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Development and Field Evaluation of a Synthetic Mosquito Lure That Is More Attractive than Humans

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Abstract

**Background:** Disease transmitting mosquitoes locate humans and other blood hosts by identifying their characteristic odor profiles. Using their olfactory organs, the mosquitoes detect compounds present in human breath, sweat and skins, and use these as cues to locate and obtain blood from the humans. These odor compounds can be synthesized in vitro, then formulated to mimic humans. While some synthetic mosquito lures already exist, evidence supporting their utility is limited to laboratory settings, where long-range stimuli cannot be investigated.

**Methodology and Principal Findings:** Here we report the development and field evaluation of an odor blend consisting of known mosquito attractants namely carbon dioxide, ammonia and carboxylic acids, which was optimized at distances comparable with attractive ranges of humans to mosquitoes. Binary choice assays were conducted inside a large-cage semi-field enclosure using attractant-baited traps placed 20 m apart. This enabled high-throughput optimization of concentrations at which the individual candidate attractants needed to be added so as to obtain a blend maximally attractive to laboratory-reared An. gambiae. To determine whether wild mosquitoes would also be attracted to this synthetic odor blend and to compare it with whole humans under epidemiologically relevant conditions, field experiments were conducted inside experimental huts, where the blend was compared with 10 different adult male volunteers (20-34 years old). The blend attracted 3 to 5 times more mosquitoes than humans when the two baits were in different experimental huts (10-100 metres apart), but was equally or less attractive than humans when compared side by side within same huts.

**Conclusion and Significance:** This highly attractive substitute for human baits might enable development of technologies for trapping mosquitoes in numbers sufficient to prevent rather than merely monitor transmission of mosquito-borne diseases.

Introduction

Mosquitoes rely on biochemical cues to find essential resources such as human hosts, mates and suitable sites to lay their eggs [1]. African mosquitoes such as Anopheles gambiae sensu stricto and Anopheles funestus are efficient vectors of human malaria because they are highly adapted to preferentially feed on humans [2]. Using their highly sensitive olfactory organs, these mosquitoes can select more attractive persons over less attractive ones by identifying chemicals present in breath, sweat and other skin emanations originating from the persons [3,4]. Though not adequately understood, these evolutionary host preferences may benefit the mosquitoes in a number of ways including the identification of hosts with more nutritive blood, or those who are less defensive against mosquito bites [3,4]. Examples of these attractive chemicals include carbon dioxide (CO2), the ultimate catabolite of vertebrate respiratory processes, L-lactic acid, which is produced through anaerobic glycolysis in human eccrine glands, and ammonia, also a component of human sweat [5,6,7]. Human skin also secretes triglycerides, which can be broken down to several behaviorally active fatty acids by skin surface microflora [3].

Many of these odorants can be synthesized in vitro and therefore they can be reformulated to produce mixtures that mimic real
humans to lure mosquitoes [8,9]. While some synthetic odor mixtures already exist which can lure host-seeking mosquitoes, evidence supporting their utility is largely limited to short range evaluations inside laboratories, where the crucial role of long-range stimuli is artificially negated [10,11,12]. Other recent successes include the formulation of synthetic odor blends which can match the attractiveness of humans for field populations of certain mosquitoes transmitting malaria [13], dengue and yellow fever [14], but none of these improves upon humans for priority vector species. The former example [13] does surpass humans for zoophagic species which transmit several neglected tropical diseases but this inevitably biases the sample of mosquitoes obtained so that they cannot be considered epidemiologically representative for surveillance of human exposure to the full range of mosquito-borne pathogens.

Here we report on the development and field evaluation of a highly attractive odor blend consisting of synthetic versions of known mosquito attractants [6,12,13,15]: carbon dioxide, ammonia, L-lactic acid and seven other aliphatic carboxylic acids, namely propionic acid, butanoic acid, pentanoic acid, 3-methylbutanoic acid, heptanoic acid, octanoic acid and tetradecanoic acid. The conceptual process followed in developing the blend is illustrated in Fig. 1.

