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BRIEF REPORT

Gametocytemia and Attractiveness of *Plasmodium falciparum*–Infected Kenyan Children to *Anopheles gambiae* Mosquitoes

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(See the editorial commentary by Cator on pages 289–90.)

It has been suggested that *Plasmodia* manipulate their vertebrate hosts to enhance parasite transmission. Using a dual-choice olfactometer, we investigated the attraction of *Anopheles gambiae* to 50 Kenyan children (aged 5–12 years) who were naturally infected with *Plasmodium falciparum* or noninfected controls. Microscopic gametocyte carriers attracted almost 2 times more mosquitoes than children who were parasite free, harbored asexual stages, or had gametocytes at submicroscopic densities. By using highly sensitive stage-specific molecular methods to detect *P. falciparum*, we show that gametocytes—and not their noninfectious asexual progenitors—induce increased attractiveness of humans to mosquitoes. Our findings therefore support the parasite host manipulation hypothesis.

Keywords. chemical ecology; olfactory behavior; malaria transmission; vector control; host finding.

Numerous parasites alter the phenotype or behavior of their hosts to increase their transmission success and fitness. In vector-transmitted parasites, the effects may be observed in the vector and/or in the vertebrate host. *Plasmodium* is known to alter the phenotype of infected mosquitoes, by increasing blood-meal size or frequency of feeding or enhancing responses to host odor [1], and of its vertebrate host, which may become more attractive to mosquitoes [2–4]. Volatile olfactory cues play a crucial role in mosquito host-seeking behavior, and it was recently shown that *Plasmodium chabaudi* infection in mice alters their odor profile in such a way that *Anopheles stephensi* mosquitoes were differentially attracted by chronically infected mice [4].

Two previous studies in humans suggest an effect of *Plasmodium* gametocytes on attractiveness to mosquitoes. South American adults infected with *Plasmodium vivax* gametocytes were significantly more attractive to *Anopheles darlingi* before antimalarial treatment than during or after medication [3]. In a study with Kenyan children naturally infected with *Plasmodium falciparum*, microscopic gametocyte carriers were significantly more attractive to *Anopheles gambiae* mosquitoes than asexual carriers or parasite-free children [2], whereas children were equally attractive after antimalarial treatment. Although these results seem to offer evidence for manipulation by malaria parasites, both studies detected *Plasmodium* by microscopy, which means that a significant proportion of infections—and, in particular, gametocytes—has probably gone unnoticed [5]. It therefore remains unclear whether the gametocytes specifically, and not asexual parasites, induce increased human attractiveness to mosquitoes and whether there is a density-dependent relationship between gametocytemia and attractiveness.

In the current study, we further explored the hypothesis that gametocytes manipulate the vertebrate host by investigating whether subclinical *P. falciparum* infection with different life-cycle stages affects the host-seeking behavior of *A. gambiae* in a dual-choice olfactometer. We used sensitive stage-specific molecular methods to detect low levels of gametocytes or parasites [6]. The attractiveness of 50 naturally infected or parasite-free control children was compared against a standardized control odor before and after antimalarial treatment with artemisinin-lumefantrine, which rapidly clears asexual parasites and also has a pronounced effect on posttreatment gametocyte prevalence and density [6].

METHODS

Study Design and Recruitment of Participants

Participants, aged 5–12 years, were recruited at schools on Rusinga Island or in Lambwe Valley (Homabay County, western Kenya). Children without malaria symptoms and with tympanic temperature <37.5°C were invited to participate when they had a microscopically confirmed *P. falciparum* infection or when they were *P. falciparum* free as shown by nested polymerase chain reaction (PCR) (see below). Exclusion criteria included the presence of malaria symptoms or another disease, the presence of a different *Plasmodium* species, and antimalarial treatment in the previous 2 weeks. Participants were recruited in the study after obtaining signed consent. The study protocol (NON SSC 389) was

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During the school visits, finger-prick-blood was used to prepare Giemsa-stained thick and thin smears and to obtain dried blood spots on filter paper (Supplementary Methods). Asexual stage and gametocyte density were determined microscopically by counting the number of parasites per 200 white blood cells. Dried blood spots were used to determine the presence of *P. falciparum* with nested PCR, based on a fragment of the 18S ribosomal RNA gene [7]. This method was used to increase the chance of including parasite-free children by excluding those that were *P. falciparum* negative at microscopy but had low levels of parasites detectable with the more sensitive nested PCR.

