

Malaria Parasites Produce Volatile Mosquito Attractants

Megan Kelly,^a Chih-Ying Su,^{b*} Chad Schaber,^a Jan R. Crowley,^c Fong-Fu Hsu,^c John R. Carlson,^b Audrey R. Odom^{a,d}

Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA^a; Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut, USA^b; Mass Spectrometry Resource, Washington University School of Medicine, St. Louis, Missouri, USA^c; Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA^d

* Present address: Chih-Ying Su, Division of Biological Sciences, University of California San Diego, San Diego, California, USA.

ABSTRACT The malaria parasite *Plasmodium falciparum* contains a nonphotosynthetic plastid organelle that possesses plant-like metabolic pathways. Plants use the plastidial isoprenoid biosynthesis pathway to produce volatile odorants, known as terpenes. In this work, we describe the volatile chemical profile of cultured malaria parasites. Among the identified compounds are several plant-like terpenes and terpene derivatives, including known mosquito attractants. We establish the molecular identity of the odorant receptors of the malaria mosquito vector *Anopheles gambiae*, which responds to these compounds. The malaria parasite produces volatile signals that are recognized by mosquitoes and may thereby mediate host attraction and facilitate transmission.

IMPORTANCE Malaria is a key global health concern. Mosquitoes that transmit malaria are more attracted to malaria parasite-infected mammalian hosts. These studies aimed to understand the chemical signals produced by malaria parasites; such an understanding may lead to new transmission-blocking strategies or noninvasive malaria diagnostics.

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Address correspondence to Audrey R. Odom, odom_a@kids.wustl.edu.

Malaria remains an enormous burden to human health worldwide. There are over 250 million cases of malaria each year, and nearly 1 million deaths (1). A single protozoan species, *Plasmodium falciparum*, is responsible for the most severe and deadly cases of this disease. Widespread and emerging drug resistance has contributed to a resurgence of malaria and to increased international attention to malaria control (2, 3). Because *P. falciparum* is transmitted through the bite of mosquitoes of the genus *Anopheles*, mosquito vector management has remained a key component of most malaria reduction efforts (4).

Female mosquitoes choose their mammalian hosts based in part on complex chemical cues. Some of these signals, such as carbon dioxide, have been well characterized on a molecular level. For example, carbon dioxide is not only a potent mosquito stimulant but also augments mosquito feeding behaviors and modulates attraction to other human body odors (5). However, *Anopheles gambiae* strains that lack functional CO₂ receptors are still capable of locating human hosts (6), indicating that additional chemical signals also drive host preference. Several recent studies have demonstrated that *Plasmodium*-infected hosts, including humans and rodents, are more attractive to *Anopheles* spp. (7–9). Analysis of volatile organic compounds emitted by *Plasmodium*-infected mice revealed an overall increase in volatiles, including host-derived compounds that enhance mosquito attraction (9).

While female mosquitoes depend on protein-rich blood meals for egg maturation, both male and female mosquitoes are also attracted to and feed from plants. Plant nectar is an important, carbohydrate-rich nutrient source that provides essential energy

for flight and, for some mosquito species, overwintering (10, 11). This phytoattraction has been successfully harnessed by malaria control efforts through “attractive nectar baiting” strategies, in which mosquitoes are lured to sugar-water blends spiked with plant volatiles and insecticides (12, 13). Suspected preferred host plants for *Anopheles gambiae* include *Asteraceae* spp. and *Ricinus communis* (14). Analysis of purified odorants from these plants has revealed enrichment of volatile compounds known as terpenes, including 10-carbon monoterpenes such as pinene and limonene. At low concentrations, these purified terpenes directly mediate attraction of *Anopheles* spp. (14).

Terpenes are low-vapor-pressure hydrocarbons that belong to a class of compounds known as isoprenoids. Over 200,000 isoprenoids have been described, and this large group of biomolecules exhibits dramatic structural and functional diversity (15). All isoprenoids are produced downstream of two common 5-carbon precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Animals and fungi generate isoprenoids through a biosynthetic route that proceeds through mevalonate. In contrast, eubacteria and plastid-containing eukaryotes use an alternate metabolic route, the nonmevalonate or methylerythritol phosphate (MEP) pathway. Plants utilize both the mevalonate and MEP pathways; however, it is the chloroplast-localized MEP pathway that is used for biosynthesis of the terpene volatiles that constitute their characteristic flavors and fragrances (16). For many species of insects, not just mosquitoes, chemodetection of plant-derived terpenes directly modulates herbivory and pollination behaviors (reviewed in reference 17).

