Effect of Seminal Fluids in Mating Between M and S Forms of Anopheles gambiae

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ABSTRACT
Previous studies have shown that sympatric populations of M and S molecular forms of Anopheles gambiae sensu stricto exhibit strong assortative mating. In the few documented cases of cross-mating between M and S forms, females that mated with a male of the alternative form were often also mated with a male of their own form. A potential explanation for the association between cross-mating and double mating could be that male accessory gland or sperm proteins that are responsible for inducing refractoriness to further mating by females have diverged between the M and S forms. This mechanism of postmating reproductive isolation would have important implications for our understanding of the speciation processes in the An. gambiae complex. We tested for this mechanism, by comparing the likelihood of mating, feeding, and laying eggs, as well as the fertility of females presented with males of their own form or the alternate form in the laboratory. We also compared the likelihood of remating in cross-mated and assortatively-mated females, and we analyzed their progeny to unravel patterns of sperm precedence. We found that cross-mated females differed from assortatively-mated females only in terms of egg-hatching rate and larval survival but that these effects could be attributed to hybrid vigor rather than differential response to seminal products. Cross-mating between forms was not associated with remating behavior. These results indicate that the sex proteins responsible for inhibiting further insemination and triggering the gonotrophic cycle in females have not diverged between these M and S populations. We discuss alternative explanations for the patterns of cross-mating and multiple mating observed in the field.

KEY WORDS assortive mating, polyandry, postmating reproductive isolation, speciation

The Anopheles gambiae complex includes the two most important vectors of malaria in Africa, An. arabiensis Patton and An. gambiae Giles. An. gambiae sensu stricto (s.s.) is highly polymorphic and has been subdivided into five forms, differing in their chromosomal inversion arrangements (Coluzzi et al. 1985, 2002) or alternatively into the M and S molecular forms based on fixed differences at the rDNA locus (Favia et al. 2001, Della Torre et al. 2002). The forms of An. gambiae s.s. are morphologically indistinguishable and co-occur in many areas of sub-Saharan Africa. Hybrids between the molecular forms of An. gambiae s.s. are rare but are thought to occur at least in some areas of Africa (Tripet et al. 2001, Edillo et al. 2002), and no postmating barriers to reproduction have been identified (Di Deco et al. 1980, Persiani et al. 1986). Because the low occurrence of hybrids cannot be attributed to hybrid inviability, it has been suggested that strong premating barriers maintain reproductive isolation between forms (Toure et al. 1998). Tripet et al. (2001) analyzed sperm collected from the spermathecae of mated females from a field population in Mali, West Africa. Analysis of the sperm and female DNA extracts using a polymerase chain reaction (PCR)-based diagnostic showed strong assortative mating between the M and S forms. Cross-mating was observed in two of 195 M females and one of 55 S sampled females. PCR amplification at four microsatellite loci also allowed the detection of sperm extracts exhibiting >2 alleles per locus thereby revealing the occurrence of multiple inseminations (Tripet et al. 2003). This analysis showed that in two of three cross-matings detected between the M and S forms, females were actually inseminated with sperm from both S- and M-form males (Tripet et al. 2001, 2003). The third cross-mated female had a partial bloodmeal, a characteristic that was significantly associated with non-mated females in that study (Tripet et al. 2003). Thus, despite the small number of cross-matings observed, these results are consistent with the hypothesis that females, when mated with a male of a different chromosomal or molecular form, would mate a second time. If true, such a mating pattern could be critical for understanding reproductive isolation within the An. gambiae complex.

In the Culicidae, as in many other insect families, male accessory gland (MAG) proteins and sperm are responsible for inhibiting further mating (Craig 1967, Klowden 1999, Gilliot 2003) and stimulate ovulation and oviposition (Leahy and Craig 1965, Yeh and Klow-
Socius and Howard 1994). Except for females of 1999). Goma (1963) reported that in double-mated reproductive isolation between these taxa (Howard 1967). However, injections of such extract in the abdomen of An. quadrimaculatus and An. albimanus Wiedemann did not seem to induce refractoriness (Klowden 2001). This could suggest a lesser potency of MAG proteins, differences in receptor sites, or more complex interactions between sperm and MAG proteins that induce refractoriness in some anopheline species. Independent of the mechanism involved, our observations of remating in field-collected females of the M and S form of An. gambiae suggest that the proteins associated with MAG and/or sperm that are responsible for rendering females refractory to subsequent mating may not prevent remating in reciprocal crosses between forms.

