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A volatile sex attractant of tsetse flies

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Abstract

Tsetse flies transmit trypanosomes, parasites that cause devastating diseases in humans and livestock across much of sub-Saharan Africa. Chemical communication through volatile pheromones is common among insects; however, whether and how such chemical communication occurs in tsetse flies remain to be elucidated. We identified methyl palmitoleate (MPO), methyl oleate, and methyl palmitate as compounds that are produced by the tsetse fly *Glossina morsitans* and elicit strong behavioral responses. MPO evoked a behavioral response in male but not virgin female *G. morsitans*. *G. morsitans* males mounted females of another species, *G. fuscipes,* when they were treated with MPO. We further identified a subpopulation of olfactory neurons in *G. morsitans* that increase their firing rate in response to MPO and showed that infecting flies with African trypanosomes alters the chemical profile and mating behavior. The identification of volatile attractants in tsetse flies may be useful for reducing disease spreading.

One-Sentence Summary:

Methyl palmitoleate is a volatile sex attractant in tsetse flies.

Chemical communication among insects is used to identify and locate suitable mating partners. Pheromones are used to recognize a conspecific in a habitat that may contain thousands of the world's millions of insect species. Volatile pheromones have been identified and their mechanisms of action elucidated in a wide diversity of species (1).

However, little is known about chemical communication among tsetse flies. One pheromone has been identified in the tsetse fly *Glossina morsitans morsitans* (*G. morsitans)*, 15,19,23-trimethylheptatriacontane (morsilure); but this molecule consists of a chain of 37 carbon atoms and its relative vapor pressure is vanishingly low (2-7).

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Tsetse flies spread disease across much of sub-Saharan Africa. They can carry African trypanosomes, which they transmit when they bite humans or animals. In humans these parasites cause African Sleeping Sickness, and in livestock they cause a disease called nagana, which has had a devastating effect on the agricultural and economic development of Africa (8, 9).

The primary means of controlling these diseases is to control the tsetse flies that spread them. The use of traps and targets containing attractive odorants derived from animal hosts has been shown to be an effective approach for controlling disease spread (10). Because pheromones have been successfully used in the control of many other insects (11), the identification of airborne chemical communication among tsetse could be useful to enhance further their control.

Tsetse flies are also of great intrinsic biological interest. Rather than laying eggs like most other insects, females give birth to larvae (12). All embryonic and larval stages occur within the female's uterus, where progeny is nourished by maternal milk. Once laid, larvae burrow into the substrate and pupate within 30 minutes. After eclosion, male and female flies feed exclusively on vertebrate blood. It is an open question whether their chemical communication is also unusual.

The antenna of tsetse contains several classes of olfactory sensilla, including trichoid sensilla (13). Sensilla in the medial portion of the *G. morsitans* antenna respond to a variety of monomolecular odorants (14). The antennae of other tsetse species have also been shown to respond to natural mixtures of plant or host animal volatiles (15, 16).

G. morsitans mating is initiated rapidly

In order to identify chemical factors affecting tsetse mating behavior, we first placed a single male and a single virgin *G. morsitans* female together in a tube and found that the male quickly mounted the female (Figs. 1A,B). The half-time to mounting was on the order of 5 seconds. Nearly all of 20 pairs began copulating within 15 seconds.

In a second experiment, we compared directly the copulation of *G. morsitans* to that of *Drosophila melanogaster* in the same assay. The mean latency of *G. morsitans* was two orders of magnitude faster than that of *D. melanogaster:* 0.15 min ± 0.02 min, v. 22.14 min ± 4.9 min (Fig. 1C, $p<0.0001$, Mann-Whitney test). *G. morsitans* also showed a much longer duration of copulation: 58.5 min ± 5.3 min, compared to only 20.3 ± 1.0 min for *D. melanogaster* (Fig. 1D, $p<0.0001$, Mann-Whitney test). These latencies and durations were calculated from the pairs that had initiated mating within one hour, *i.e.* 18/20 pairs for *G. morsitans* and 14/20 pairs for *D. melanogaster.*

In contrast to the rapid copulation that ensued when a male *G. morsitans* encountered a virgin female, no copulation was observed when a male was paired with a non-virgin female (0%, n=20)(Fig. 1E).

Thus, tsetse showed copious mating behavior in this paradigm, with males mounting virgin females quickly and copulating for approximately an hour. Based on these data, we

hypothesized that multiple sensory modalities could contribute to tsetse mating behavior, and we investigated whether olfaction played a role.

Female but not male hexane extracts attract *G. morsitans* males

We established a behavioral paradigm to measure the attraction of *G. morsitans* males to a tsetse-sized decoy (Fig. 2A) (5). We dosed the decoy with a chemosensory stimulus of interest, placed it in a chamber with a single male, and observed for one hour. In some cases males landed on the decoy and stayed attached, often for the remainder of the experiment. We scored this response as the percentage of male flies that landed on the decoy and remained attached for at least five minutes.

We prepared extracts from virgin females, mated females, virgin males, and mated males by soaking flies in hexane with gentle shaking for 10 minutes. This procedure has been used previously to isolate pheromones from the cuticle of other insects (17-20). None of these extracts elicited attraction in this paradigm (Fig. S1A). We then considered the possibility that tsetse flies might contain attractive compounds that were temporarily sequestered beneath the surface of the fly, either in internal oenocytes or in internal glands such as those of other insects that synthesize and eventually secrete attractive pheromones (21-23). Accordingly, we prepared hexane extracts by soaking flies in hexane with gentle shaking for 24 hours, a procedure that has been successfully used to isolate pheromones from *Drosophila* and other insects (24-27).

Extracts from virgin females elicited a behavioral response in 60% (9 of 15) of cases (Fig. 2B). Mated female extracts elicited a response in 27% (4 of 15) of cases. Neither male extract evoked any response in male tsetse flies. This gender-specificity in the effect of extracts suggested a sexually dimorphic composition of effective compounds. In order to identify these compounds, we carried out a Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the extracts.

G. morsitans hexane extracts contain candidate attractants

We injected *G. morsitans* extracts into a GC-MS instrument and among the compounds identified were three fatty acids and three fatty acid methyl esters that are pheromones in other insects (22) (28) (29) (26, 30-32). (Fig. 2C) (4). These compounds were methyl palmitoleate (MPO), methyl palmitate (MP), palmitoleic acid (POA), palmitic acid (PA), methyl oleate (MO), and oleic acid (OA).

The relative abundance of these six compounds in the 24 hour extracts depended on sex and mating status (Fig. 2D); POA was more abundant in females than in males; PA, MO and OA were more abundant in females than in virgin males. We did not detect the six compounds from an extract that was prepared by a brief immersion of flies in hexane for 10 minutes (Fig. S1B).