The malaria parasite *Plasmodium falciparum* contains an unusual plastid organelle called the apicoplast, which is of a similar endosymbiotic evolutionary origin as plant chloroplasts (18). While the apicoplast retains several plant-like metabolic pathways, evidence suggests that the MEP pathway may be the only essential function of this organelle during intraerythrocytic development (19–21). In this work, we examined the possibility that, like plants, *Plasmodium falciparum* parasites might utilize the MEP pathway to produce terpenes. We determined the volatile chemical composition of headspace gas from cultured *P. falciparum* and thus identified parasite-produced terpene molecules that represent known mosquito phytoattractants. In addition, we established the molecular identity of the *Anopheles gambiae* odorant receptors that respond to these plant-like terpenes. Together, our studies provide evidence that malaria parasites produce specific volatile compounds, and anopheline mosquitoes that transmit malaria contain the cellular machinery necessary for detecting and responding to these compounds. Thus, plant-like terpenes produced by *P. falciparum* may represent semiochemicals for mediating anopheline mammalian host preference.

RESULTS

Plant-like volatile compounds in *Plasmodium falciparum* headspace gas. We hypothesized that malaria parasites might produce volatile organic compounds, including terpenes. We therefore evaluated the chemical composition of the headspace gas above asynchronous *P. falciparum* parasites cultured in human red blood cells (RBCs). Because previous studies of volatile emissions from *Plasmodium berghei*-infected mice (average blood volume, 2 to 4 ml) (9) or low-volume *P. falciparum* cultures (22) did not detect malaria parasite-specific volatiles, we utilized large-volume cultures (200 ml) to increase the likelihood of detecting small quantities of *Plasmodium*-produced compounds. In addition, because terpenes are present at low levels in human serum (23), we utilized medium supplemented with a lyophilized serum substitute (Albumax; Invitrogen) which does not contain detectable terpenes. For headspace sampling, we employed solid-phase microextraction (SPME) fibers, which selectively bind and concentrate nonpolar organic compounds, as is typically performed to evaluate plant-derived volatiles (reviewed in reference 24).

Fibers were exposed to a controlled atmosphere conditioned by *P. falciparum* for 48 h and then were desorbed and analyzed via electron impact (EI) gas chromatography-mass spectrometry (GC-MS). As is typical of complex volatile samples, component peaks overlapped and were not well resolved by visual inspection (see Fig. S1 in the supplemental material for representative traces). For this reason, resulting chromatograms were deconvoluted to isolate overlapping peaks and to extract and annotate component mass spectra. When distinguishing parasite-specific compounds, we aimed to identify compounds qualitatively present in parasite-infected samples compared to controls. Therefore, we conservatively selected compounds present in a majority of independent biological replicates of parasite-infected RBC samples and excluded entities also present in either uninfected RBC samples or blank controls that contained neither RBCs nor medium. Four compounds specific to parasite-infected samples were thus identified, including two terpenes (Fig. 1). These identified compounds have previously been identified as typical components of plant essential oils and/or fungal volatile profiles (25–28).

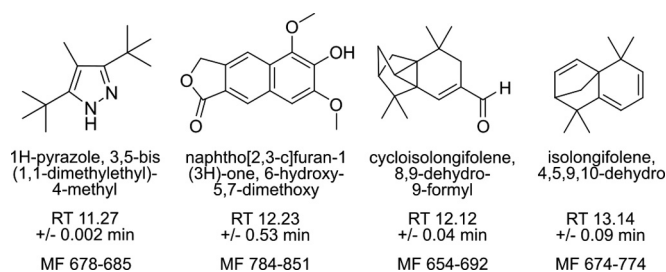


FIG 1 *Plasmodium*-specific volatile organic compounds. Compounds annotated in three or more *P. falciparum*-infected SPME sampling replicates (total $n = 5$) and not in uninfected red blood cell samples ($n = 3$) or blank controls ($n = 6$). For each compound, the average retention time (RT) and the range of match factors are indicated. Match factors (MF, 0 to 999) describe how well a sample spectrum agrees with the database spectrum. Values of >650 indicate close identity.