After each school visit, up to 4 children were invited to participate in olfactometer experiments to determine their attractiveness to mosquitoes. Selected children had an additional 50 µL of finger-prick blood stored in 250 µL of RNAprotect cell reagent (Qiagen) for molecular detection of *P. falciparum* with 18S quantitative PCR (qPCR) and gametocyte-specific Pfs25 (Gene ID: PF3D7_1031000) quantitative nucleic acid sequence–based amplification (QT-NASBA) [6]. Samples with an estimated parasite density <0.02/µL were considered parasite negative.

Olfactometer Assay

Two dual-choice olfactometers were used (Supplementary Figure 1 and Supplementary Methods), placed inside a 7 × 11-m screen house to test attractiveness of up to 4 children per evening (18:30-21:30). Participant sex, age, weight, tympanic temperature, and hemoglobin level (HemoCue Hb 301) were recorded, and children were dressed in clean cotton T-shirts and shorts. A child was positioned in one tent and the standard control odor, consisting of strips of worn nylon socks (Supplementary Methods) in the other tent of an olfactometer, with positions alternated in subsequent runs to minimize positional bias.

Uninfected mosquitoes from a laboratory-reared colony of *A. gambiae s.s.* originating from Mbita were used. Eight hours before an olfactometer assay, 100 female mosquitoes (3–7-days old) with no prior access to a blood meal were transferred to small holding cups, with water on cotton wool. Mosquitoes were released into the choice chamber of the olfactometer and their preference for odor from either tent was recorded after 30 minutes.

Immediately after the experiment, infected participants were treated by weight-based dosing of artemisinin-lumefantrine (Coartem-D; Novartis). Three weeks after antimalarial treatment, attractiveness to *A. gambiae* was tested again according to the same procedures, with the same individuals placed in the same tent as in the first visit. Finger-prick blood was obtained for *P. falciparum* detection by means of microscopy and molecular methods.

Statistical Analyses

A generalized linear mixed model (GLMM; Binomial distribution, logit link function) was used to investigate the main effects of parasitological status and sampling time point (before vs after antimalarial treatment) and their interaction on attractiveness as fixed-effect terms, with participant identity as a random-effect term (Supplementary Methods). Levels of parasitemia and gametocytemia (by microscopy and 18S qPCR), age, weight, tympanic temperature, and hemoglobin levels were compared between parasitological status groups by means of 1-way analysis of variance for data collected before and after antimalarial treatment separately. This analysis accounted for unbalanced designs and was followed by Bonferroni tests for pairwise comparisons between categories. All analyses performed with GenStat software,18th edition (VSN International).

RESULTS

Study Population

The 50 participating children were categorized as parasite free, as confirmed by 18S qPCR (n = 12); asexual *P. falciparum* carriers, as confirmed by microscopy and/or 18S qPCR without gametocytes by QT-NASBA (n = 9); submicroscopic gametocyte carriers confirmed by QT-NASBA (n = 10); or microscopic *P. falciparum* gametocyte carriers (n = 19) (Supplementary Figure 2). After antimalarial treatment, all children were microscopically negative for *P. falciparum*. Molecular parasite and gametocyte prevalence also dropped considerably (Table 1), although parasites were still detectable by 18S qPCR in both groups of former gametocyte carriers (3 of the 6 former submicroscopic and 10 of the 15 former microscopic gametocyte carriers). Only 3 children (1 in the submicroscopic and 2 in the microscopic gametocyte group) still had submicroscopic gametocytes by QT-NASBA after antimalarial treatment. Before antimalarial treatment, there was a significant association between parasitological status and tympanic temperature (Table 1; analysis of variance, $P = .04$), with a significantly higher tympanic temperature in participants of the asexual group than in those of the submicroscopic gametocyte group (Bonferroni pairwise comparisons, $P < .008$).