We note that the abundance of a certain methyl branched alkane was higher in mated females than in virgin females (Fig. 2E; $p = 0.029$, Mann-Whitney test; $n = 4$), suggesting that this compound, which was present at relatively high levels in males, was transferred

from males to females during copulation. We speculated that this compound could reduce the attractiveness of mated females, thereby contributing to the lack of copulation observed among previously mated females (Fig. 1E). It could also explain why male extracts do not elicit a response from males in our decoy paradigm (Fig. 2B). Transfer of other compounds, some inhibitory, during mating has previously been shown or suggested in species of *Drosophila* (33-36).

Taken together, these results suggested the possibility that some of the six new compounds might have a behavioral effect on tsetse flies.

MPO, MP, and MO attract *G. morsitans* males

We measured the response of *G. morsitans* males to the tsetse-sized decoy perfumed with each of the six compounds (Fig. 3A). MO, MPO, and MP all elicited a strong response (Fig. 3B). MPO elicited a response in 87% (13 of 15) of cases when tested at a 10^{-3} dilution. MO elicited a response at concentrations spanning three orders of magnitude. The other three compounds did not elicit a response. We also tested nine other compounds, including three known attractants of *G. morsitans*, 1-octen-3-ol, 4-methylphenol, and acetone (10), and found that none of these compounds elicited a response in this paradigm at a 10^{-2} dilution (Fig. S2).

We further investigated MPO, which elicited the greatest response in the decoy paradigm. We tested virgin females with MPO and found no response at any concentration (Fig. 3C). Thus, MPO elicited a male-specific response in this paradigm. Surgical removal of the antennae eliminated the response, suggesting that the response relied on the olfactory system (Fig. 3D).

In insect chemical ecology, compounds are described as arrestants, aphrodisiacs, and/or attractants (37) based on the elicited behavior. Compounds can belong to more than one category. The long periods of time that males spend on a decoy dosed with MPO are reminiscent of the behavior elicited by an arrestant. However, we tested the possibility that MPO also acted as aphrodisiac and attractant.

We paired a *G. morsitans* male with a *G. fuscipes* female perfumed with MPO diluted 10^{-3} in hexane (Fig. 3E). *G. fuscipes* is a close relative of *G. morsitans* and accounts for the greatest number of cases of human African trypanosomiasis (38). In control experiments using hexane solvent alone, *G. morsitans* males made no attempts to mate with *G. fuscipes* females during a one hour observation period, *i.e.* 0% (Fig. 3F; n=20). By contrast, when paired with a *G. fuscipes* female perfumed with MPO, in 60% (9 of 15) of cases the *G. morsitans* male mounted the *G. fuscipes* females in an apparent attempt to copulate (Fig. 3F). In no case did the coupling persist; in all cases the female separated from the male shortly after mounting. These data suggest that MPO acts as an aphrodisiac for *G. morsitans* males.

To test whether the compounds identified acted as attractants, we measured olfactory attraction in a T-maze assay (Fig. 3G). In this paradigm flies made a choice between two tubes, of which one contained a volatile odor and the other contained a diluent control.

Flies that entered either tube rarely left. We tested all six compounds in this assay at concentrations spanning five orders of magnitude. MO, MPO, and MP all elicited an attractive response at one or more concentrations (Fig. 3H).

In summary, MPO elicited behavior expected of an arrestant, an aphrodisiac, and an attractant. MO and MP also elicited behavioral responses from males. Next, we asked whether there are olfactory neurons in the *G. morsitans* antenna that might mediate these responses.

G. morsitans olfactory neurons respond to extracts and to MPO

We first asked whether the extracts themselves elicited electrophysiological responses from ORNs of the tsetse antenna (Fig. 4A). We focused on trichoid sensilla because in *Drosophila* volatile pheromones elicit responses primarily from trichoid sensilla (26).

Using single-sensillum electrophysiology and a modified stimulus delivery system in which an airflow carried odors to the antenna from a source 24 cm away (Fig. 4B), we recorded from trichoid sensilla located on the lateral face of the antenna (Fig. 4A). We tested hexane extracts from virgin females, mated females, virgin males, and mated males.

Each of the four extracts elicited responses from a number of sensilla, and each of the four groups of flies (virgin female, mated female, virgin male, mated male) contained sensilla that responded to extracts (Fig. S3). Overall, 15% (12 of 82) of the tested sensilla responded to a least one extract with a response of ≥10 spikes/s.

We next tested the six compounds against trichoid sensilla of the antenna. We tested trichoid sensilla from males (n=56) and virgin females (n=50). Delivering the compounds via an airflow using the system shown in Fig. 4B, we found that MPO elicited excitatory responses from a subset of sensilla (Fig. 4C). The responding sensilla have two ORNs, one designated the "A" neuron, which produces spikes of large amplitude, and one, the "B" neuron, which produces spikes of small amplitude (Fig. 4D). The responses to MPO were from the B neuron (Fig. 5C,E). Responses were observed in sensilla of both males and virgin females (Fig. 4C,E); 20% of tested male sensilla (11 of 56) and 26% of female sensilla (13 of 50) responded with >20 spikes/s at the tested dose. We observed few if any responses from the tested trichoid sensilla with the other five compounds using this delivery system (Fig. 4E).

The response magnitudes to MPO were greater in males than in virgin females (Fig. 4F). To determine if responses were dose-dependent we tested a range of concentrations. Since trichoid sensilla were heterogeneous in their responses to MPO we carried out this analysis on two individual sensilla, one in males and one in virgin females, which are located on the proximal portion of the antenna and had produced the highest responses in our survey. Increasing doses produced increasing responses in both cases (Figs. 4G,H).

In order to identify and classify the ORNs that respond to MPO, we tested the sensilla with a panel of volatile compounds. Trichoid sensilla in *G. morsitans* on the opposite side of the antenna (the medial side) respond to odorants (14). We therefore included in our analysis of the trichoid sensilla nine odorants in addition to the six compounds from the

tsetse extract. The nine odorants included three that are known to attract *G. morsitans:* 1-octen-3-ol, acetone, and 4-methyl phenol (also known as p-cresol) (10).

We then classified the ORNs based on their responses to the 15 compounds, using a hierarchical cluster analysis. Neurons classified as "A" fell into three functional classes, whereas neurons classified as "B" fell into four functional classes, in both males (Fig. 5A) and females (Fig. S4). In some cases the classes appeared qualitatively similar but with different response magnitudes (*e.g.* B2 and B3 in Fig. 5B). The response profiles of most male ORNs, *e.g.* B1, have female counterparts that appear similar. (In the case of B1, the male and female counterparts were indistinguishable; $p>0.05$, ANOSIM test).