Examples from the Culicidae, Drosophilidae, Tenebrionidae, and Gryllidae also show that in multiple mating involving conspecific and heterospecific mates from closely related taxa, sperm use by females may be modulated by complex interactions between MAG proteins and sperm (Howard 1999, Klowden 1999, Gilliot 2003). Interactions between male seminal products and the female reproductive tract can result in females effectively being able to preferentially use conspecific sperm. Conspecific sperm precedence has been demonstrated between the fruit flies Drosophila simulans Sturtevant and closely related species (Price 1997, Price et al. 2001), between the beetles Tribolium castaneum Herbst and Tribolium freemani Hinton (Robinson et al. 1994), and between the ground crickets Allomemosus fasciatus De Geer and Allomemosus socius Scudder (Howard and Gregory 1993, Gregory and Howard 1994). Except for Tribolium, single heterospecific mating between the related taxa resulted in the production of viable hybrid progeny; hence, sperm precedence plays an active role in processes of reproductive isolation between these taxa (Howard 1999). Goma (1963) reported that in double-mated females of An. gambiae the sperm of one male seemed to be used preferentially and suggested that this mechanism could play a role in reproductive isolation between closely related members of this species complex. Strong second-male sperm precedence was later shown in this species in sequential forced mating of females with sterile hybrid males and nonsterile males (Bryan 1968, 1972). If An. gambiae females effectively remate after mating with a male of alternative form, they could decrease the proportion of “hybrid” progeny through sperm dilution and, potentially, further decrease it by sperm precedence (Tripet et al. 2003). Such mechanisms could explain the discrepancy between the estimated rate of cross-mating and the frequency of adult hybrids between forms found in natural populations (Lanzaro and Tripet 2003).

The hypothesis that the M and S molecular form have diverged with regard to proteins associated with MAG and/or sperm was investigated through laboratory studies of behavioral responses and reproductive success of females either cross-mated or assortatively mated. In a first experiment, we compared the likelihood to take a bloodmeal and lay eggs of M and S females after mating with a male of their own form or the alternative form. We also compared the fertility of cross-mated versus assortatively-mated females. In a second experiment, we investigated remating patterns and sperm utilization of cross and assortatively mated females by genetic analyses of the sperm content of their spermatheca and their progeny. The results are important for understanding processes of speciation in the An. gambiae complex and shed light on potential factors responsible for the low frequencies of polynuclear observed in wild mosquito populations.

Materials and Methods

Mosquito Strains. Preliminary attempts at cross-mating F1 offspring from field-collected M and S form females from sympatric populations in Mali failed because of the low cross-mating acceptance and oviposition rates characterizing F1 progeny in the laboratory (F.T., unpublished data). For these reasons, we used colonized strains with better mating and egg-laying characteristics. Blood-fed females from the N’Gabacoro Droit population in which remating was described previously (Tripet et al. 2003) were collected in January and isolated for oviposition in the laboratory at the Malaria Research Training Center, National School of Medicine and Pharmacology, Bamako, Mali. After oviposition, female carcasses and their live eggs were shipped to the University of California Davis where DNA was extracted from females by using a standard extraction protocol (Post et al. 1993) and a polymerase chain reaction (PCR)-based diagnostic (Scott et al. 1993) was used to discriminate between An. arabiensis and An. gambiae. Each individual was assigned to the rDNA-IGS molecular form M or S, by using the PCR diagnostic developed by Favia et al. (2001). In Mali, the M molecular form characterizes the Mopti chromosomal form, and the S molecular form of rDNA IGS characterizes both the Savanna and Bamako chromosomal forms. The eggs of 12 M-form females were hatched, and their larvae were pooled and reared to the adult stage to start the M-form strain. Cross-mating experiments were conducted using the ninth and 10th generation of this M strain, and double-mating experiments were conducted using the 12th generation. The S-form from that area is extremely difficult to colonize, and all our attempts failed. Consequently, we opted for an established S-form strain colonized from mosquitoes collected in the area of Kismu in Kenya and maintained at the Malaria Research & Reference Re-
source Center (Manassas, VA) (MR4) repository at the Center for Disease Control (strain reference MRA-762). Before its use, the strain was double-checked for potential contamination with the M form by running the rDNA diagnostic on a subsample of 40 females and 40 males (Favia et al. 2001) Cross-mating experiments were conducted using the 35th and 36th generation of the S strain and double-mating experiments using the 38th generation.

