ARTICLE

Mating Alters the Cuticular Hydrocarbons of Female *Anopheles gambiae* sensu stricto and *Aedes aegypti* (Diptera: Culicidae)

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ABSTRACT The cuticular hydrocarbons of female *Anopheles gambiae* Giles sensu stricto and *Aedes aegypti* (L.) mosquitoes were analyzed before and after they mated. In *An. gambiae*, the proportions of the two cuticular hydrocarbon components, *n*-heneicosane and *n*-tricosane, were significantly reduced as the female aged and after it mated. There were no changes in the hydrocarbon composition of males after they mated. Hydrocarbon extracts from mated and unmated *An. gambiae* females as well as those from males caused a reduction in the rates of female insemination when they were applied to unmated females. Female *Ae. aegypti* showed significant changes in the proportions of *n*-heptadecane, *n*-pentacosane and *n*-hexacosane in their cuticles after mating. These data suggest that cuticular hydrocarbons may play some role in chemical communication during mosquito courtship.

KEY WORDS *Anopheles gambiae*, *Aedes aegypti*, mosquitoes, mating behavior, cuticular hydrocarbons

THE FEMALES OF many mosquito species are monogamous, or at the very least, show a reduction in their tendency to mate once they have successfully been inseminated. Although the control of mosquito mating behavior is generally believed to be modulated by male accessory gland components that are transferred during mating (Craig 1967), the mating behavior of several anopheline species was recently shown to be independent of male accessory gland substances (Klowden 2001). Unlike most aedine mosquitoes that generally mate only once due to the action of these male substances, the anophelines that have been studied are not prevented from mating once male accessory gland substances are introduced. It has yet to be determined whether anopheline females do indeed routinely mate multiply in the field in the absence of these effects from male accessory gland substances, or if other mechanisms exist that might control mating. Several laboratory and field studies have suggested that a limited amount of multiple mating may occur in anophelines (Mahmood and Reisen 1980, Gomulski 1990, Scarpassa et al. 1992, Yuval and Fritz 1994).

The mating behavior of female *Drosophila* is a good example of the complexity of the regulatory mechanisms that are involved. In these insects, mating is governed by the physiological activity of male accessory gland substances (Wolfner 1997) as well as their formation of mating plugs (Polak et al. 1998), sperm contained within the spermatheca (Manning 1962), and changes in cuticular hydrocarbons (CHC) (Antony and Jallon 1982, Scott 1986, Scott et al. 1988, Ferveur and Jallon 1996, Cobb and Ferveur 1996). All of these appear to play some role in the complex behavioral interactions that occur between female and male *Drosophila* during a mating event.

Many dipterans use contact sex pheromones for species and sex recognition (Blomquist et al. 1993), and these substances often are altered with physiological state and age (Trabalon et al. 1988, Pomonis 1989). The changes that occur in the cuticular hydrocarbons of *Drosophila melanogaster* (Meigen) affect their mating behavior. During copulation, males acquire 7,11-heptacosadiene from the female (Antony and Jallon 1982, Antony et al. 1985), and females receive 7-tricosene from the male (Scott 1986). The presence of the 7,11-heptacosadiene in the cuticle of mated males decreases female receptivity even in small quantities (Mane et al. 1983, Zawitowski and Richmond 1986); whereas in females, the elevated levels of 7-tricosene decrease their attractiveness to males (Scott 1986). There is a sexual dimorphism of CHCs among several dipterans including *Drosophila* (Jackson and Bartelt 1986, Jallon and David 1987), *Glossina* (Carlson and Langley 1986), and *Musca domestica* (L.) (Nelson et al. 1981, Gibbs et al. 1995, Mpuru et al. 2001). An exception is the lack of any sexual dichotomy of the cuticular hydrocarbons in the blow fly *Phormia regina* (Meigen) (Stoffolano et al. 1997). The sexual dimorphism, when it exists, suggests that the differing compounds may play a role in mating behavior.