Neurons that responded to MPO, the B1 neurons, also responded to known *G. morsitans* attractants: two of the strongest responses were to the attractants 4-methylphenol and 1-octen-3-ol (Fig. 5B). These results suggest that MPO may mediate attractive behaviors at least in part via these ORNs.

MO and MP activate trichoid neurons at close range

MO and MP elicited behavioral responses from *G. morsitans* (Figs. 3B,H), but not electrophysiological responses in our initial analysis (Fig. 4E). We reasoned that MO and MP might elicit physiological responses from sensilla on untested regions of the large and complex tsetse antenna (13). Alternatively, it is possible that given the low volatility of these long-chain compounds, the dosage or odorant dynamics used in the preceding survey were not adequate to elicit responses. To address this latter possibility, we modified the odor delivery system (Fig. 5C). We reduced the distance from the odor source to the antennal preparation, to ~1 mm, and we eliminated the airflow, analogous to an approach successfully used with pheromones in *Drosophila* (39). This close proximity of odor source to antenna is reminiscent of the proximity of two mating flies in a natural context.

The diluent control produced no firing among the tested male trichoid sensilla, and MPO elicited responses from B neurons but not A neurons (Fig. 5D,E), consistent with our earlier results (Fig. 4C). However, using this new delivery system, MP elicited responses from B neurons, and MO activated both A and B neurons.

These results indicate that MP and MO, as well as MPO, activate olfactory neurons, consistent with their activity in attracting and arresting *G. morsitans* males.

G. fuscipes differs from *G. morsitans* in responses to MPO, MP, MO and other odorants

We next examined the response of *G. fuscipes* trichoid sensilla to MPO, MP, and MO, using the same close-range delivery system used for *G. morsitans*. We again focused on trichoid sensilla that cover the lateral side of the male antenna. In contrast to our results with *G. morsitans,* the tested methyl esters elicited little if any response from any tested sensilla when used at the same concentrations (Fig. S5).

Consistent with these physiological results, behavioral testing did not reveal a response to these compounds, in either the decoy paradigm (Fig. S5B) or the T-maze preference paradigm (Fig. S5C). We also measured the abundance of MPO, MP, MO and the other three compounds in virgin female, mated female, virgin male, and mated male *G. fuscipes*. In almost all cases the relative abundance of these compounds in *G. fuscipes* was lower than that in *G. morsitans* (Fig. S6).

The lack of responses to MPO, MP, and MO motivated us to ask whether these *G. fuscipes* sensilla respond to other odorants, and if so whether the response profiles to these odorants differ from those of *G. morsitans.* Accordingly, we surveyed the trichoid sensilla on the lateral face of the male antenna with the same 9 odorants tested against *G. morsitans.*

Strong olfactory responses were recorded in *G. fuscipes* (Fig. S5D). The A neurons could be divided via a cluster analysis into four distinguishable classes, as opposed to three in *G. morsitans;* the B neurons fell into four classes in both species. In both species there are classes that responded to none of the tested odorants. Testing with more odorants might allow more detailed classification. Inhibitory responses were observed in *G. fuscipes,* but not in *G. morsitans.* One class of B neurons, B4, was strongly inhibited by several odorants, including 6-methyl-5-hepten-2-one, which excited another class of B neuron, B3.

Trypanosome infection affects chemical profiles and mating behavior

We next investigated the impact of trypanosome infection on olfactory physiology, profiles of body-wash extracts, and mating. All previous experiments in this study were conducted with uninfected tsetse flies. We infected *G. morsitans* with *Trypanosoma brucei brucei* (strain RUMP 503) under laboratory conditions and compared them to uninfected controls.

We first asked whether olfactory responses underwent major alterations after infection. From infected flies we measured the responses of 27 trichoid sensilla on the lateral surface of the antenna to a panel of 10 compounds, including MPO (Fig. S7A). We then compared neuronal spaces constructed from the neuronal responses of infected (Fig. S7B) and uninfected *G. morsitans* (Fig. 5). We found many common features and much overall similarity ($p=0.08$, $R=0.0103$ for A neurons; $p=0.04$, $R=0.8$ for B neurons; ANOSIM based on Bray-Curtis similarity)(Fig. S7B).

We then compared chemical profiles of infected and uninfected flies. Hexane extracts of infected mated males and females both contained 21 small volatile compounds, including α-pinene, that were not observed in uninfected, control flies or in the solvent control (Fig. 6A). These 21 compounds were specific not only to infected flies, but to mated flies: we did not observe them in either infected or uninfected virgin males or females (Fig. S7C). To test the robustness of these results we individually examined 19-29 flies of each of the eight types (infected and uninfected mated males, infected and uninfected mated females, infected and uninfected virgin males, infected and uninfected virgin females) and found a high degree of consistency: the appearance of these compounds depended on both infection and mating.

Finally we investigated whether infection had any effects on mating behavior. We placed a virgin female in a tube with two males, one infected and one uninfected. The female mated with the two kinds of males with equal frequency (Fig. 6B, $p>0.05$, Chi-squared test, $n=20$). However, when an uninfected virgin male was placed with two females, one infected and one uninfected, in all of 20 trials the male mated with the uninfected female (Fig. 6C); the infected females were much less receptive to the males.

Discussion

G. morsitans males mounted females very quickly upon coming into each other's vicinity. Although behaviors in laboratory settings are imperfect representations of behaviors in natural settings, in the same laboratory paradigm, pairs of *G. morsitans* had mounting latencies that were 1/100th those of *D. melanogaster.* Whereas *D. melanogaster* males engage in a prolonged and elaborate series of courtship behaviors prior to copulation (40, 41), *G. morsitans* males were quick to mount.

Once initiated, copulation lasted much longer in *G. morsitans* than in *D. melanogaster.* Moreover, the copulation time of *G. morsitans*, 58 minutes, exceeded that of all but one of 82 species of *Drosophila* considered in an analysis (42). Our measurement of mean copulation time is consistent with the observation of long-lasting copulation in studies of *G. morsitans* insemination and ovulation (43), and of interactions between males with decoys (44).

These differences between the two fly species raise questions about whether the molecular, cellular and circuit basis of sexual behavior differs markedly between tsetse flies and *Drosophila*, whose ecology, physiology, and behavior are quite distinct from those of tsetse flies (40, 41). We have carried out behavioral, chemical, and electrophysiological analysis of tsetse in an effort to gain insight into the underpinnings of tsetse fly sexual behavior.

We have found evidence that volatile compounds can affect mating behavior in tsetse flies. There has been little evidence to support a role for volatile pheromones in these animals. The compound 15,19,23-trimethylheptatriacontane has previously been identified as a contact sex pheromone in *G. morsitans* (5, 45). Five-day-old females contain more than 4 mg of 15,19,23-trimethylheptatriacontane, and when microgram quantities were placed on dead males they elicited copulatory responses from other males (7). Although widely considered a contact pheromone, this compound was reported in one study to elicit an olfactory response from the antenna (46); however, this finding has been controversial (2, 3), in part because the compound consists of a chain of 37 carbon atoms and its relative vapor pressure has been calculated to be 12 orders of magnitude lower than that of (Z)-9-tricosene, a volatile sex pheromone of houseflies (47).