Effect of Parasitological Status on Human Attractiveness to Mosquitoes

Mosquito response to children was significantly affected by parasitological status, sampling time point, and their interaction (Figure 1, Supplementary Figure 3, and Supplementary Table 1) (GLMM, $P < .001$ for parasitological status, sampling time point, and their interaction). Children who harbored microscopic gametocytes attracted almost twice as many mosquitoes as children in the other 3 groups before antimalarial treatment (pairwise comparisons, $P < .001$). The presence of submicroscopic gametocytes or asexual stages of *P. falciparum* did not increase the attractiveness of children compared with parasite-free children before antimalarial treatment (pairwise comparisons, $P = .66$ and $P = .52$, respectively).

After antimalarial treatment, children attracted on average 24–29 mosquitoes of the 100 released, and mosquito responses to the 4 groups of children did not differ significantly (Figure 1,

Table 1. Overview of Study Population Categorized by Parasitological Status Before Antimalarial Treatment According to Microscopy, 18S qPCR, and Gametocyte-Specific QT-NASBA

	Before Antimalarial Treatment					After Antimalarial Treatment				
		Mean (SEM) [No. of Replicates]					Mean (SEM) [No. of Replicates][b]			
Parameter	Children, No.[a]	Parasite Free	Asexual Parasite Carriers	SG Carriers	MG Carriers	Children, No.	Parasite Free	Asexual Parasite Carriers	SG Carriers	MG Carriers
Total parasite density by 18S qPCR, No./µL[c]	41	0 [12]	10 628 (9968 [6]	101 203 (74 303) [10]	156 180 (102 432) [13]	40	0 [11]	0 [8]	23 547 (23 529) [6]	29 (20) [15]
Asexual parasite density by microscopy, No./µL[c]	49	0 [12]	1102 (404) [9]	1360 (721) [10]	342.2 (215.9) [18]	50	0 [12]	0 [9]	0 [10]	0 [19]
Gametocyte density by microscopy No./µL[c]	50	0[d] [12]	0[d,e] [9]	0[d,e] [10]	162 (57)[e] [19]	50	0 [12]	0 [9]	0 [10]	0 [19]
Age, y	50	7.8 (0.6) [12]	8.9 (0.7) [9]	9.7 (0.6) [10]	9.1 (0.5) [19]	50	7.8 (0.6) [12]	8.9 (0.7) [9]	9.7 (0.6 [10]	9.1 (0.5) [19]
Body weight, kg	50	24.1 (1.7) [12]	28.3 (1.8) [9]	29.3 (3.1) [10]	28.6 (1.9) [19]	50	23.4 (1.7) [12]	28.1 (1.8) [9]	29.3 (3.1) [10]	28.5 (1.9) [19]
Hemoglobin, mmol/L	46[f]	7.80 (0.37) [11]	7.69 (0.33) [9]	7.04 (0.39) [10]	6.84 (0.20 [16]	50	7.64 (0.25) [12]	7.49 (0.26) [9]	7.15 (0.44) [10]	7.12 (0.14) [19]
Tympanic temperature, °C	50	36.1 (0.1)[d,e] [12]	36.5 (0.3)[e] [9]	35.6 (0.2)[d] [10]	36.1 (0.1)[d,e] [19]	50	36.3 (0.2) [12]	36.1 (0.3) [9]	36.1 (0.2) [10]	36.1 (0.1) [19]

Abbreviations: MG, microscopic gametocyte; qPCR, quantitative polymerase chain reaction; QT-NASBA, quantitative nucleic acid sequence–based amplification; SG, submicroscopic gametocyte.

[a]The sex breakdown by status group, before treatment, was as follows: parasite free, 7 male and 5 female children; asexual, 6 male and 3 female; SG, 4 male and 6 female; and MG, 11 male and 8 female.

[b]The same categorization was used after antimalarial treatment; parasite free, asexual, SG, and MG denote the child's parasitological status before antimalarial treatment and not at the second time point.

[c]Zero values are included in calculation of mean parasite and gametocyte densities.

[d,e]Significant difference in the means between parasitological status groups within time point (1-way analysis of variance, Bonferroni tests, $P < .008$). Parasitological status did not affect any other parameters within the time point (1-way analysis of variance, $P > .05$).

[f]Four outliers from the hemoglobin data were excluded from analysis because they were due to a machine malfunction that day.