**First Experiment. Mating Frequencies.** Eighty males and 60 virgin females of the M and S forms were placed in 3.8-liters (1-gal) cardboard cages either with mates of their own molecular form or the alternative form, resulting in four experimental groups (M females with M males, M females with S males, S females with S males, and S females with M males). All individuals were reared under similar conditions and provided finely ground Tetramin fish food (Tetra Werke, Germany) ad libitum. Sexes were separated at the pupal stage and kept separate until they were 1–3 d old, at which time the experiment was initiated. The four cages were kept in proximity on the same shelf in the insectary, and their position (left or right, front and back) was randomly changed daily. After 3 d, 15 females were randomly picked from each cage, and their spermathecae were inspected for the presence of sperm to calculate insemination rates.

**Feeding Behavior, Oviposition, and Hatching Rate.** One day before blood-feeding, we removed the sugar-water from each cage. The next day, we counted the number of dead individuals in the experimental cages and blood-fed the remaining females (45 minus mortality) on the arm of a human host. The number of females landing on the arm was recorded at 1-min intervals. The four cages were sequentially exposed to the same surface area of the arm for a 15-min period. The next day, the groups were fed again, this time without behavioral observations, and after 3 d females were placed individually in plastic tubes for oviposition. Two days later females that laid eggs were collected, and their eggs were counted. Individual female egg batches were transferred to rearing trays, and after 3 d the number of first instars was counted to determine hatching rates.

**Fertility, Larval Survival, and Sex Ratio.** We maintained all larvae as individual families, reared them to the adult stage, and calculated the sex ratio of adults as well as the survival rate from first instars to the imaginal stage. Because, oviposition rates were much lower in the recently established M-form strain, we replicated the experimental groups involving M-form females cross-mated and assortatively mated. This allowed us to increase sample sizes for comparing the fertility, hatching rate, and survival rates between the four mating combinations. We performed exactly the same manipulations on these two extra groups, but we did not record feeding behavior nor did we include the number of females that laid eggs in those two groups in our comparison of egg-laying rates between the first four experimental groups.

**Second Experiment.** Fifty males and 50 virgin females of the M and S forms were placed in 3.8-liters cardboard cages either with mates of their own molecular form or the alternative form, resulting in four experimental groups similar to those described above. Rearing conditions, pupal sexing, and cage locations were as described in the first experiment. After 3 d, we separated males and females and immediately recombinated the groups to obtain four remating schemes. After the sixth day of mating (3 d with first males and 3 d with second males), we removed the sugar water and blood-fed the females twice over the next 2 d. Three days later, we placed females in individual tubes for egg laying. We collected females that laid eggs and stored them in 70% ethanol for genetic analyses. Individual egg batches were transferred in rearing trays as described in the first experiment. Second instars were collected and stored in ethanol for genetic analyses.