We have found that *G. morsitans* flies produce MPO, MO, and MP; these volatile compounds have chain lengths of only 16 carbons, much shorter than the 37 carbon chain length of 5,19,23-trimethylheptatriacontane, or the 23 carbons of of (Z)-9-tricosene. MPO, MO, and MP had an arrestant effect in a decoy assay and an attractive effect in a T-maze assay.

MPO is the compound for which we found the most evidence to support a role as a volatile pheromone in *G. morsitans.* There are several arguments in favor of such a role. MPO acts as a pheromone in other species (22, 28). It elicited a response from males but not virgin females in the decoy assay. MPO showed characteristics of an aphrodisiac: *G. morsitans* males mounted *G. fuscipes* virgin females that were perfumed with MPO, but not with control *G. fuscipes* females, consistent with a role for MPO in driving male *G. morsitans* sexual behavior. MPO elicited greater responses from ORNs of males than virgin females. It elicited little if any response from any tested sensilla in *G. fuscipes* (Fig. S5A). While we cannot exclude the possibility of responses from untested sensilla, we observed no behavioral responses to MPO in *G. fuscipes*, in either of two paradigms.

However, MPO was recovered from a 24 hour hexane extract but was not detectable in a 10 minute extract, as one might have expected of a cuticular pheromone. The 24 hour extracts have two salient properties. They showed sexual dimorphism in their effects in the decoy paradigm, and the number of peaks in the 24 hour extract did not greatly exceed the number in the 10 minute extracts. Thus, among the universe of small molecules within a fly, very few were detected in the 24 hour extract, and MPO is among them.

We do not know the typical distribution of MPO within the fly. MPO might be synthesized and stored in an internal gland, such as those of Tephritid fruit flies, cockroaches, and a variety of hymenopterans and lepidopterans, which produce pheromones and then release them during certain behaviors, often via a duct to a pore in the cuticle (48-51); in some species a pheromone is released only when needed (48, 52). Alternatively, MPO might reside largely in internal oenocytes that produce pheromones and that deliver them to the cuticle only when the fly is in certain nutritional or behavioral states (53-57). However, the absence of detectable MPO in the cuticular layer of flies fed and maintained under standard laboratory conditions may explain why it has not been identified previously as a pheromone in tsetse.

Insect pheromones are extremely diverse in many ways. Some, such as the classic example of bombykol in moths, act in long-distance attraction, are secreted specifically by females, elicit electrophysiological responses from narrowly tuned male-specific ORNs, and elicit male-specific behavioral responses (58, 59). By contrast, other insect pheromones i) elicit other behaviors (60); ii) are secreted at comparable levels by both sexes (26, 39); iii) elicit electrophysiological responses from broadly tuned ORNs in both sexes (5, 26, 61); or iv) elicit behavioral responses from both sexes (26). The sensitivity of ORNs to their cognate pheromones also varies greatly: one molecule of bombykol is sufficient to elicit a nervous impulse from a male moth ORN (62, 63); by contrast, a 10^{-2} dilution of *cis*-vaccenyl acetate was required to elicit responses from its cognate at1 neuron of *Drosophila* (64), similar to the sensitivity of tsetse trichoid sensilla to MPO that we have observed.

MPO has shown characteristics of both an attractant and an arrestant in laboratory tests. We speculate that as an attractant produced by females it might contribute to the initiation of mating; whereas, as an arrestant it might contribute to the maintenance of mating, preventing its premature termination.

MPO activates neurons that also respond to the olfactory attractants 4-methylphenol and 1-octen-3-ol. These results support the interpretation that MPO activates a circuit that mediates olfactory attraction. Mating encounters of *Glossina* generally occur on or near hosts (9), and 4-methylphenol, and 1-octen-3-ol are all host odors (10). The circuit might be activated more strongly when both fly and host cues are presented together. We note precedent for the dual response of neurons to pheromones and odorants: in *Drosophila* the ab9A ORNs respond both to the pheromone (Z)-4-undecenal and to food odors (61). These ORNs express two receptors, one of which responds to (Z)-4-undecenal and the other to food odors.

In addition to promoting or maintaining mounting, MPO could have roles in any of a series of stereotyped sexual behaviors that occur after mounting (65). MPO could also affect female receptivity.

Our results support a role for olfactory cues in tsetse mating behavior. The overall role of olfactory cues in tsetse mating, however, remains to be determined. Multiple sensory modalities are likely to influence mating behavior in tsetse, as in many other animal species (3). Moreover, there is evidence that *G. morsitans* males from which antennae have been surgically removed are able to mount conspecific females (2). These results are consistent with those from *Drosophila*, in which cues of several modalities act in mating behavior, but mating does not require the presence of all (66).

We found that infection with trypanosomes changed the chemical profile of mated males and mated females: 21 volatile compounds were identified in the body-wash extracts of infected flies but not in their uninfected counterparts. This finding raises a series of questions, including whether these compounds are synthesized by the fly or by the parasites. Malaria parasites that infect mosquitoes produce a variety of volatile compounds, including α-pinene, which is one of the 21 compounds we identified (67). Although trypanosomes could synthesize the 21 identified compounds directly, infection also causes a wide variety of effects on the gene expression of tsetse flies (68, 69), some of which could promote the production of volatile compounds by the fly. Perhaps the appearance of these compounds requires mating because it allows their transport from the interior of the fly to the external surface. In support of this possibility, evidence indicates that the act of mating exposes pores in the ninth tergite of the male, through which compounds could be released (70).

Previous data have shown that a *G. morsitans* odor receptor, GmmOr35 responds to α-pinene (13), supporting the possibility that the infection or mating status of a fly could be discerned by another fly via olfaction; α-pinene also elicited antennal responses from three other species of *Glossina* (16).

Infection with trypanosomes reduced mating receptivity in females. Animals fighting infections may invest less in reproduction, as there is a trade-off between these processes due to limited energy resources (71). Consistent with this argument, fecundity of infected female tsetse flies is reduced (72), and mosquitoes infected with malaria parasites produce fewer eggs (73, 74).