Cuticular hydrocarbons acting as pheromones may also modulate mating behavior in mosquitoes. Gjullin

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et al. (1967) reported that benzene extracts of *Culex quinquefasciatus* Say, *Cx. tarsalis* Coquillett, and *Cx. pipiens* L. were attractants for conspecific females. Lang (1977) found that a contact pheromone is present on the legs of *Culiseta inornata* (Williston) that also may allow males to recognize conspecific females. Similar contact sex pheromones are used for sex recognition in *Stegomyia* mosquitoes (Nijhout and Craig 1971).

With evidence that many dipterans rely on chemical cues during mating, and that changes in the amount or types of pheromones present before and after mating can influence courtship, the lack of an obvious role for male accessory gland substances in *Anopheles gambiae* led us to examine whether its CHCs are altered after insemination and if they might play a role in their mating behavior.

Materials and Methods

Mosquito Rearing Procedures. Laboratory stock colonies used in these experiments were *Anopheles gambiae* Giles sensu stricto, Kilimanjaro strain, from insects collected in the northern region of Tanzania ≈1990 (courtesy of C. F. Curtis) and colonized in our laboratory for >6 yr, and *Aedes aegypti* (L.) Bora Bora strain, colonized in our laboratory for >15 yr. Two-hundred larvae were reared in aluminum pans with 450 ml of water, at 27°C and a photoperiod of 14:10 (L:D) h. *An. gambiae* larvae were fed a diet of ground Tetrami fish food (Tetra Werke, Germany), and *Ae. aegypti* were fed a water suspension of standard diet of brewer's yeast, lactalbumin hydrolysate, and finely ground rat chow (1:1:1 by weight) in increasing amounts as they aged. Males and females were separated at the pupal stage and adults were maintained on 10% sucrose from cotton wicks at 27°C, 80% RH, and a photoperiod of 14:10 (L:D) h. All mosquitoes were between 1–2 d old when used for mating experiments.

Mated females used to extract cuticular preparations for application experiments were not separated as pupae and females were allowed to mate freely for 5 d. Allowing females to mate for 5 d ensured that a greater proportion of them would be mated for the extraction. When extracts of mated mosquitoes were required for gas chromatography/mass spectrometry (GC/m) extract analysis, pupae also were not separated and adults were allowed to mate for 5 d after emergence for comparison to extracts of unmated mosquitoes of the same age.

Preparation of Extracts for Gas Chromatograph/Mass Spectral Analysis. CHCs were extracted from 20 mosquitoes in 200 μl hexane for 5 min in 15-ml conical glass centrifuge tubes. Extracts were then concentrated under a stream of nitrogen. Immediately before their injection into the GC/MS, the concentrated extracts were reconstituted in 15 μl hexane, and 5 μl of 1-octadecanol was added as an internal standard. All samples were maintained at −7°C and analyzed within 48 h of preparation.

Gas Chromatograph/Mass Spectral Analysis and Cuticular Hydrocarbon Determination. GC/MS was performed using a Hewlett–Packard 6890 Series gas chromatograph and a Hewlett–Packard 5973 mass selective detector (Palo Alto, CA). The GC was fitted with a split/splitless injector operated in splitless mode, and a Hewlett–Packard-5MS 5% phenyl methyl siloxane capillary column (30 m by 250 μm by 0.25 μm nominal). Oven temperature was programmed from 50 to 310°C at a rate of 10°C/min, with a final isothermal hold of 10 min, and the injector temperature was set to 250°C. One-microliter injections were made with hexane as the carrier solvent. Helium was used as the carrier gas with a flow rate of 2.0 ml/min. Hydrocarbon determination used a Hewlett–Packard 5973 electron mass selective detector controlled by MS ChemStation software.

Retention times and chain length equivalent values were obtained by comparison with known *n*-alkane standards (Howard and Infante 1996). CHC components were identified by their characteristic EI-MS (70 kev) fragmentation patterns and chain length equivalent values (Jackson and Blomquist 1976, Nelson 1978; Howard and Infante 1996). The peak area of each CHC component was calculated as a percentage of the total peak areas detected by the instrument for each sample. A Student's *t*-test was used to compare differences in the relative percentages of each CHC component between mosquitoes of different sex, age, or mating status.