**DNA Extractions and Species/Form Diagnostic.** Females that laid eggs were dissected to separate their head and thorax from the abdomen. DNA extractions were conducted on the female heads and thoraxes and on 20 larvae from each family (Post et al. 1993). We dissected the abdomens to extract the spermatheca and collect its sperm content as described in Triplet et al. (2001). Sperm DNA extracts were used as template for multiple displacement amplification (MDA) by using the protocol and reagents provided in the GenomePhi DNA amplification kit (Amersham Biosciences Inc., Piscataway, NJ). MDA is a whole-genome amplification technique that uses random hexamer primers and bacteriophage polymerase to generate high-molecular-weight double-stranded DNA (Dean et al. 2002, Gorrochotegui-Escalante and Black 2003). The resulting highly concentrated DNA solution was diluted 100 times before being used as template in subsequent PCR reactions.

**Microsatellite DNA Analysis.** Commonly used M- and S-form diagnostics based on the IGS ribosomal DNA locus on the X chromosome (Favia et al. 2001, Fanello et al. 2002) are not very sensitive for the detection of unequal sperm contribution of males of the two forms (F.T., personal observation). Consequently, we used the microsatellite locus AGXH678, which is linked to the rDNA locus (Wang et al. 2001) and for which the two strains exhibited characteristic alleles (alleles 130, 154, and 162 bp for the M strain and a single 160-bp allele for the S-form strain). This locus does not suffer from slippage, allowing for unambiguous scoring of the two molecular forms. Primer sequences and PCR conditions for locus AGXH678 were used as described by Zheng et al. (1996). The locus was PCR amplified using fluorescent primers and an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA). PCR products were mixed with a Genescan (PerkinElmer Life and Analytical Sciences, Boston, MA) size standard and analyzed on an ABI 3100 capillary sequencer (PerkinElmer Life and Analytical Sciences). Allele sizes in base pairs were calculated using the ABI PRISM Genescan Analysis Soft-
ware and Genotyper DNA Fragment Analysis Software (PerkinElmer Life and Analytical Sciences).

Estimation of Paternal Allelic Contributions in Progeny. To verify the contribution of M and S males in the progeny of females in which sperm from alternative males was detected, we genotyped 20 larvae per female by using the microsatellite locus AGH678 linked to the rDNA locus. In potential cases of remating, we analyzed all larvae. Female larvae have two copies of the X chromosome and inherit one maternal allele and one paternal allele. Male larvae have only one copy of the X chromosome; hence, a single maternal allele. Because we did not know the sex of the larvae when analyzing our microsatellite gels, we could not distinguish single maternal allelic bands found in male larvae from the single bands that would characterize homozygote female larvae indicative of remating in females that produced mixed M-S progeny. In contrast, heterozygous M-S female larvae feature a maternal conspecific allele and the alternative paternal allele and are readily distinguishable on gels. For every family, we calculated the proportion of heterozygous female larvae found in their progeny and compared it with that predicted for cross-mating and remating. For cross-mated females, the expected proportion of heterozygous larvae in unsexed larvae is 1:2 or 0.5. In remated females, assuming the two males contribute equally to the progeny, the proportion of heterozygous female larvae in unsexed larvae is 1:4 or 0.25 (all others will be single banded mixtures of males and female larvae). If sperm precedence favored the second mating as some evidence suggests, the expected proportion of heterozygous larvae should be even lower (Bryan 1972).

All statistical analyses were performed using the JMP statistical software (Sall et al. 2004). Statistical tests performed (with test statistic in parentheses) were the one-tailed Fisher’s exact test (P value), Kruskal–Wallis (H), and Mann–Whitney (U).

Results

First Experiment. Mating Frequencies. The percentage of inseminated females after 3 d in mating cages was 93.3% (14 of 15 females) for S females mated with S males, 100% (15 of 15) for S females with M males, 53.3% (8 of 15) for M females mated with M males, and 73.3% (11 of 15) for M females mated with S males. Thus, S females kept with males of their own form were not more likely to be inseminated than those kept with M males (P = 0.934), and M females kept with M males were not more likely to be inseminated than those kept with S males (P = 1.000).