Materials and Methods

Mosquitoes

The semi-field assays were conducted using laboratory-reared An. gambiae s.s., which had originally been obtained from Njagi village in Kilombero district, southern Tanzania. This colony had originally been established in 1997, but was recently restocked in 2008. The mosquito larvae were fed on Tetramin® fish food and maintained at a temperature of 27 ± 1°C. The adult mosquitoes were kept inside a separate room, where temperatures were maintained at 27°C and relative humidity at 70–90% and were fed on 10% glucose solution. To sustain the colony, the adult female mosquitoes were fed also on human blood (by way of adult volunteers regularly inserting their arms inside the mosquito cages for 5–10 minutes every two days). The insectary was set to a photoperiod of 12 hours darkness and 12 hours light.

The Semi-Field System

All behavioral assays aimed at the formulation and optimization of the synthetic odor blend were conducted within a semi-field enclosure (screen house), at the Ifakara Health Institute [16]. The screen house has three 200 m² compartments, one of which was used for this research.

Formulation and Optimization of the Synthetic Odor Blend

The blend was developed through a sequential procedure (Fig. 1), starting with a weakly attractive primary mixture consisting of 2.5% ammonia solution and 500 ml/min of CO₂ gas. Onto this primary mixture, L-lactic acid was first added followed by the other aliphatic carboxylic acids initially one at a time to determine their optimal concentrations, and then jointly at their optimal concentrations to create the final blend. Whenever each of the candidate compounds were added, we iteratively varied its concentrations until the point when the resulting mixture was maximally competitive, in terms of its attractiveness to laboratory reared mosquitoes relative to the original mixture. This final concentration was considered the optimum for each respective candidate compound.

L-Lactic acid was the first to be added onto the primary mixture. The treatment trap was baited with the primary mixture plus different concentrations of L-lactic acid. The control trap on the other hand was baited with only the primary mixture. For each concentration of L-lactic acid, four replicates were conducted each lasting six hours, and between which we rotated the positions of the traps so as to minimize directional bias. The most significant improvement in attractiveness was determined to occur when L-lactic acid was added at 85% concentration (which was the undiluted formulation of L-lactic acid as purchased from the manufacturer).

Figure 1. A conceptual model summarizing the development of the odor blend. The process began with a weakly attractive mixture containing 2.5% aqueous ammonia and CO₂ gas flowing at 500 ml/min, which was enhanced by adding 85% L-lactic acid (LA). Onto the resulting mixture, each of the other aliphatic carboxylic acids was added separately, each of them at their optimally attractive concentrations. The blend therefore consisted of the CO₂ gas plus hydrous solutions of ammonia (2.5%) and L-lactic acid (85%), and the other aliphatic carboxylic acids at their respective optimum concentrations as follows: propionic acid (C3) at 0.1%, butanoic acid (C4) at 1%, pentanoic acid (C5) at 0.01%, 3-methylbutanoic acid (3mC4) at 0.001%, heptanoic acid (C7) at 0.01%, octanoic acid (C8) at 0.01% and tetradecanoic acid (C14) at 0.01%. Finally, a variant of the blend was formulated by removing 3mC4.

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Previously, the synergistic effect of L-lactic acid when combined with ammonia and a blend of carboxylic acids, has been demonstrated [17,10]. Therefore in the rest of our assays, all other aliphatic carboxylic acids were all tested in combination with 83% L-lactic acid, each time comparing the resulting mixture with the original mixture as before. In the case of these other carboxylic acids, the treatment trap was therefore baited with: 1) the primary mixture, 2) undiluted L-lactic acid and 3) an iteratively selected concentration of a selected carboxylic acid, while the control trap was baited with only the primary mixture. For each carboxylic acid, the optimal concentration was determined by iterating until the point when the resulting mixture was maximally attractive relative to the primary mixture.