Terpenes are present in malaria-infected erythrocytes. We identified several entities that were annotated as terpenes and were present exclusively in the headspace gas of malaria parasites and not that of control uninfected erythrocytes or blank samples. Since terpenes with closely related chemical structures give rise to similar mass spectra, variability in compound annotation is typical and expected. The dominant malaria parasite-specific terpenes were annotated as a 15-carbon sesquiterpene (4,5,9,10-dehydroisolongifolene) and its close derivative (8,9-dehydro-9-formyl cycloisolongifolene) (Fig. 2; see also Fig. S2 in the supplemental material). No commercial standards or known synthesis routes

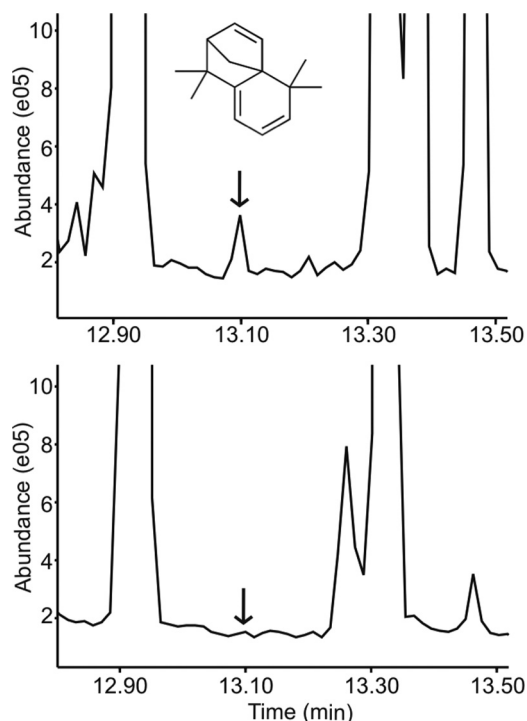


FIG 2 4,5,9,10-Dehydro-isolongifolene is present in the headspace gas of *Plasmodium*-infected RBCs. (Top) Total ion chromatogram (TIC) of SPME fibers conditioned with headspace gas from *P. falciparum*-infected human RBCs. Arrow, retention time of 13.101 min (typical of 4,5,9,10-dehydroisolongifolene). (Bottom) TIC of SPME fibers conditioned with headspace gas from uninfected human RBCs.

have been described for either compound; however, the structural annotations are supported by consistent database match factors, from 654 to 774.

In addition, each malaria parasite-infected sample contained at least one 10-carbon monoterpene. Monoterpene annotations varied between samples but included the structurally related compounds limonene and pinanediol (an α -pinene derivative) (see Fig. S3 in the supplemental material). To confirm the identity of these monoterpenes, we extracted nonpolar organic compounds from cultured *P. falciparum* and performed GC-MS analysis. *P. falciparum*-infected cultures, but not uninfected RBC or blank controls, contained a single peak suggestive of a monoterpene with a retention time of 2.39 min, identical to that of an α -pinene (monoterpene) standard (Fig. 3). Comparison of the mass spectra of the observed parasite-specific peak with that of a purified standard established that the parasite-specific compound is α -pinene (Fig. 3E), a terpene compound previously shown to be produced by *Anopheles*-preferred plant species and attractive to *A. gambiae* (14).

Terpenes are produced by *de novo* isoprenoid biosynthesis in malaria parasites. To evaluate whether terpenes in malaria parasite-infected samples were produced *de novo* by the parasite, we utilized fosmidomycin, a phosphonic acid antibiotic that inhibits the first dedicated enzyme of the MEP pathway, deoxyxylulose phosphate reductoisomerase (19). Previous metabolic profiling of fosmidomycin-treated parasites has established that fosmidomycin reduces concentrations of isoprenoid precursors in *P. falciparum* (29). Upon fosmidomycin treatment of cultured *P. falciparum*, pinene peak abundance decreased dramatically (Fig. 3D). Proteomic studies of mature RBCs have indicated that these cells do not possess the enzymatic machinery to produce the isoprenoid precursors required for terpene synthesis (30, 31). In addition, RBCs do not appear to contain substantial stores of IPP, since malaria parasites that cannot produce IPP themselves are unable to survive (21, 29). Together, this evidence strongly supports that the monoterpenes emitted by *Plasmodium*-infected RBCs arise from the MEP pathway of the malaria parasite.