The identification of volatile pheromones in tsetse flies may in the long term have important implications for disease control. One of the most effective means of controlling tsetse flies is with traps and targets, which historically have included attractive host odors. Our results now suggest the possibility of using tsetse odors, in combination with long-range attraction to host odors, for controlling tsetse flies and disease spreading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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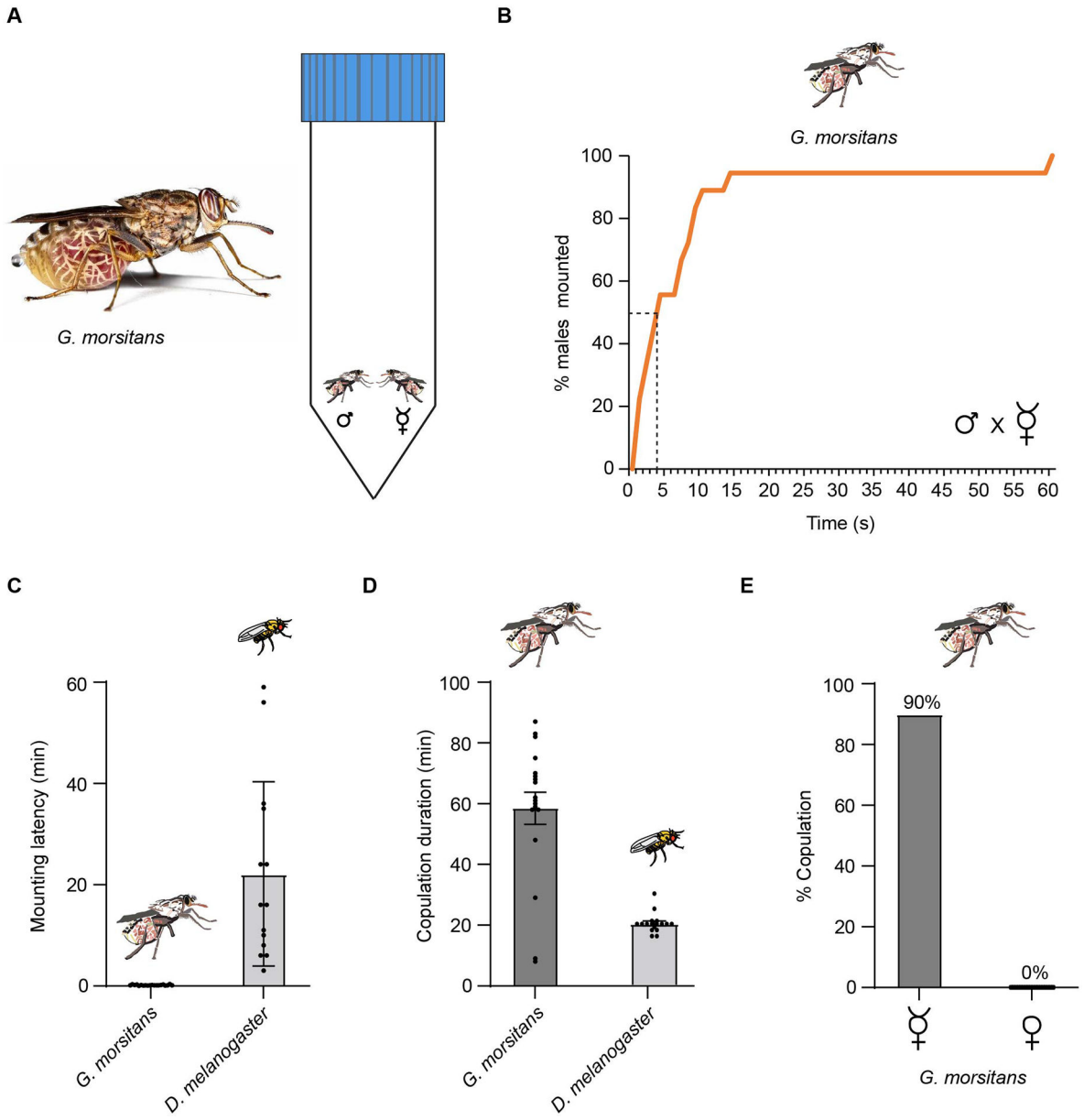

Figure 1. Mating of *G. morsitans*.

(**A**) The mating assay. A male and a female are placed together in a 50 ml tube, 11.5 cm in height. (**B**) Percentage of *G. morsitans* males that had mounted a virgin female in the mating assay as a function of time. n=20. (**C**) Copulation latencies of *G. morsitans* and *D. melanogaster* males with a virgin female. 14/20 pairs of *D. melanogaster* and 18/20 pairs of *G. morsitans* mated; n=14 for *D. melanogaster* and n=18 for *G. morsitans.* Error bars are SEM. (**D**) Copulation duration. n=14 for *D. melanogaster* and n=18 for *G. morsitans.* Error bars are SEM. (**E**) Percentage of copulation of *G. morsitans* males with either virgin females or mated females. n=20

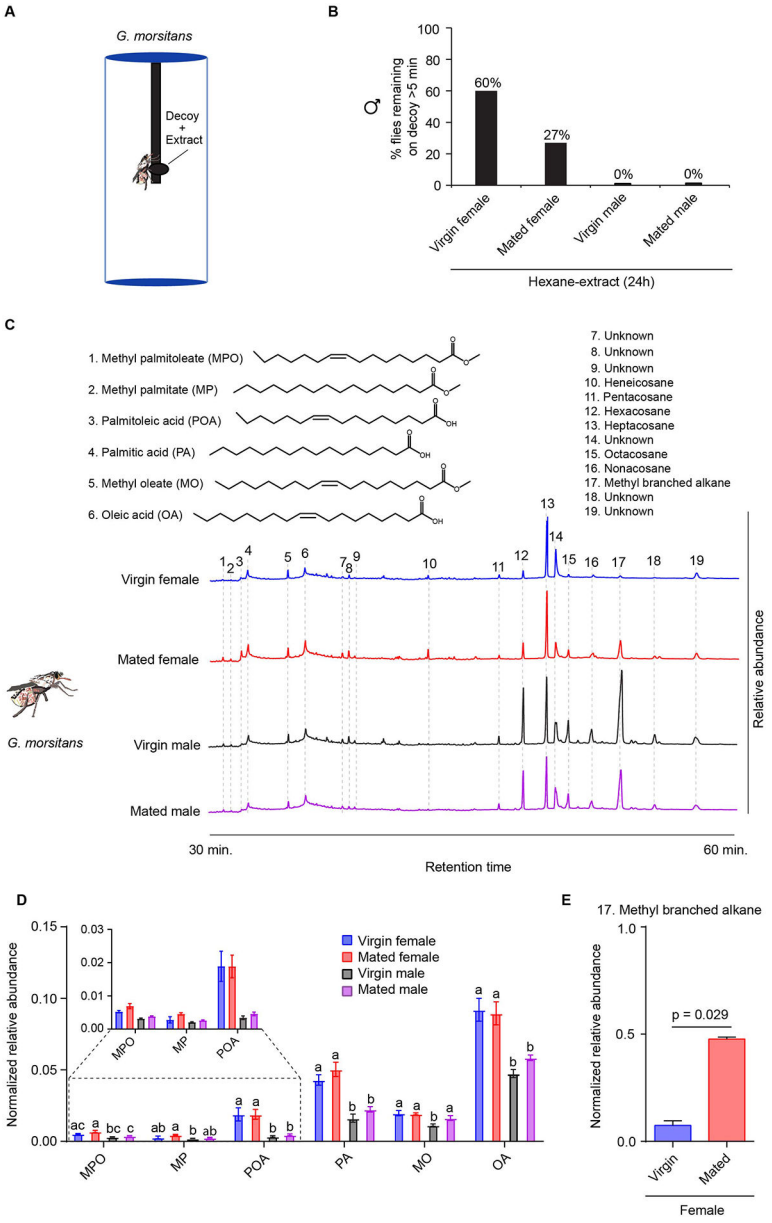

Figure 2. *G. morsitans* hexane extracts contain candidate attractants.