Supplementary Figure 3, and Supplementary Table 1; pairwise comparisons, $P > .29$). Clearance of microscopic gametocytes after antimalarial treatment (Table 1) reduced mosquito responses approximately 2-fold (pairwise comparison, $P < .001$).

Results of the analyses of mosquito choice between children and the standard control odor were similar to those of mosquito responses (Figure 1, Supplementary Figure 4, and Supplementary Table 2) (GLMM, $P = .004$ for parasitological status, $P = .052$ for sampling time point, and $P < .001$ for their interaction). This means that children in the microscopic gametocyte group, compared with the other groups, attracted more mosquitoes and a larger proportion of trapped mosquitoes relative to the standardized control odor.

DISCUSSION

Microscopic gametocyte carriers attracted almost twice as many *A. gambiae* mosquitoes as children without microscopic gametocytes attracted, confirming earlier findings [2]. Interestingly, gametocytemia below the microscopic detection threshold did not contribute significantly to attractiveness, suggesting a density-dependent effect of gametocytes on attractiveness. The presence of asexual parasites did also not affect attractiveness in the absence of gametocytes. Indeed, children with asexual stages or submicroscopic densities of gametocytes had similar attractiveness as parasite-free children. Importantly, we used highly sensitive 18S qPCR (detection limit for parasite density, 0.02 /µL) to determine the absence of parasites in this control group.

After antimalarial treatment and clearance of gametocytes below the microscopic detection threshold, the attractiveness of former microscopic gametocyte carriers dropped to the level of attractiveness of children without gametocytes, which remained the same before or after treatment. Within our study population of 50 subclinical, 5–12-year-old children, gametocyte-induced attractiveness was independent of sex, age, and body temperature. Efforts to quantify a density-dependent effect of gametocytes on host attractiveness, or determine the minimum gametocyte density to manipulate mosquito choice, will require a larger sample that may be purposefully selected to include a range of gametocyte densities. The combined findings support our hypothesis that *P. falciparum* gametocytes, and not asexual stages, mediate the attractiveness of human hosts to *A. gambiae*,