Preparation of Extracts for Application. CHCs were extracted in hexane at a ratio of 2:1 μl of hexane to whole mosquitoes for 5 min in 15 ml conical glass centrifuge tubes with centrifugation at 3,400 × *g*. No fewer than 80 mosquitoes were used in each extract. The extract was pipetted from the 15-ml centrifuge tubes and centrifuged again for 5 min in 6 by 50-mm glass tissue culture tubes at 9,000 ×g. The extract was then evaporated under a gentle stream of nitrogen gas, and reconstituted in acetone at a ratio of 1:1, 1:2, or 1:3 μl acetone to whole mosquitoes used in the initial extraction. These concentrations represented 0.5, 1.0, or 1.5 mosquito equivalents of CHCs when applied in 0.5 μl. Because of its better properties as an organic solvent, hexane was used for the extraction, but it could not be used for bioassay application because of its toxicity to mosquitoes.

Unmated females were lightly anesthetized with ethyl ether. Using a Hamilton syringe, 0.5 μl of extract, containing 0.5, 1.0, or 1.5 mosquito equivalents of hydrocarbons, was applied in successive droplets to the tip of the abdomens of females in experimental groups. Controls received 0.5 μl of acetone applied in the same manner.

Treated females were placed in 30 by 21.5 by 15-cm plastic cages with males added at a ratio of 1:1 for 24 h. There were no more than 100 mosquitoes per cage. Females were then dissected and the presence or absence of sperm in the spermathecae was used as the criterion for determining their mating status. Statistical differences between the percentage of females

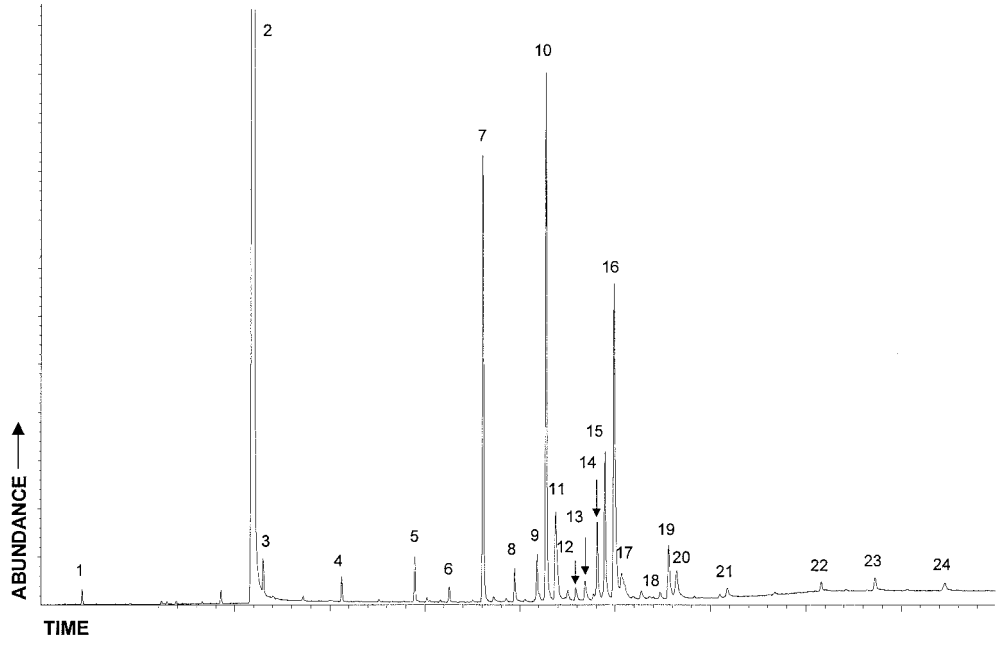

Fig. 1. Chromatogram of unmated 5-d-old *Anopheles gambiae* adult females.

mated in control and experimental groups were determined by a *z*-test.