***Anopheles* odorant receptors respond to malaria parasite-produced terpenes.** *P. falciparum* is transmitted person to person through the bite of anopheline mosquitoes. To locate plant and mammalian nutrient sources, *A. gambiae* detects volatile compounds via signals through ligand-gated voltage channels known as odorant receptors (AgORs) (32). Electrophysiological and behavioral studies have indicated that *A. gambiae* detects and is attracted to plant volatiles. While high concentrations of terpenes often repel mosquitoes, pinene and limonene at low concentrations directly attract *A. gambiae* and are the dominant volatile organic compounds found in the extracts of mosquito-preferred plant species (14).

To determine the biochemical mechanism by which *A. gambiae* detects plant- and malaria parasite-produced terpenes, we assayed a panel of mosquito odorant receptors (AgORs) for pinene and/or limonene ligand-activated electrical activity. Using the *Drosophila melanogaster* “empty neuron” *in vivo* expression system (33, 34), we found that AgOR75 was dramatically stimulated by (+)-limonene, while AgORs 21 and 50 were substantially stimulated by pinene (Fig. 4; see also Fig. S4 in the supplemental material). These odorant receptors are differentially expressed in *Anopheles* chemosensory tissues. Specifically, AgORs 21 and 50 are highly expressed in both male and female antennae (35). These

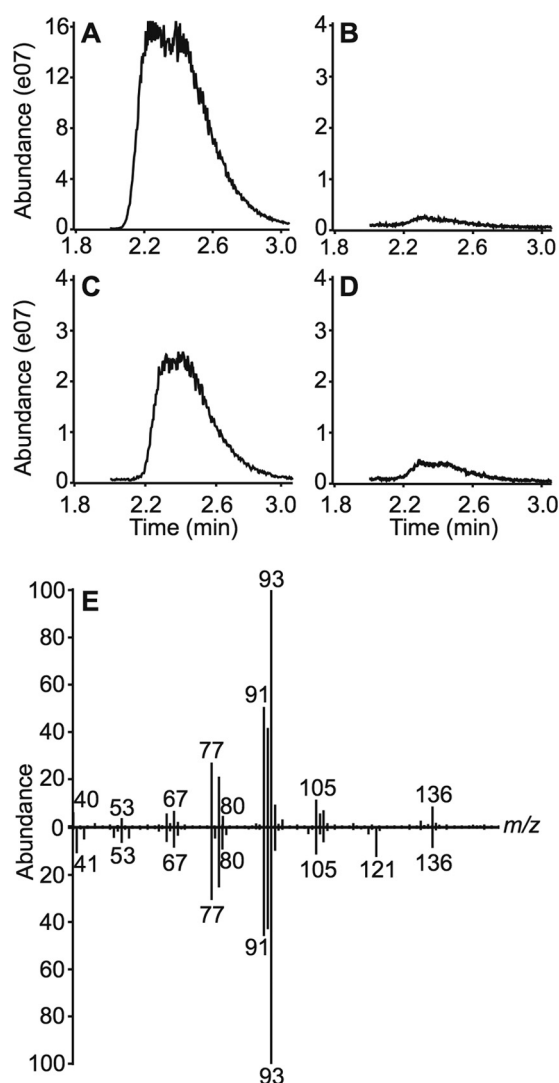


FIG 3 Pinene is produced in malaria parasites by *de novo* isoprenoid biosynthesis. (A to D) Extracted ion chromatograms (EIC) of the total ion chromatograms (TIC) at m/z 93, the base peak in the ion spectrum of pinene. (A) α -(+)-Pinene standard (positive control); (B) uninfected RBCs (negative control); (C) *Plasmodium*-infected RBCs; (D) *Plasmodium*-infected RBCs treated with 5 μ M fosmidomycin (inhibitor of parasite isoprenoid biosynthesis). (E) EI mass spectra of the observed pinene peak from *P. falciparum* RBCs in panel C (top) compared to the purified α -(+)-pinene standard in panel A (bottom).

studies confirmed that the primary African malaria vector mosquito can distinguish monoterpenes produced by *P. falciparum*. In addition, our studies establish the molecular identity of the monoterpene-specific odorant receptors of *A. gambiae*.

