 2003, 2006). However, in many species, swarms represent one of two alternative mating strategies (Thornhill and Alcock 1983). The ceratopogonid *Culicoides nubeculosus* usually swarms, but males also perch on the host (cows) and initiate mating with females who land to blood feed (Downes 1955). Unlike large chironomid midge males that swarm, smaller conspecific males mate with females aggregated in vegetation near swarms (McLachlan and Neems 1989). *Aedes aegypti* males form various types of swarms but also approach females near the host (Hartberg 1971). *An. labranchiae atroparvus* swarms outdoors and indoors, yet most mating events occur in sheltered resting sites (Cambounac and Hill 1940).

Whether indoor mating represents an alternative mating tactic in *An. gambiae* is the focus of our study. The evidence supporting this hypothesis consists of (1) observing mating of 2- to 6-d-old virgin F_1 females,

released into natural houses over a single day when they can leave the house through exit traps and without prior adaptation to laboratory conditions; (2) males and virgin females are naturally found in houses from sunrise and until an hour after sunset (throughout the day in Niono); and (3) the rate of insemination in samples of the M form from exit traps was higher than in those from entry traps, implicating that some mating occurred indoors. However, assortative mating of the molecular forms was not observed indoors. Because assortative mating is a key characteristic of the molecular forms (Tripet et al. 2001) and because hybrids are rare in Mali (Tripet et al. 2001, della Torre et al. 2005), indoor mating may occur in one but not in both forms. Notably, the evidence showing that indoor mating is an alternative mating strategy of *An. gambiae* pertains primarily to the M molecular form. Indoor MRR experiments showed substantially higher insemination rate in the M molecular form and *An. arabiensis* compared with the S form (15 versus 6%). In these experiments, mated females were smaller than virgin females; but this difference was only found in the M form. The higher insemination rate in females from exit versus entry traps was also found in the M form. This evidence remains short of direct observation of naturally occurring mating couples indoors and therefore is not definite. However, observing mosquito couples under dark settings on the background of mud walls and thatch roof is not a trivial task.

The relationships between indoor mating and density, sex ratio, form composition indoors, and conditions indoors and outdoors are not known. The insemination rate in the indoor MRR experiments ($\approx$10%/d) represent a unique subset of these parameters and therefore may not provide a "general" rate of indoor mating. The difference between exit and entry traps in Niono ($\approx$5%/d) is a more meaningful estimate for natural indoor mating under these conditions. Because virgin females constituted $\approx$40% of the total females in Niono, indoor mating constituted $\approx$12% (5/40) of total mating, whereas 88% occurred in outdoor swarms.

Mating systems are shaped by the species phylogenetic history, evolutionary constraints, and unique ecological factors (Emlen and Oring 1977, Thornhill and Alcock 1983, Yuval and Fritz 1994, Yuval 2006). Alternative mating strategies are commonly used by males that have low prospects to mate under "standard" conditions because of competitive disadvantage, such as smaller body size (Thornhill and Alcock 1983, Yuval et al. 1993). Accordingly, indoor mating may have evolved to circumvent low prospects of certain males to mate in swarms. That smaller M form females mated indoor more frequently than larger females provides some support to this explanation, if smaller males mate more successfully with smaller females. Possibly, indoor mating may have evolved because it enhances prospects of mating under conditions that are not favorable for swarms, such as dry, hot, and windy weather (e.g., during the period of Harmattan). Moreover, such conditions prevail when population density decreases, so swarms are probably rare and

localizing them would require higher energetic expanse and increased risk of predation for both sexes. Under such conditions, meeting at the resting sites, which are also the females "feeding grounds," is expected to reduce the above costs. Consistent with that explanation, the arid-adapted *An. arabiensis* and the M form of *An. gambiae* mated indoors in MRR experiments more frequently than the humid-adapted S form. However, mating in swarms probably provides females with better mate choice. To better understand how both mating strategies are maintained, more information is needed on the relative contribution of indoor mating during different seasons and population densities as well as on the differences between the males and females that participate in indoor versus outdoor mating activities during varied conditions.

Indoor mating provides insights on important aspects of *An. gambiae* mating behaviors. The low specificity of indoor mating indicates that chemical cues such as pheromones and cuticular hydrocarbons do not play a major role in form recognition, unless such cues are not released or perceived away from a swarm. Likewise, flight tone is not a primary form recognition cue (Tripet et al. 2004), unless flying is not involved in couple formation indoors. Because we used laboratory-reared F_1s in our experiments, we cannot rule out that larvae growing in natural habitats acquire cues or capacity to respond to differences between adults that promote assortative mating. However, larvae of both forms naturally cohabit many larval sites (Edillo et al. 2002), and the F_1 larvae were raised in separate pans according to molecular form. Alternatively, these results suggest that form specificity relies on spatial segregation of mating arenas; once sexually active males and females are brought together into close range, their mating is not assortative. This is supported by the absence of specificity in experiments without exit traps (specificity experiments) compared with the 2.5-fold higher indoor mating rate of the M versus S forms in houses with exit traps (MRR experiments). Supposedly virgin females of the S form depart the house before indoor mating or are less receptive to mating indoors. Female receptivity in *Ae. ageypti* determined whether insemination will occur after coupling (Gwadz et al. 1971).

Spatial segregation between swarms of the molecular forms was described previously (Diabate et al. 2006), and recent results in Donéguébougou showed striking spatial segregation between swarms of the molecular forms (A. Diabate, A. D., A.S.Y., A. Adamou, R. Gonzalez, N. Manoukis, S.F.T., R. Gwadz, and T. L., unpublished data). Indoor mating might reflect the plasticity of mating behavior, which probably has implications for the evolution of mating barriers and for introgression events between sibling species and molecular forms. Rare situations where males and females of different molecular forms (and sibling species) are confined in houses may result in introgression events even if specificity in outdoor swarms is absolute. Rare introgression events could have important ramifications for evolution of these species. We believe that these results will encourage further work on mating behavior of *An. gambiae* and focus attention on indoor mating as a possible alternative mating strategy in this complex of mosquito species. Evaluating the relative importance of these mating strategies in different conditions and understanding the reproductive isolation mechanisms between siblings and incipient species and the conditions, where cross-mating occurs, are key aspects for future research.

Acknowledgments

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