Results

Analysis of CHC profiles of *Anopheles gambiae* Over Time and After Mating. We identified 23 CHCs for comparison in *Anopheles gambiae* (Fig. 1). The main constituents were long-chain saturated hydrocarbons with between 17 and 33 carbons, and methyl-branched hydrocarbons between C29 and C45 (Table 1). Of the peaks identified in the GC/m analysis, two were altered with age and mating status. The relative percentage of peak 3 on the cuticle of unmated females, identified as *n*-heneicosane, decreased from 5.56 ± 0.48% to 2.40 ± 0.13% during the 5 d after emergence. The percentage of *n*-heneicosane in 5-d-old mated females was significantly lower than that found on the cuticle of 5-d-old unmated females (t = 6.46, df = 5, P = 0.0013, n = 4) (Fig. 2A). Peak 4, *n*-tricosane, also decreased over the 5 d after emergence from 2.84 ± 0.32% on day 0 to 0.87 ± 0.06% on day 5. The percentage of *n*-tricosane in the cuticles of 5-d-old mated females was significantly reduced to 0.53 ± 0.06%, as compared with the percentage found on the cuticle of unmated females of the same age (t = 3.25, df = 5, P = 0.023, n = 4) (Fig. 2B). There were no significant differences between the unmated and 5-d-old mated males. Unsaturated hydrocarbons comprised a very small percentage or were completely absent from 5-d-old males.

***Anopheles gambiae* Mating Bioassay.** To determine if they played a role in mating behavior, CHC extracts from males, unmated females, or mated females were applied to unmated females that were then exposed to males. The application of extracts of unmated males to the females at a concentration of 1.0 mosquito equivalents resulted in a significant decrease in female insemination rates compared with controls (z = 2.46; P = 0.014, n = 100) (Fig. 3A), but higher and lower concentrations were not significantly different. Extracts of both mated and unmated females at 1.5 mosquito equivalents resulted in significant reductions in mating compared with the insemination rates of control females (z = 3.21; P = 0.001, n = 100) (Fig. 3B).

Table 1. Cuticular hydrocarbons of adult female *An. gambiae*

Peak no.	Retention time (min)	Hydrocarbon(s)
1	12.79	*n*-C17
2	16.43	1-octadecanol (internal standard)
3	16.59	*n*-C21
4	18.24	*n*-C23
5	19.77	*n*-C25
6	20.50	*n*-C26
7	21.21	*n*-C27
8	21.87	*n*-C28
9	22.35	C29:1
10	22.54	*n*-C29
11	22.74	13-MeC29;15-MeC29
12	23.16	*n*-C30
13	23.35	13-MeC30;14-MeC30;15-MeC30
14	23.61	C31:1
15	23.77	*n*-C31
16	23.97	13-MeC31;15-MeC31
17	24.12	11-MeC31;13-MeC31
18	24.93	*n*-C33
19	25.11	13-MeC33;15-MeC33;17-MeC33
20	25.27	9,11-DiMeC33;13,15-DiMeC33
21	26.34	11,13-DiMeC35
22	28.32	11,13-DiMeC39
23	29.44	13-MeC41
24	30.92	13-MeC43

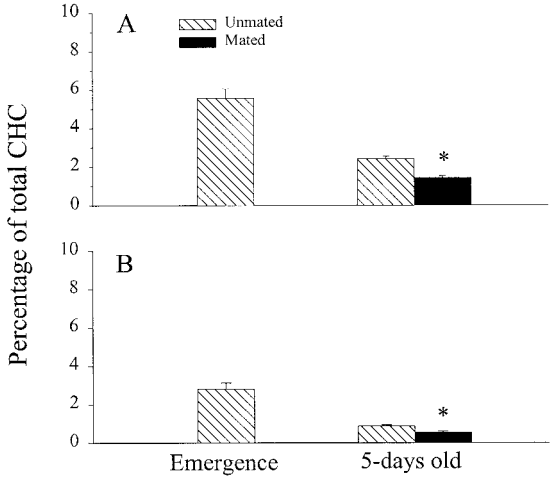

Fig. 2. Changes in peak 3, *n*-heneicosane (A) and peak 4, *n*-tricosane (B) with age and mating in *Anopheles gambiae* adult females. The asterisks indicate significant differences between relative percentages of hydrocarbons in unmated and mated 5-d-old females ($P < 0.05$). Vertical lines represent standard errors ($n = 4$).