DISCUSSION

Our studies indicated that *Plasmodium falciparum* malaria parasites produce a repertoire of plant-like volatile compounds. These compounds may represent interspecies chemical signals, or semiochemicals, that modulate the attraction of vector mosquitoes to hosts. Among the parasite-specific compounds we identified, terpenes are bioavailable molecules that readily pass through membranes and partition into alveolar gas in the lung. Terpenes, likely

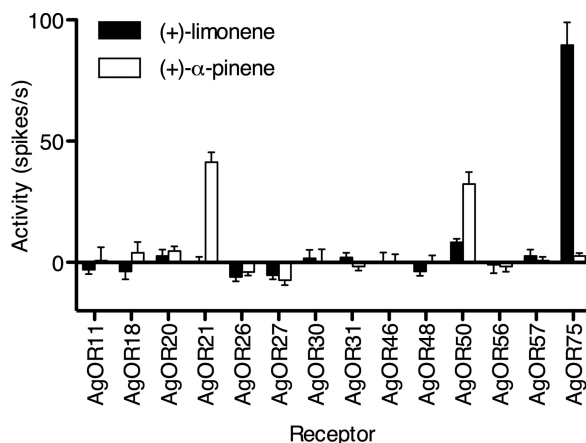


FIG 4 *Anopheles gambiae* odorant receptors respond to malaria terpenes. *Anopheles* odorant receptors were expressed in the *Drosophila* empty neuron *in vivo* expression system and exposed to (+)-limonene and α-(+)-pinene. (+)-Limonene specifically activated receptor 75. α-(+)-Pinene activated receptors 21 and 50 ($n = 6$; error bars indicate standard errors of the means).

from dietary sources, have previously been identified in exhaled breath samples of humans (36). Upon malaria parasite infection, parasite-produced terpenes are likely to be detected outside infected individuals, since the total number of parasites in a typical infected human well exceeds the number sampled in culture in such studies (37, 38).

Previous studies have suggested that *P. falciparum* infection of *Anopheles* spp. mosquitoes may reduce fitness and alter feeding behaviors (39–41). Over time, selective pressures might enrich for mosquitoes with a decreased tendency to feed from malaria parasite-infected individuals. Therefore, any chemical signals that increase attraction of mosquitoes to infected individuals must be difficult to select against and resistant to evolutionary pressures. This hypothesis is consistent with the finding that malaria infection increases production of typical mammalian host odorants (9). Our studies suggest an additional strategy by *P. falciparum* for overcoming selection against biting infected hosts, in which the malaria parasite compensates by imitating the volatile components of plants preferred by *Anopheles* spp. The parasite thus hijacks a highly selected signaling response that is necessary for mosquito nectar feeding behavior and survival. Since *Plasmodium* infection increases nectar attraction in *Anopheles* (42), the parasite appears to facilitate transmission both by generating a mosquito chemoattractant and by sensitizing the mosquito to detect this signal. Interruption of parasite-mediated volatile signaling to mosquitoes will be a potent means of blocking this critical step in the malaria life cycle.

P. falciparum has well-characterized biosynthetic machinery to produce isoprenoid building blocks and prenyl diphosphates (43–45). In other systems, such as plants, terpenes are produced by terpene synthases, which generate terpenes by catalyzing intramolecular cyclization of prenyl diphosphate substrates (46). This promiscuous reaction typically produces a variety of chemically related terpene variants from a single enzyme, a cardinal feature of this enzyme class (47). Consistent with this product diversity, the large protein family of terpene synthases (Pfam 01397) exhibits remarkable sequence diversity. Our studies strongly suggest that terpenes are produced *de novo* in *P. falciparum*, since chemical inhibition of parasite-specific isoprenoid biosynthesis reduces ter-

pene production. No unambiguous terpene synthase ortholog is present in *P. falciparum*, based on domain or phylogenetic analyses, but is likely to be represented among the nearly one-half of the parasite genes that remain unannotated. The diversity of terpenes present in *P. falciparum*-conditioned gas suggests that there is at least one monoterpene and one sesquiterpene synthase.

Here, we have reported a repertoire of volatile organic compounds that are specific to *P. falciparum*-infected cultures. These compounds are not likely to represent all possible malaria parasite-specific volatiles, because our conservative data filtering necessarily excluded compounds that are parasite specific but exhibit significant biological variability. The volatile fingerprint of *P. falciparum* represents not only a target for the development of inhibitors that will interrupt malaria transmission, but also an untapped strategy for malaria diagnostics. The parasite-specific compounds we have identified may represent volatile biomarkers of malaria infection. Ongoing studies will establish the presence and identity of these compounds in human *P. falciparum* infection.