After the optimal concentrations for all the carboxylic acids had been determined, all the compounds were added to the primary mixture at those respective concentrations to form the final synthetic odor blend. The synthetic blend therefore consisted of CO₂ gas flowing at 500 ml/min plus hydrous solutions of ammonia (2.5%), L-lactic acid (85%), and the other aliphatic carboxylic acids: propionic acid (C3) at 0.1%, butanoic acid (C4) at 1%, pentanoic acid (C5) at 0.01%, 3-methylbutanoic acid (3mC4) at 0.001%, heptanoic acid (C7) at 0.01%, octanoic acid (C8) at 0.01% and tetradecanoic acid (C14) at 0.01%. Finally, a variant of the blend was formulated by removing the unpleasant smelling 3mC4 in an attempt to improve the appeal of the blend to potential users.

Semi-Field Experiment Conducted to Compare the Attractiveness of the Synthetic Odor Blend with the Attractiveness of Natural Host Odors

The blend was first tested against a combination of CO₂ gas and natural human foot odors collected in worn nylon socks, a technique previously used to trap An. gambiae s.s in a similar semi-field system [19]. To collect the foot odors, the nylon socks were worn for 10 hours by a 26 year old male volunteer prior to the experiment. Binary tests were conducted in which one trap was baited with the synthetic blend and the other trap baited with a worn nylon sock and 500 ml/min of CO₂. Fourteen replicates, baited with the synthetic blend and the other trap baited with a human volunteer, were set up in all the experimental huts to catch mosquitoes that enter huts in which there are human volunteers. To compare the synthetic odor blend with whole humans under epidemiologically relevant conditions, and determine whether wild mosquitoes would also be attracted to this it, field experiments were conducted in which the blend was compared with 10 different adult male volunteers (20–34 years old). We used 4 specially-designed experimental huts (Fig. S4) located at 10 to 100 metres apart in a malaria-endemic village in rural Tanzania. The experimental huts were located at the edge of the village, so that they were between the main breeding grounds (a perennial rice field) and the human houses, and also so that they were at least 50 metres away from the nearest human house. Each night, 2 human volunteers slept under non-insecticidal bed nets inside 2 separate huts. In the third hut, the synthetic odor blend was dispensed by evaporation in a continuous air plume, also from under a non-insecticidal bed net. The fourth hut had a variant of this blend which lacked one constituent, 3-methyl-butanonic acid (3mC4). This constituent was the most unpleasant smelling, so it was expected that its exclusion would improve the acceptability of the odor blend. The blend and its variant were dispensed using nylon strips inserted in the attractant plume tube of an MM-X® trap (a counter flow geometry trap made by American Biophysics Corporation) and CO₂ gas was added to the trap using rubber tubing (Figs. S1-S3). This odor dispensing method has been described in detail in Okumu et al [21].

In these field tests, the MM-X® was used only to dispense the blend but not to trap mosquitoes; its collection tube was closed and the exhausted fan disabled so as to eliminate suction. Instead, a different trap, the Centres for Disease Control Light Trap (CDC-LT), was set up in all the experimental huts to catch mosquitoes that enter. The light traps were placed beside the bed nets (from under which the odor cues from human or blend were emanating), such that it was one metre from the floor. To minimize any positional bias, the set up was rotated nightly so that at the end of a four-day rotation, the blend, its variant, and each volunteer had been to every hut. Also by the end of each rotation, the blend and its variant had been tested against humans four different times. Then a new pair of volunteers was recruited and the test repeated for another four nights. This way, the blend and its variant were each tested against 10 different humans (5 pairs) over 20 different nights. All experiments were conducted between 7.00pm to 7.00am, during which time mosquitoes entering the huts were trapped; and each morning, the mosquitoes were sorted into different taxa and counted.

Field Experiments Conducted to Evaluate the Blend against Wild Mosquitoes

**Study village.** The study field was conducted in Lupiro Village (8.01°S and 36.63°E), Ulanga District, in the south eastern part of Tanzania. The village lies 300 metres above sea level, and experiences very high malaria transmission, recently reported to be at least 50% in 2007 (3mC4) at 0.001%, heptanoic acid (C7) at 0.01%, octanoic acid (C8) at 0.01% and tetradecanoic acid (C14) at 0.01%. Finally, a variant of the blend was formulated by removing the unpleasant smelling 3mC4 in an attempt to improve the appeal of the blend to potential users.