Analysis of CHC Profiles of *Aedes aegypti* Over Time and After Mating. We identified 16 CHCs for comparison in *Ae. aegypti* adults (Fig. 4). The main constituents were long-chain saturated hydrocarbons with between 17 and 31 carbons, and methyl-branched hydrocarbons with between 29 and 43 carbons (Table 2). Of these CHCs, the relative amounts of five of the peaks changed with mating. The percentage of peak 1, *n*-heptadecane, on the cuticle of unmated females decreased from 0.79 ± 0.01% on day 0 to 0.62 ± 0.01% on day 5 after emergence. Mated 5-d-old females had a significantly greater relative percentage of *n*-heptadecane, 0.74 ± 0.04%, as compared with unmated females of the same age ($t = -3.20$, $P = 0.049$, $n = 3$) (Fig. 5A). Peak 5, *n*-pentacosane decreased from 5.83 ± 0.47% on day 0 to 5.15 ± 0.06% on day 5 after emergence. The relative percentage of *n*-pentacosane on the cuticles of 5-d-old mated females, 8.25 ± 0.06%, was significantly greater than that on the cuticles of unmated 5-d-old females ($t = -18.8$, $P < 0.0001$, $n = 3$) (Fig. 5B). Peak 6, *n*-hexacosane also decreased with age in unmated females, from 3.82 ± 0.26% to 2.69 ± 0.09% over 5 d after emergence. Five-day-old mated females had a significantly greater relative percentage of *n*-hexacosane, 3.25 ± 0.10%, as compared with levels found in the cuticles of unmated females of the same age ($t = -4.16$, $P = 0.014$, $n = 3$) (Fig. 5C). The percentage of peak 7, *n*-heptacosane, decreased in unmated females from 27.81 ± 1.85% on day 0 after emergence to 22.95 ± 0.70% on day 5 after emergence. The relative percentage of *n*-heptacosane on the cuticles of 5-d-old mated females, 26.66 ± 0.76%, was significantly greater than that on the cuticle of unmated 5-d-old females ($t = -3.58$, $P = 0.023$, $n = 4$) (Fig. 5D). Peak 14, a mixture of 13-methylhentriacontane and 15-methylhentriacontane increased as unmated females aged over 5 d after emergence from 10.97 ± 0.10% to 17.05 ± 0.16%. Five-day-old mated females had a significant reduction in the relative percentage of peak 14, 12.72 ± 0.96%, when compared with the percentage found in the cuticles of unmated females of the same age ($t = 4.43$, $P = 0.011$, $n = 3$) (Fig. 5E). There were no significant differences in males of the relative percent composition of CHCs with age or with mating.