(**A**) The decoy paradigm. The decoy consists of a knot in a length of black yarn, dosed with extract. (**B**) Percentage of males that landed on the decoy, when dosed with a 24 hour extract, and remained on it continuously for more than 5 minutes. n=15. (**C**) Examples of total ion current chromatograms of 24 hour hexane extracts. Peak numbers correspond to the identified compounds. Additional data are shown in Fig. S1. (**D**) Normalized relative abundance of compounds identified in 24 hour hexane extracts. Normalization is to the sum of the areas of all peaks. One-way ANOVA followed by Tukey's multiple comparison test; n = 4. Values indicated with different letters are significantly different. Error bars are SEM. (**E**) Normalized relative abundance of methyl branched alkane (compound # 17) in virgin and mated females. Mann-Whitney test; n = 4.

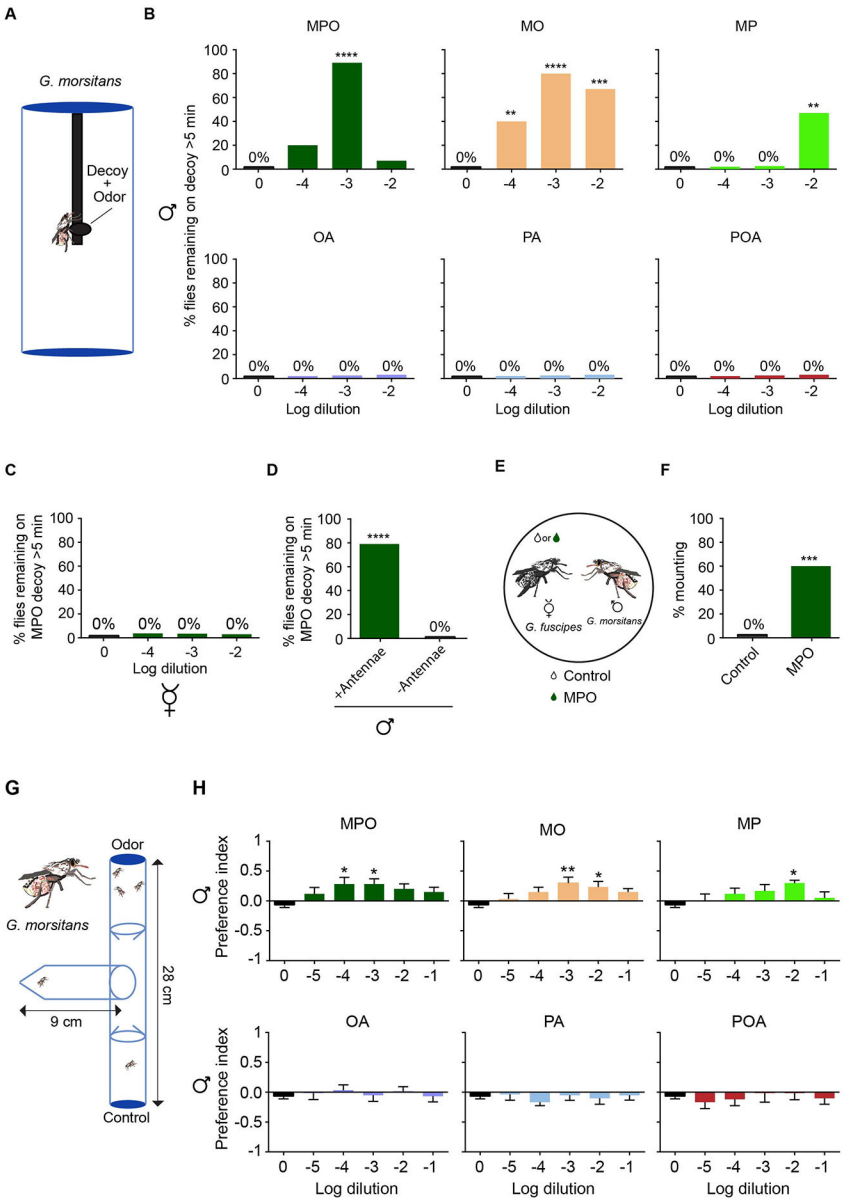

Figure 3. MPO acts as an aphrodisiac pheromone in *G. morsitans* males.

(**A**) The decoy paradigm. The decoy is dosed with a candidate pheromone or odor. (**B**) Percentage of males that landed on the decoy and remained on it continuously for more than 5 minutes. n=15. Chi-squared test. (**C**) Percentage of *G. morsitans* virgin females that stayed for more than 5 minutes on a decoy dosed with a range of dilutions of methyl palmitoleate. n=15. (**D**) Dependence of *G. morsitans* response to decoy on antennae. The decoy was dosed with a 10^{-3} dilution of MPO in paraffin oil. n=15. Chi-squared test. (**E**) Schematic of a test with a perfumed *G. fuscipes* virgin female and a *G. morsitans* male. (**F**) Percentage of mounting when a *G. fuscipes* virgin female is perfumed with a 10^{-3} dilution of MPO, compared to a female perfumed with diluent alone. n=15. Chi-squared test. (**G**) The T-maze paradigm. Flies are initially in the tube shown at the left. The apparatus is placed horizontally on a benchtop. (**H**) Behavioral responses of *G. morsitans* males in the

T-maze paradigm. *p<0.05,**p<0.01, Wilcoxon Signed Ranked Test, n=12. Error bars are SEM.