Feeding Behavior. Females from the four groups did not differ significantly in terms of the time it took them to land on the human host and feed (H = 2.16, df = 3, P = 0.539). The mean landing times ± SD were 3.1 ± 3.8 min for S females mated with S males, 1.5 ± 0.6 for S females with M males, 2.1 ± 1.8 for M females mated with M males, and 2.2 ± 2.7 for M females mated with S males (Fig. 1). S-form females that were kept with M males were not significantly more likely to feed than those kept with M males (P = 0.091), and M females kept with M males of their own form were not more likely to feed than those kept with S males (P = 0.988) (Fig. 2).

Oviposition and Hatching Rate. The percentage of females that oviposited, referred to here as oviposition rate, was 57.9% for S females mated with S males, 52.8% for S females with M males, 22.2% for M females mated with M males, and 9.0% for M females mated with S males. Oviposition rates were not significantly higher for assortatively-mated S-form females than for cross-mated females (one-tailed, P = 0.751) nor were they higher in assortatively-mated than in cross-mated M-form females (P = 0.974) (Table 1). There was no significant difference in the number of eggs laid by S females in those groups (U = 0.7, df = 1, P = 0.496) nor in M females (U = 0.0, df = 1, P = 1.000) (Table 1). Eggs were fertile in most cases except for two batches in S females kept with M males and two batches in S females kept with M males. Eggs from assortatively-mated females had a significantly lower hatching rate (unfertile batches not included) than those

Fig. 1. Distribution of female landing times, or time taken to land and feed when presented with a host, in M- and S-form An. gambiae females kept with males of their own form or the opposite form. Landing times were recorded for a 15-min period. Sample sizes were 45 females minus mortality in each group (range 30–45).

Fig. 2. Proportion of females that took a bloodmeal in a 15-min period in experimental cages where M- and S-form females (M and S) were kept with males of their own form (groups M/M and S/S) or males of the opposite form (groups M/S and S/M). Sample sizes were 45 females in each group.
resulting from cross-mating in S females ($U = 2.6, df = 1, P = 0.010$) and tended to have a lower hatching rate in M females ($U = 1.9, df = 1, P = 0.059$).

**Fertility, Larval Survival, and Sex Ratio.** Assortatively-mated S females tended to produce fewer first instars than cross-mated females ($U = 1.7, df = 1, P = 0.084$). There was no statistical difference between those two groups in M-form females ($U = 0.8, df = 1, P = 0.402$) (Table 1). The survival of larvae to the imaginal stage was significantly higher for cross-mated than assortatively-mated females in S females ($U = 3.6, df = 1, P < 0.001$) but not in M females ($U = -1.5, df = 1, P = 0.139$). The resulting number of adult offspring produced for assortatively-mated female versus cross-mated ones was significantly lower in S-form females ($U = 2.3, df = 1, P = 0.020$) but not for M females ($U = 0.0, df = 1, P = 1.000$) (Table 1). There was no significant difference in mean sex ratio between the four experimental groups ($H = 9.8, df = 3, P = 0.298$) (Table 1).

**Second Experiment. Matting and Remating Behavior.** Seventy-three percent of females that laid egg batches (67 of 90) mated with the first group of males they were presented (Table 2). Twenty-two percent of females did not mate for 3 d and mated with males from the second male group (20 of 90). In three cases (3.3%), the sperm extract was typed as M and S form, thus indicating potential remating (Table 2). All three cases were found in the experimental group where S females did not mate for 3 d and mated with males of their own form. The proportion of females mating with the first group of males was independent of whether they first mated with males of their own form or not ($\chi^2 = 0.646, df = 1, P = 0.421$). The likelihood that a female mated with the first male group tended to be higher in S form females but was independent of the form of males (logistic regression: $R^2 = 0.04, df = 2$; effect female form $\chi^2 = 3.0, P = 0.082$; effect male form $\chi^2 = 0.6, P = 0.447$).