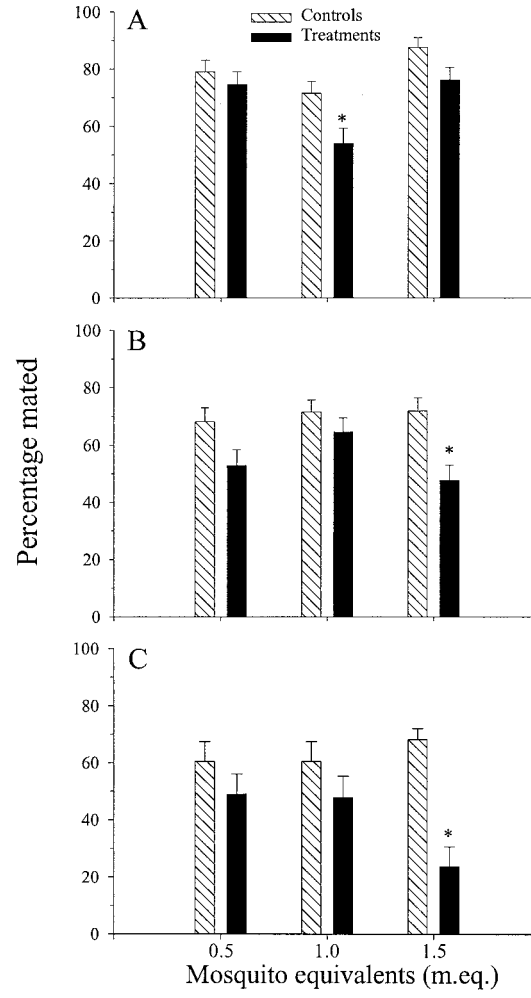

Fig. 3. Extracts of 1.5-d-old *Anopheles gambiae* unmated males (A) and females (B), and 5-d-old mated females (C) were applied to 1.5-d-old unmated females in varying concentrations. The asterisks represent significant differences between the responses of experimental and control groups ($P < 0.01$). Vertical lines represent standard errors. Sample size ranged from 50 to 100 mosquitoes.

Discussion

The relative composition of CHCs in both *An. gambiae* and *Ae. aegypti* females was altered after mating occurred. Of the 24 CHC peaks we identified in *An. gambiae*, there was a significant decline in unmated

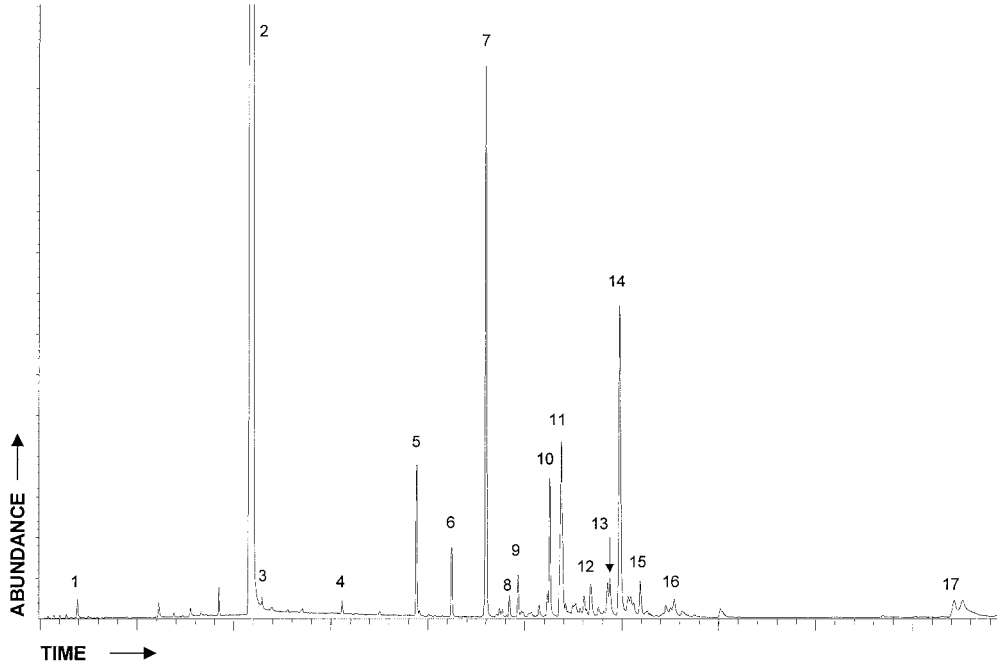

Fig. 4. Chromatogram of unmated 5-d-old *Aedes aegypti* adult females.

females of the relative percentages of only *n*-heneicosane (peak 3) and *n*-tricosane (peak 4) over time, accompanied by a significantly enhanced decrease in the percentages of these two components in mated 5-d-old female mosquitoes compared with unmated females of the same age. The topical application of extracts from unmated males and females, in certain concentrations, apparently disrupted chemical communication sufficiently to significantly reduce mating. Both unmated and mated *An. gambiae* males at 5 d old had little or completely lacked these two unsaturated hydrocarbons found in females, and mating had no significant effect on their CHC composition.

The major CHC constituents of *An. gambiae* that we observed are the same as those reported by Carlson and Service (1979, 1980). However, they reported no differences between the sexes for saturated, unsaturated, or branched hydrocarbons (Carlson and Service 1979), whereas we found that unmated 0–5 d old and mated 5-d-old males have very little or completely lack both of the unsaturated components found in females. In *D. melanogaster*, 7,11-heptacosadiene and 7-tricosene, the CHC components exchanged during mating, are both unsaturated hydrocarbons. Perhaps the absence of alkenes in male CHCs, or the presence of these unsaturated components in females, conveys additional information between the mosquitoes.