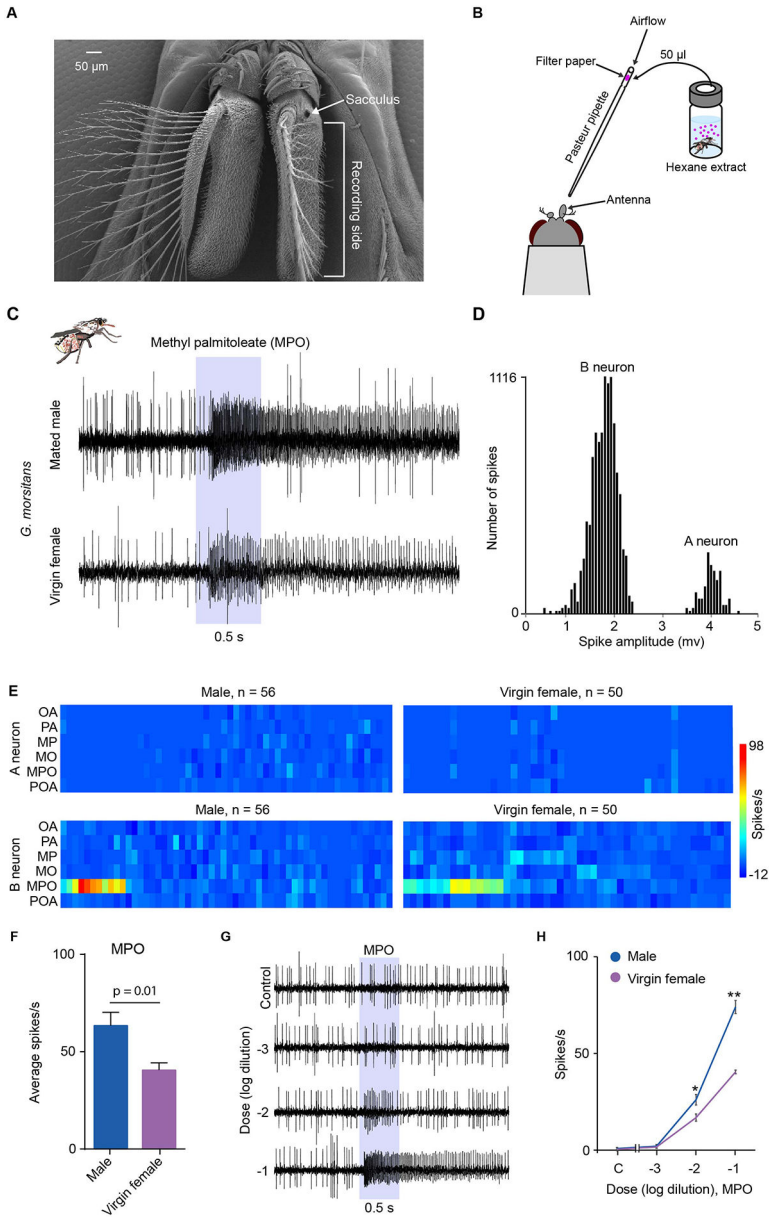

Figure 4. MPO activates ORNs in certain antennal sensilla.

(**A**) Scanning electron micrograph of antennae showing the region from which electrophysiological recordings were taken from trichoid sensilla. (**B**) The stimulus delivery system. (**C**) Example traces of electrophysiological responses of trichoid sensilla in males and virgin females to a 10^{-1} dilution of methyl palmitoleate. Shaded area indicates the 0.5s odor stimulus. (**D**) The bimodal distribution of spike amplitudes in trichoid sensilla that respond to MPO in mated males. “A” and “B” indicate subpopulations of spikes attributed to neurons A and B. (**E**) Heatmap of electrophysiological responses of trichoid sensilla to compounds identified in body-wash extracts. Each rectangle shows the response magnitude of one neuron, in spikes/s, each from a different trichoid sensillum, when tested with a 10^{-1} dilution of each compound. (**F**) Mean responses to a 10^{-1} dilution of MPO in males and virgin females. Mann-Whitney test, n=11 for males and n=15 for virgin females. Error bars

are SEM. (**G**) Example traces of electrophysiological responses of trichoid sensilla in virgin females to a range of dilutions of methyl palmitoleate. In the control trace the stimulus was the diluent control. (**H**) Dose-response curves of trichoid sensilla to a range of dilutions of methyl palmitoleate. *$p < 0.05$; **$p < 0.01$, Mann-Whitney test; $n = 5$. Mean ± SEM. "C" = control diluent.