Using the microsatellite locus AGH678 linked to the rDNA locus, we genotyped 20 larvae per female, except in cases where the eggs did not hatch or when there were fewer than 20 larvae available for analyses. Among the 70 analyzed batches of larvae, we found no cases where the molecular type of the larva was incompatible with the molecular type of the sperm or that of the mother. In one of the three cases of potential remating in S-form females, the eggs were not fertile, and we could not conduct further analyses. In the other two cases, we analyzed all available larvae (73 and 74, respectively) to calculate the proportion of heterozygous female larvae in the progeny (see Materials and Methods). This proportion was then compared with the expected proportion of heterozygous larvae for single cross-mating or remating. We found that the mean estimated proportion of heterozygous female larvae in cross-mated S and M females, and in the two potentially remated S females, did not significantly differ from the value of 0.5 expected for single cross-mating (signed rank test, $P > 0.5$ for all groups) (Fig. 3). Thus, in two of three potential cases of remating, genotyping of the progeny did not confirm remating but rather suggested contamination of the sperm extract with female tissue or strong sperm precedence in favor of the first male.

**Discussion**

Our results do not support the hypothesis of a differential response to cross-mating induced through a lack of or partial cross-reactivity of proteins associated with the male accessory gland or sperm in the M and S form of An. gambiae. Cross-mating did not affect the likelihood of females to take a bloodmeal or oviposit.

### Table 1. Number of egg batches, number of eggs per batch, number of larvae, hatching rate, number of imagoes, survival rate, and offspring sex ratio for females of the M and S form mated assortatively or cross-mated with the opposite form

<table>
<thead>
<tr>
<th>Egg batches</th>
<th>Female S/male S</th>
<th>Female S/male M</th>
<th>Female M/male M</th>
<th>Female M/male S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eggs/batch</td>
<td>22</td>
<td>19</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Larvae/family</td>
<td>49.9 ± 22.2</td>
<td>54.6 ± 22.2</td>
<td>78.8 ± 19.4</td>
<td>76.6 ± 27.4</td>
</tr>
<tr>
<td>Hatching rate (%)</td>
<td>76.0 ± 26.7</td>
<td>91.5 ± 17.0</td>
<td>83.2 ± 12.4</td>
<td>97.1 ± 2.9</td>
</tr>
<tr>
<td>Imagoes/family</td>
<td>30.3 ± 21.6</td>
<td>47.7 ± 23.6</td>
<td>58.8 ± 24.5</td>
<td>61.6 ± 22.3</td>
</tr>
<tr>
<td>Survival rate (%)</td>
<td>75.5 ± 18.4</td>
<td>92.8 ± 15.7</td>
<td>89.0 ± 20.7</td>
<td>83.4 ± 5.9</td>
</tr>
<tr>
<td>Sex ratio (male %)</td>
<td>53.1 ± 11.1</td>
<td>47.2 ± 11.5</td>
<td>46.8 ± 6.6</td>
<td>50.9 ± 13.6</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation.

### Table 2. Number and percentage (in parentheses) of spermathecae in which M-form, S-form, or M- and S-form sperm was found

<table>
<thead>
<tr>
<th>Mating scheme/sperm type</th>
<th>M sperm</th>
<th>S sperm</th>
<th>M and S sperm</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. M females + M males + S males</td>
<td>7 (70)</td>
<td>3 (30)</td>
<td>0 (0)</td>
<td>10</td>
</tr>
<tr>
<td>2. M females + S males + M males</td>
<td>4 (40)</td>
<td>6 (60)</td>
<td>0 (0)</td>
<td>10</td>
</tr>
<tr>
<td>3. S females + S males + M males</td>
<td>6 (17)</td>
<td>30 (83)</td>
<td>0 (0)</td>
<td>36</td>
</tr>
<tr>
<td>4. S females + M males + S males</td>
<td>24 (71)</td>
<td>7 (20)</td>
<td>3 (0)</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>46</td>
<td>3</td>
<td>90</td>
</tr>
</tbody>
</table>

Females were either presented for 3 d with males of their own form followed by 3 d with males of the wrong form (mating scheme 1 and 3) or with the reverse combination (mating scheme 2 and 4).
could result from the breakup of coadapted gene complexes translating into disruption of adaptations to local environment. Although there is currently no direct evidence of outbreeding depression, the maintenance of discrete forms in the absence of any mechanism of postmating barriers to reproduction and despite evidence of gene flow between forms suggests that it plays an important role (Lanzaro and Tripet 2003).