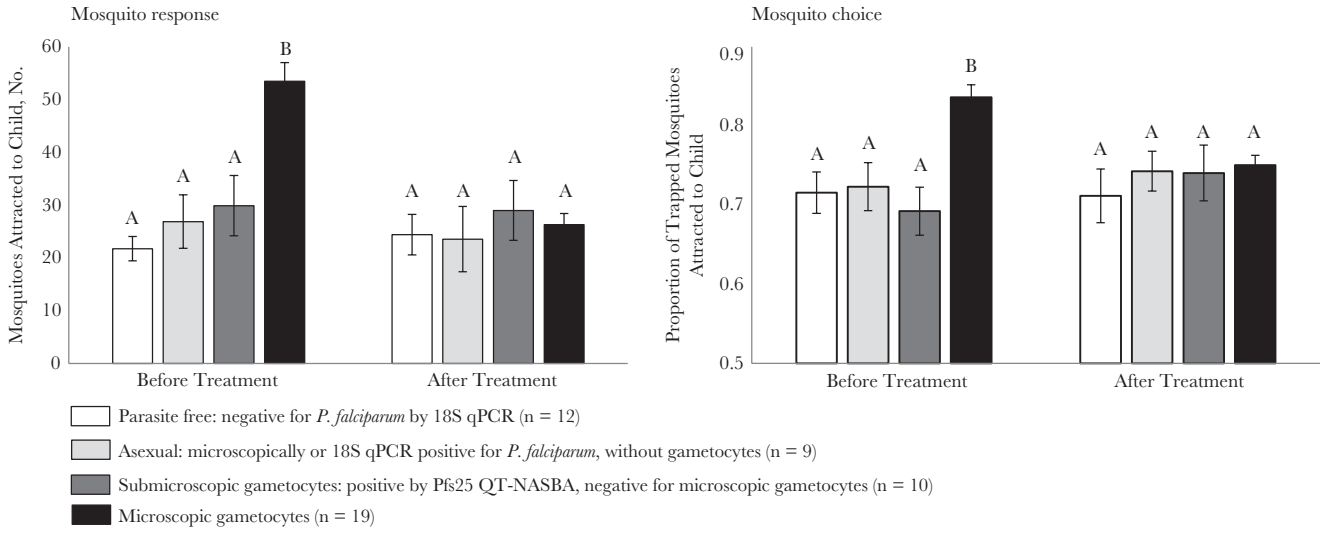

Figure 1. Attractiveness of *Plasmodium falciparum*–infected or parasite-free children to *Anopheles gambiae* mosquitoes in the dual-choice olfactometer expressed as mosquito response (number of mosquitoes attracted to a child) or mosquito choice (proportion of mosquitoes attracted to children relative to all trapped mosquitoes). Experiments were done twice for each child: before and after treatment of infected children with antimalarials. Means and standard errors of the mean are provided. Different letters above bars indicate pairwise significant differences between respective groups (generalized linear mixed model [GLMM] pairwise comparisons, $P < .05$; see Supplementary Figures 3 and 4 for predicted and back-transformed means derived from the model and Supplementary Tables 1 and 2 for *t* probabilities of pairwise comparisons). Bars with the same letter are not significantly different. More mosquitoes were trapped later in the year, and the date of the experiment was included in the final models on mosquito response and mosquito choice (GLMM, $P < .001$ and $P = .007$, respectively). qPCR, quantitative polymerase chain reaction; QT-NASBA, quantitative nucleic acid sequence–based amplification.

and they suggest a threshold gametocyte-density above which this phenomenon occurs.

Gametocyte-induced attractiveness could lead to increased exposure of infectious humans to mosquitoes. Because higher gametocyte densities also result in higher mosquito infection rates [8], *P. falciparum* may signal its presence to vectors at a stage when the chance of successful infection is the greatest. These 2 density-dependent effects may amplify each other and lead to a disproportionately large contribution of microscopic gametocyte carriers to malaria transmission. Should further investigation reveal that gametocyte-infected persons are indeed bitten more under field conditions, it will be important to consider heterogeneous biting related to gametocyte-mediated attractiveness and density-dependent infection rates in epidemiological models of malaria transmission [9].

Gametocyte-induced attractiveness is probably mediated by changes in host odor [2]. Skin odor is known to be crucial in host location behavior of malaria mosquitoes, and its composition is associated with differences in attractiveness between healthy individuals [10]. In mice, *P. chabaudi* infection led to an altered odor profile and elevation of specific volatile compounds was shown to increase attractiveness to *A. stephensi* [4]. Breath may also contribute to relative attractiveness of humans to mosquitoes [11], and it is known to be influenced by *P. falciparum* infection, with specific thioethers emitted from infected (nongametocytemic) persons [12]. Finally, host odor manipulation by *Plasmodium* may occur through direct emission of cues from malaria parasites.

Plasmodium falciparum cultures produce terpenes [13], and red blood cells treated with a key metabolite of *P. falciparum* emit enhanced levels of terpenes and aldehydes that are attractive to *A. gambiae* [14]. It is not yet known whether these compounds are emitted through the breath or skin of infected hosts and whether these effects depend on the life-cycle stage of *Plasmodium*, as observed in our experiment. To start unraveling the mechanisms of *Plasmodium* manipulation of vertebrate hosts, the relative roles of breath and body odors of naturally infected children should be investigated.

In conclusion, the current findings support the hypothesis that *P. falciparum* manipulates human attractiveness to *A. gambiae* and that this effect is stage-specific and density dependent because increased attraction was found only in microscopic gametocyte carriers. The ecological relevance of this finding needs to be studied to determine how gametocyte-induced attractiveness affects malaria transmission in the field. Second, odor profiles of infected and parasite-free humans should be analyzed to identify compounds that enable malaria mosquitoes to differentiate between gametocytemic and nongametocytemic individuals. Such compounds may contribute to improved odor-baited traps that were recently shown to be a promising addition to the vector control toolbox [15].

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the

authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. J. G. L., R. W. S., and W. T. conceived the study. A. O. B., N. O. V., W. T., and J. G. d. B. designed the experiment. A.O.B. was the main investigator. C. K. M. and D. M. coordinated the overall planning and implementation of the study. T. B. coordinated the molecular analyses. A. O. B., T. B., and J. G. d. B. analyzed the data. A. O. B. and J. G. d. B. wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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