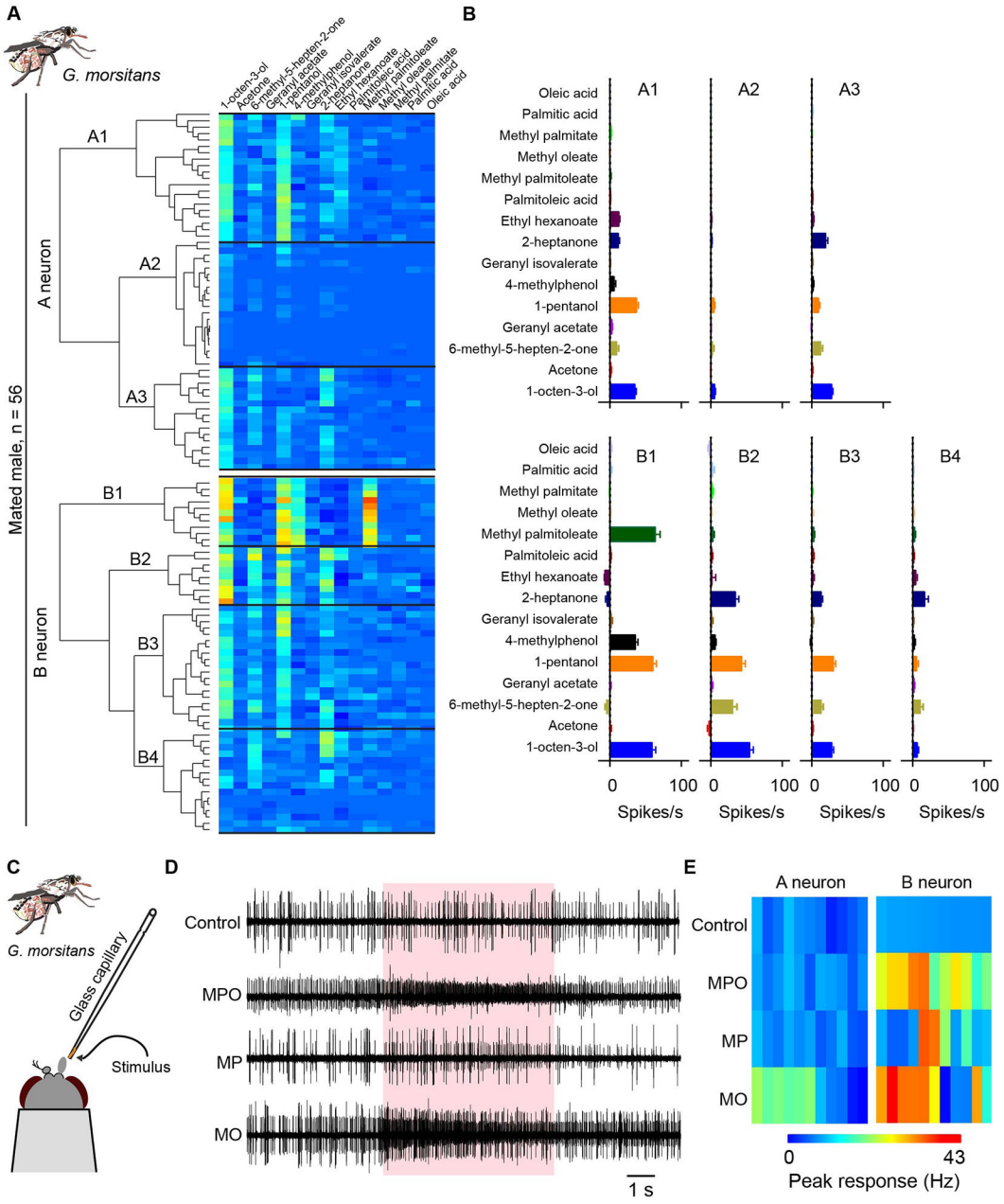

Figure 5. ORNs that respond to MPO also respond to known *G. morsitans* attractants

(**A**) Heatmap based on hierarchical cluster analysis of responses of male trichoid sensilla to a panel of 9 odorants and the 6 newly identified compounds. In the heatmap, each horizontal row represents 1 trichoid sensillum, and each vertical column represents 1 of the olfactory stimuli. The classification was carried out with Ward's method. The 9 odorants were diluted 10^{-2} in paraffin oil; the six compounds were diluted 10^{-1} in paraffin oil. (**B**) Response profiles of neuronal classes in trichoid sensilla; means ± SEM. n values range from 9-20, as shown in panel A. (**C**) The close-range stimulus delivery system. The stimulus is placed ~1 mm from the antenna, at the end of a glass capillary. (**D**) Example traces of electrophysiological responses of male trichoid sensilla to MPO (neat), MP (neat), and MO

(10^{-1} dilution). MPO and MP elicit an increase in the frequency of the small spikes (B neurons); MO elicits an increase in the frequency of both small and large spikes (B and A neurons). (**E**) Heatmaps of electrophysiological responses of A and B neurons to MPO (neat), MP (neat), and MO (10^{-1} dilution) in males. Responses are peak responses.

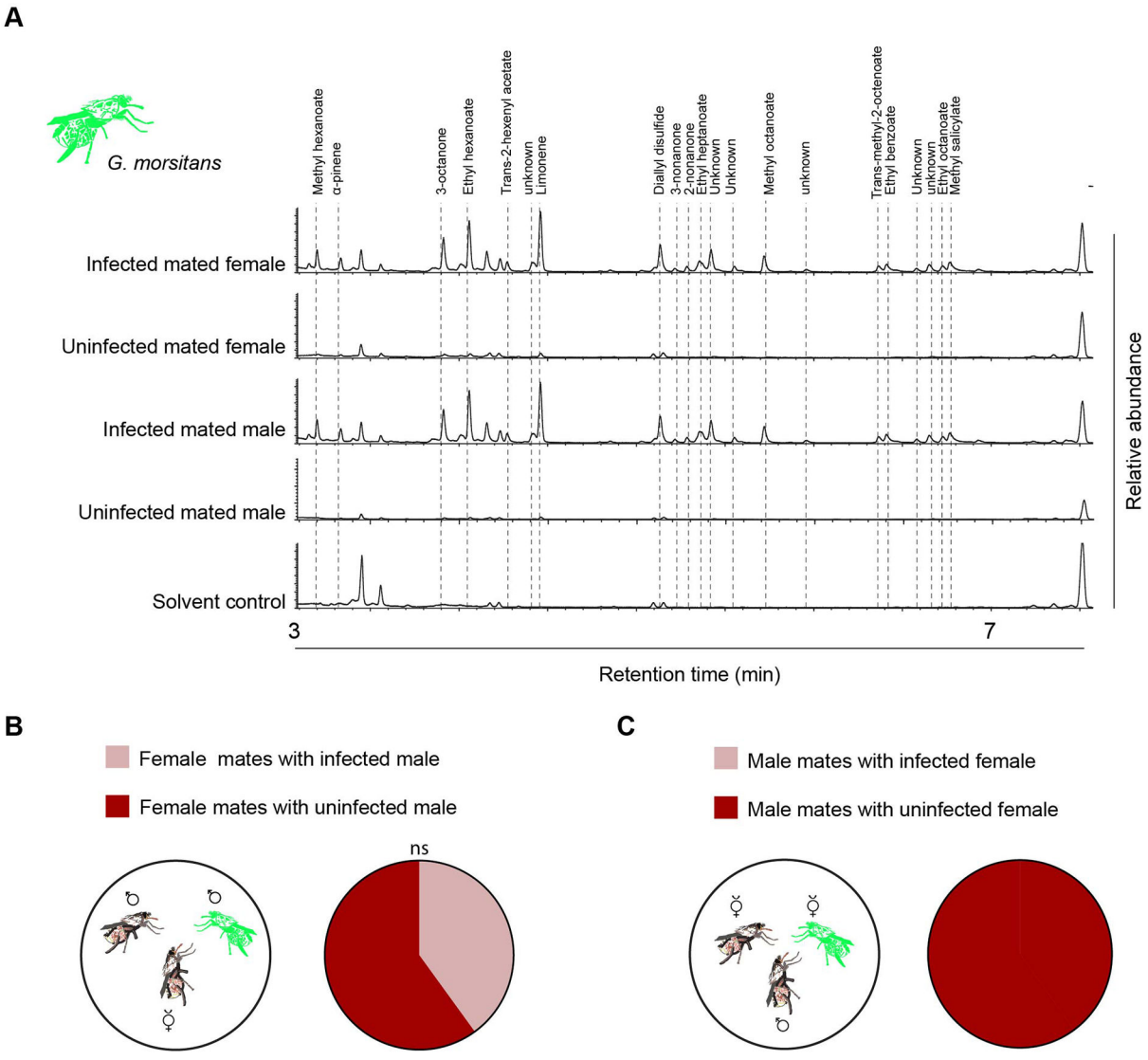

Figure 6. Trypanosome infection changes chemical profile and behavior.

(**A**) Total ion current chromatograms of 24h hexane extracts of infected and healthy mated *G. morsitans*. All flies were 14 days old. (**B**) Healthy females mate with both infected and healthy males. ($p > 0.05$, Chi-squared test, n=20). All flies are virgins, and all pairs copulated. (**C**) Healthy males mate with healthy but not infected females. All flies are virgins, and all pairs copulated. n=20.