MATERIALS AND METHODS:

***Plasmodium falciparum* culture and strains.** All *P. falciparum* strains were cultured *in vitro* in human erythrocytes (48) at 2% hematocrit. The culture conditions were as described previously (29), with the following modifications: we used a 5% O₂–5% CO₂–90% N₂ atmosphere in RPMI 1640 medium supplemented with 27 mM sodium bicarbonate, 11 mM glucose, 5 mM HEPES, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 0.01 mM thymidine, 0.25 mg/ml gentamicin (Goldbio), and 0.5% Albumax (Invitrogen). Wild-type strain 3D7 (MRA-102) was obtained from the Malaria Research and Reference Reagent Resource Center (MR4). 3D7-IG was kindly provided by Daniel Goldberg, Washington University School of Medicine.

Headspace sampling. *Plasmodium falciparum* strain 3D7-MR4 was cultured in a cell bioreactor bag (GE Life Sciences) for 48 h with a volume of 200 ml at 2% hematocrit and 2% parasitemia (infected erythrocytes/total erythrocytes). The culture was injected into the bag via syringe through a liquid injection port in a sterile environment. Uninfected samples contained erythrocytes and medium, and blank controls represented sampling from empty bags without medium or erythrocytes. The two injection ports with attached airtight filters were then used to fill the bag with a 5% O₂–5% CO₂–90% N₂ atmosphere. The biobag was secured to a tilting plate and connected to 0.63-in. sterile plastic tubing (Cole Parmer) through two injection ports. The ends of the tubing were connected to Luer pieces, which were secured to the biobag ports by using Parafilm. Of note, the biobag ports do not contain Luer locks, but all other pieces of tubing in the system are connected with interlocking Luer pieces. One piece of tubing was connected directly to a Bio-Rad Econo pump, and the other was fed through an airtight hole in a 250-ml medium bottle (Kimax). The bottle also contained openings for fiber insertion and outgoing plastic tubing. This tubing was connected to the other end of the peristaltic pump, completing the closed loop. A carboxen-polydimethylsiloxane SPME microfiber (Sigma-Aldrich), inside a manual holder, was placed through an adaptor into the medium bottle. Parafilm was used to secure the fiber and fiber holder in place and provide an airtight seal. Each experiment was performed in a temperature-maintained 37°C room for optimal malaria parasite growth. Sampling was initiated by opening the clamps on the two biobag injection ports, initiating peristalsis, and extending the fiber from inside the holder to its exposed position in the bottle. The fiber was exposed to the sampling conditions for 48 h. After sampling, the fiber was resheathed and analyzed by GC-MS as detailed below.

GC-MS analysis of SPME fiber extracts. Samples were analyzed on an Agilent 7890A gas chromatograph interfaced with an Agilent 5975C mass spectrometer. The GC column used for the study was an Agilent HP-5MS column (30 m, 0.25-mm inner diameter [i.d.], 0.25-μm film thickness). Samples were injected in a splitless mode with injector and transfer line temperatures set at 300°C. A linear temperature gradient was started with

an initial temperature of 60°C, held for 2 min, increased to 300°C at 10°C/min, and held for 1 min. The ion source temperature, electron energy, and emission current were set at 230°C, 70 eV, and 300 μ A, respectively, to obtain EI mass spectra.

Manual analysis of GC-MS data. The raw data were analyzed by using the automated mass spectral deconvolution and identification system (AMDIS), which provides an output of the GC trace with a deconvoluted mass spectrum extracted from each trace. Each mass spectrum represents a potential compound at a specific point in the trace. Every sample (head-space gas and parasite extract) yielded an average of 700 mass spectra per analysis. The structures of the compounds in each GC peak were identified by database search (NIST Mass Spectrum Library) using AMDIS software. Background peaks that represented known biologically irrelevant contaminants, such as polysiloxane arising from SPME fibers, were excluded from further analysis, as were compounds that did not possess consistent, parasite-unique ion spectra at given retention times.

Saponin lysis of *P. falciparum* cultures. Parasites were freed from RBCs through lysis with saponin at a final concentration of 0.1% (vol/vol), followed by centrifugation at 2,500 rpm and resuspension in 4 ml of phosphate-buffered saline (PBS). Pellets were washed in an additional 1 ml of PBS, followed by centrifugation at 14,000 for 1 min. Dry pellets were stored at -80°C until analysis.