Comparing the number of mosquitoes attracted to the synthetic odor blend with natural human foot odors showed that the blend was less effective at attracting mosquitoes than the foot odors. When both the synthetic blend and the foot odors were available to mosquitoes, the foot odors were more attractive than the blend, but when only the blend was available, the mosquitoes were equally attracted to both. These results suggest that the synthetic blend may not be a suitable alternative to natural human foot odors.

Ethics statement. After a full explanation of the risks involved and the objectives of the study, written informed consent was obtained from the volunteers, all of whom were male and aged between 20 to 34 years old. When inside the huts during the experimental nights, all volunteers slept under bed nets and were guaranteed immediate access to treatment if needed. Weekly screening for malaria parasites by light microscopy and treatment with artemether-lumeartin did not result in any participants being affected during the study. Ethical approval for the study was obtained from the Medical Research Coordination Committee of the National Institute for Medical Research of the United Republic of Tanzania (NIMR/HQ/R.8a/Vol.IX/710).
between 7.00pm and 7.00am. The mosquitoes were sorted into different taxa and counted daily. These experiments were repeated for a total of sixteen nights.

Molecular Analysis
A total of 600 *An. gambiae sensu lato* mosquitoes were selected for further identification using ribosomal DNA-polymerase chain reaction [22]. To constitute this total sample, 200 females were selected at random from catches obtained with each bait type.

Data Analysis
Data from the semi-field experiments were analyzed by logistic regression using SPSS version 15 (SPSS Inc., Chicago). With regards to the data from the field experiments, we compared the arithmetic means of all the mosquito catches per hut per night and illustrated these using simple bar graphs with error bars showing 95% confidence intervals. Further analysis was conducted using General Linear Models (GLM) as follows as follows: Mosquito catches were modelled as a function of two fixed factors, bait and hut, treating day as a random variable to reflect daily fluctuations in mosquito numbers. Also, due to the heterogeneity of the mosquito counts, the data was log transformed to make it amenable to assumptions of the standard normal distribution.

Results
High-throughput determination of optimal concentrations of individual candidate attractants was successfully achieved through the binary choice assays in the semi-field enclosure. These were the concentrations at which the individual candidate attractants needed to be added so as to obtain a blend maximally attractive to laboratory-reared *An. gambiae s.s.*. When the resulting blend was tested within the semi-field enclosure against a combination of natural human foot odors collected in worn nylon socks and CO₂ gas, it caught 46.5% (38.0%–55.9%) of all responding mosquitoes, indicating no difference between the two odor sources ($P = 0.408$).

In the field experiments conducted to compare our synthetic blend with humans, more mosquitoes were trapped inside the huts with either variant of the synthetic blend than inside the huts in which human volunteers slept ($F = 66.025$, $P < 0.001$). With or without 3mC4, the blend attracted approximately 3.9 times more *An. gambiae sensu lato* (morphologically indistinguishable members of the species complex of which *An. gambiae* is a member), 3.1 times more *An. funestus*, 5.2 times more of other *Anopheles* species, 4.2 times more *Culex* mosquitoes and 2.7 times more *Mansonia* mosquitoes than an average human volunteer (Fig. 2).

In the other separate experiment, where the full blend was placed inside the same huts occupied by human volunteers, the number of mosquitoes of any species entering those huts was increased compared to huts in which there were only human volunteers. There were 2.6 times more *An. gambiae s.l.*, 5.5 times more *Culex* and 4.6 times more *Mansonia* mosquitoes (Fig. 3A). However, once the mosquitoes had entered the huts, significantly more *An. gambiae s.l.* ($P < 0.001$), *Culex* ($P = 0.045$) and *Mansonia* mosquitoes ($P < 0.001$) were attracted to the humans than to the synthetic blend and similar but non-significant trends were observed for *An. funestus* ($P = 0.179$) and the assortment of other *Anopheles* mosquitoes ($P = 0.82$) present in the study village (Fig. 3B).