That cross-mated females were not more likely to remate with males of their own form further suggests that refractoriness to further mating was effective in these experimental groups. Successful mating in An. gambiae results in the transfer of sperm and of a mating plug that acts as a physical barrier deterring other males from coupling and contains the accessory gland proteins (Giglioli and Mason 1966, Craig 1967). Because mating plugs dissolve within 48 h (Gillies 1956), we designed our experiment to give 3 d to the females with each group of males, thereby maximizing the number of mated females that could effectively remate with the second group of males. In the field, mating plugs seem to effectively prevent remating and double mating plugs indicative of double mating within a short time are extremely rare (Giglioli and Mason 1966). It is unknown whether effective sperm transfer is achieved in those rare cases when two matings occur in short sequences. Our genetic analysis of sperm extracts from females revealed only three cases of potential remating. However, the proportion of M and S alleles in the progeny suggested that these were either contaminations by female tissues or that remating led to strong first male sperm precedence. The latter possibility would eliminate the benefits of remating and again argue against our hypothesis of adaptive divergence of MAG or sperm proteins.

The absence of a mechanism of postmating reproductive isolation due to a differential response to cross-mating between forms observed here begs the question as to what causes the correlation between cross-mating and remating found in a field population (Tripet et al. 2003). Remating in the Culicidae is thought to happen mostly after a mating with incomplete sperm and accessory gland protein transfer (Gwandz and Craig 1970, Mahmood and Reisen 1980). Although capable of mating more than five times within a short period of time, male anopheline mosquitoes can only produce two mating plugs within the same time period (Giglioli 1963, Giglioli and Mason 1966). In cage experiments, some males mate so frequently that their accessory gland and testes become depleted and cannot induce refractoriness (Mahmood and Reisen 1980). This phenomenon provides an explanation for the higher frequencies of multiple mating observed in the laboratory compared with those reported from wild populations (reviewed in Tripet et al. 2003). Both Goma (1963) and Mahmood and Reisen (1980) used genetic markers to study remating frequencies in crowded cage experiments. These experiments led to estimates of remating frequencies as high as 27.5% in An. gambiae and 9.5% in An. culicifacies.
We assumed that our M strain, colonized from a population in which remating was previously observed, would remate if cross-mated with any S-strain. However, secondary sex-protein divergence could create complex patterns of cross-reactivity between the M-form and different S-form populations. For example, females could be using the same sensory cues used in mate choice to cross-mate and remate according to these same cues. Currently, little is known about factors involved such that the sex proteins of our S strain originating between the M-form and different S-form populations could create complex patterns of cross-reactivity between the M-form and different S-form populations such that the sex proteins of our S strain originating from a distant Kenyan population fully cross-reacted with our M strain. There is clearly a need for evaluating the importance of cross-mating and remating in other areas where the two molecular forms co-occur.

There are other mechanisms that could explain remating in nature that would not require sex protein divergence. For example, we know that the forms use some unknown cues to mate assortatively. If for some reason cross-mating avoidance fails, females could be using the same sensory cues used in mate choice to assortatively mate and remate according to these same cues. Currently, little is known about factors involved in complex behavioral mechanisms such as the initiation and completion of mate search and mate choice (Klowden 2001, Gilliot 2003). Rather than be the consequence of an incomplete or alternative first mating, remating in nature could simply be due to genetic and/or environmental variation influencing mate search and mating. For example, female mating preference and level of polyandry may vary due to genetic and environmental factors that constantly generate a percentage of females that are less choosy and/or less monandrous than others. Thus, our understanding of mating behavior in An. gambiae and the Culicidae in general will depend on identifying the sensory and hormonal pathways relevant to the mating process and the major genes and environmental influences that impact them.

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