Table 2. Cuticular hydrocarbons of adult female *Ae. aegypti*

Peak no.	Retention time (min)	Hydrocarbon(s)
1	12.80	*n*-C17
2	16.43	1-octadecanol (internal standard)
3	16.60	*n*-C21
4	18.25	*n*-C23
5	19.79	*n*-C25
6	20.51	*n*-C26
7	21.22	*n*-C27
8	21.71	3-MeC27
9	21.88	*n*-C28
10	22.53	*n*-C29
11	22.78	13-MeC29;15-MeC29
12	23.38	13-MeC30;14-MeC30;15-MeC30
13	23.78	*n*-C31
14	23.97	13-MeC31;15-MeC31
15	24.41	11-MeC32
16	25.11	13-MeC33;15-MeC33;17-MeC33
17	30.89	13-MeC43

Many dipterans rely on chemical cues during mating, and the changes that occur in the amount or types of contact pheromones present after mating can influence male courtship. This chemical communication may be complex and is critical for the release of behaviors required for successful insemination. Tompkins and Hall (1981) demonstrated that extracts of mated *D. melanogaster* females stimulated less courtship than extracts of unmated flies as a result of the reduction in one component. They concluded that the decrease in attractiveness after mating was the result of the reduced level of a sex stimulant, in combination with small amounts of a courtship inhibitor. However, Scott (1986) found that the decrease in attractiveness was due to the receipt and later release of 7-tricosene after mating. Our results are consistent with the scenario presented by Tompkins and Hall (1981). The significant decrease in the relative percentages of *n*-heniecosane and *n*-tricosane in *An. gambiae* females after mating may reduce their attractiveness to courting males. This decrease in attractiveness may play a

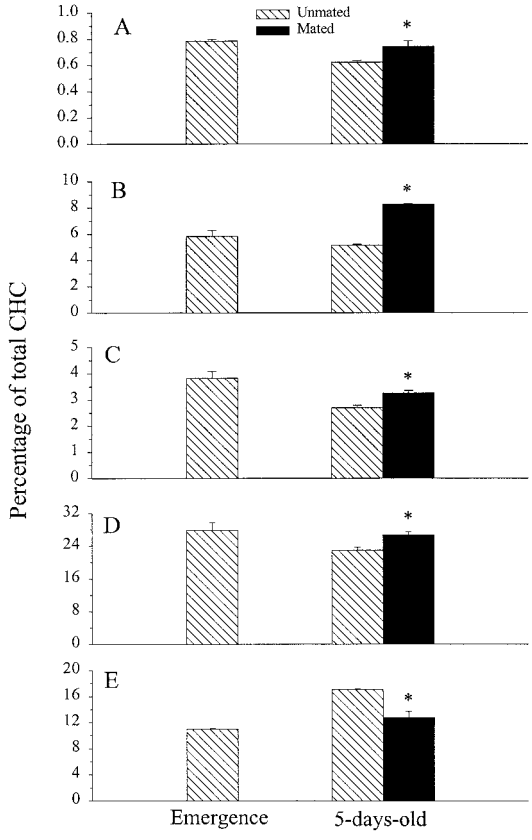

Fig. 5. Changes in peak 1, *n*-heptadecane (A), peak 5, *n*-pentacosane (B), peak 6, *n*-hexacosane (C), peak 7, *n*-heptacosane (D), and peak 14, a mixture of 13-methylhentriacontane and 15-methylhentriacontane (E) with age and mating in *Aedes aegypti* adult females. The asterisks indicate significant differences between relative percentages of hydrocarbons in unmated and mated 5-d-old females ($P < 0.05$). Vertical lines represent standard errors ($n = 3$)

role in the reduced mating of already inseminated females. However, it cannot be assumed that the changes in CHC percent composition play a role in the reduction in mating after insemination. It is possible that subsequent mating is inhibited as a result of diminished female receptivity unrelated to the change in CHC composition, or that mechanisms independent of CHCs act to inhibit future mating.