Organic extraction. Extraction of isolated parasite cells was performed as described for the original Folch procedure (49), with the following modifications. Saponin-lysed parasite pellets were suspended in 1 ml of 2:1 (vol/vol) chloroform-methanol. The suspensions were sonicated for 30 s and then iced for 30 s for 3 cycles. Samples were vortexed at 600 rpm for 1 h after sonication. Samples were then centrifuged at 14,000 rpm, and the supernatant was recovered. Three hundred microliters of 0.9% NaCl was added to induce phase separation of the sample. The organic phase was recovered for analysis. The organic phase was not evaporated after extraction, in order to avoid loss of volatile compounds.

GC-MS analysis of extracted samples. GC-MS analyses were conducted on a Thermo ISQ 1300 GC-MS system with the Xcalibur operation system (San Jose, CA, USA). Separation was achieved with a Thermo 30-m TG SQC column (0.25-mm i.d., 0.25- μ m film thickness) at a flow rate of 1 ml/min with He as the carrier gas. The GC temperature was started at 50°C for 2 min, raised to 150°C at 10°C/min, and then to the final temperature of 300°C at a rate of 20°C/min. The samples were injected in a splitless mode, and the EI mass spectra were acquired in the mass range of 40 to 450 Da at a rate of 0.2 s/scan. The injector, transfer line, and ion source temperatures were set at 240°C, 250°C, and 210°C, respectively.

Single-unit electrophysiological recordings. All experiments were performed on adult female flies, 5 days after eclosion. Flies were reared at 25°C in an incubator with a 12-h light-dark cycle. “Empty neuron” recordings were from flies of genotype *w* Δ halo/ Δ halo *Or22a-GAL4/UAS-AgOrX*. The *ab3A* mutant flies and *Or22a-GAL4* and *UAS-AgOr* transgenic lines were described previously (32). Fourteen AgOrs (AgOr11, -18, -20, -21, -26, -27, -30, -31, -46, -48, -50, -56, -57, and -75), previously found to respond to terpenes (32), were selected to test their responsiveness to additional terpene compounds [α -(+)-pinene, Sigma-Aldrich catalog no 26870; β -(+)-pinene, no. 80607; α -(-)-pinene, no. 305715; β -(-)-pinene, no. 402753; *R*-(+)-limonene, no. 183164; *S*-(-)-limonene, no. 218367]. Odorants were diluted in paraffin oil (10^{-2} , vol/vol), and odor stimuli (50 μ l applied to a filter disc) were delivered from a Pasteur pipette via a 500-ms pulse of air (200 ml/min) into the main air stream (2,000 ml/min), as described previously (32). Extracellular single-unit recordings were performed essentially as described elsewhere (32). Briefly, electrical activity of the olfactory receptor neurons (ORNs) was recorded extracellularly by placing a sharp electrode filled with Ringer solution into a sensillum, and the reference electrode filled with the same Ringer solution was placed in the eye. AC signals (300 to 2,000 Hz) were recorded on an Iso-DAM amplifier (World Precision Instruments) and digitized at 5 kHz with an Axoscope 10.2 apparatus (Molecular Devices). ORN spike responses were quantified offline and averaged from 6 differ-

ent neurons. Baseline spike frequency (calculated from spike activity 1 s prior to odor stimulus) was subtracted from the result.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00235-15/-DCSupplemental>.

Figure S1, TIF file, 0.1 MB.

Figure S2, TIF file, 0.1 MB.

Figure S3, TIF file, 0.6 MB.

Figure S4, TIF file, 0.1 MB.

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For the experiments carried out in *Plasmodium falciparum*, A. Odom and M. Kelly conceived and designed the experiments; A. Odom, M. Kelly, Jan Crowley, F.-F. Hsu, and C. Schaber performed the experiments and analyzed the data. For the *Anopheles gambiae* odorant receptor experiments, J. R. Carlson and C.-Y. Su conceived and designed the experiments, C.-Y. Su performed the experiments, and C.-Y. Su and J. R. Carlson analyzed the data. M. Kelly, C.-Y. Su, J. R. Carlson, C. Schaber, and A. Odom wrote the manuscript.

We declare we have no conflicts of interest.

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