Anopheline mosquitoes are believed to be the more ancestral mosquito group compared with culicine mosquitoes (Besansky et al. 1992). The formation of the mating plug in these mosquitoes may be a remnant of sperm transfer via dipteran spermatophores as seen in ancestral nematocerans such as simuliids (Davies 1965, Giglioli and Mason 1966). A mating plug is also formed in *Drosophila* after insemination. In contrast, *Aedes* mosquitoes do not form a mating plug as a barrier to reinsemination, and have evolved the use of internal transfer of active proteins, the male accessory gland substances, to reduce the occurrence of remating.

Cuticular hydrocarbons may be an ancestral means that dipteran females have evolved to convey information about their mating history. Changes in cuticular hydrocarbon composition do occur after mating in *D. melanogaster* as well as in *Ae. aegypti* and *An. gambiae*, but in both *Ae. aegypti* and *D. melanogaster* additional mechanisms exist to reduce the possibility of multiple mating. In both *Aedes* and *Drosophila*, male accessory gland substances are responsible for initiating mating inhibition. A short-term inhibition of mating appears to result from male accessory gland components, and a longer-term inhibition is due to the presence of sperm in the spermatheca (Manning 1962, Tram and Wolfner 1998). The formation of a mating plug after insemination in *Drosophila* may be an ancestral mating inhibitory mechanism, while the use of cuticular hydrocarbons as chemosensory cues during mating may be the ancestral mechanism for communication of mating status, with male accessory gland substance induced refractoriness developing later phylogenetically in Diptera. The examination of the CHCs of *Ae. aegypti* was intended to be a control that represented a species that, unlike *An. gambiae*, used male accessory gland substances to control mating. However, we were surprised to find that the CHCs in this species were also altered after mating. We do not understand the significance of this alteration and whether CHCs are at all involved in the mating behavior of *Aedes.*

Although a number of studies have suggested that *An. gambiae* females are likely to be monogamous (Goma 1963, Bryan 1968, Charlwood and Jones 1979) there is also evidence that anophelines may not be rendered completely monogamous following insemination. Gomulski (1990) demonstrated, using sex-linked recessive eye color mutations, that *An. gambiae* females fertilized eggs with sperm from more than one male before the first gonotrophic cycle. Yuval and Fritz (1994), using enzyme electrophoresis, reported that multiple mating occurs at low frequencies in field populations of *An. freeborni.* It may be that the mechanism for female refractoriness or decreased attractiveness after mating in anophelines is less complete, possibly due to differences in mating habits that warrant less stringent barriers to reinsemination. Charlwood and Jones (1979) speculated that the different mating habits of *Ae. aegypti* and *An. gambiae* are due to the location and timing of mating in each species. In *Ae. aegypti*, both males and females are attracted to the host because both blood feeding and mating occur in the host's vicinity (Hartberg 1971). Therefore, males would commonly encounter both unmated and mated females in this location. In *An. gambiae* however, blood feeding and mating occur at different times and locations (Jones and Gubbins 1978). It is less likely that mated *An. gambiae* females would reenter the mating swarm once inseminated. It has been proposed that it is costly to evolve mechanisms that inhibit mating once a female is inseminated. If this assumption is true, *Ae. aegypti* females may benefit more from development of additional mechanisms that inhibit further mating once inseminated than would *An.*

gambiae females, since in their case the chance of being courted once they have mated is greater.

Although our data indicate that CHCs are altered after mating in both *An. gambiae* and *Ae. aegypti* females, it is still unclear if this change plays a role in the subsequent mating of these mosquitoes. As with other pheromone systems, the blend of components that presents a specific pattern to the central nervous system of the receiver is important in releasing the particular behaviors. A behavioral bioassay using applications of the specific components that differed with mating would be useful in determining their influence on male and female behavior before and after mating. Certainly, more behavioral observation and physiological experimentation are required before the role of CHCs in mosquito mating behavior is fully understood.

Acknowledgments

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