Molecular identification of *An. gambiae s.l.* mosquitoes caught in the experiment described in Fig. 1 showed that 97.8% (405/414) were *An. arabiensis*, with the remainder being *An. gambiae s.s.*, and this proportion did not vary with bait type ($F = 2.96$, $P = 0.53$). Furthermore, unlike previous blends tested in Africa [13], the proportions of mosquitoes caught which were accounted for by the various mosquito taxa were essentially identical to those observed with human baits ($\chi^2 = 2.94$, $P = 0.938$), so these synthetic lures appear to representatively sample a wide range of human-biting mosquitoes with high sensitivity (Table 1).

Discussion
This study greatly improves upon some of the recent successes with odor based technologies [10,13,14] and therefore represents a significant advancement in attempts to develop synthetic lures, which would effectively compete against humans for host seeking mosquitoes in actual field settings. Similar to previous field evaluations of synthetic blends for attracting *Aedes aegypti*, the principle vector of yellow fever and dengue [10,14,23], and *Anopheles gambiae*, the most important vector of malaria globally [13], our findings confirm that it is possible to formulate synthetic odor blends which attract as many mosquitoes as human odors, even without including all the biologically active components naturally found in the human emanations. In this study, we did not assume that a ‘surrogate human’ would consist of only the few compounds that we included. Instead, we expect that it would be possible to improve upon this simple mixture of carboxylic acids, ammonia and carbon dioxide by adding other attractants from different chemical groups, for example ketones and aldehydes, as well as physical cues such as heat and color.

Also, it appears that similar to results of previous studies [10,24], preferences of mosquitoes towards any one of two different stimuli are dependent upon distances between those stimuli, i.e. whether the stimuli are in direct short range competition or whether they are far apart and have completely separated odor plumes (so that they are not perceived by the host seeking mosquitoes as two competing odor sources). Whereas huts baited with the synthetic blend or its variant had more mosquitoes than those huts with humans, many of the vectors when presented with the two odor sources side by side within the same hut appeared to retain their preferences for humans. On the basis of this observation and also because the experimental huts were located 10–100 metres apart,
An. gambiae sensu stricto although the synthetic blend was originally optimized using only
and relevant across Africa (Ogoma et al., Unpublished). Therefore, the synthetic blend was originally optimized using only
laboratory-reared An. gambiae sensu stricto, it can be considered a
general attractant that is equally effective for use against a range of
other important human-biting mosquito species in the field.
This highly attractive and broad spectrum odor blend has many
potential applications because it can substitute for human subjects
as baits in mosquito traps, or as decoy hosts to lure away
mosquitoes which would otherwise bite and possibly transmit
pathogens to humans. The availability of such a consistent and
effective bait for representative sampling a wide range of host-
seeking mosquitoes means that it should be possible to measure
human exposure to mosquito-borne infections without the need
for human volunteers. The necessity to use human volunteers as
baits in mosquito traps inevitably limits their compactness,
consistency, cost and safety, and has restricted the development
of sustainable mosquito surveillance systems, especially in
resource-limited countries [23].

The attractants might also be applied for mass trapping of
mosquitoes so as to reduce, rather than merely monitor,
transmission of the various pathogens that the mosquitoes carry.
Such mass trapping operations would simultaneously suppress
nuisance bites from abundant Culicine mosquito species, thereby
enhancing community uptake of such technologies. Another
potential application of this odor blend is to combine them with
existing malaria control tools using push-pull strategies similar to
those commonly practiced in agricultural pest management
[26,27]. For example, where people use insecticide treated nets
(ITNs) or indoor residual spraying with insecticides (IRS) which
apart from being lethal targets also deter mosquitoes from entering
houses, odor baited traps could be strategically located so as to
trap the mosquitoes deterred from dwellings. Similarly, this odor
blend could be used to lure host-seeking vectors to lethal outdoor
targets such as surfaces treated with insecticides, a strategy that has
been successfully applied to control tsetse flies [28].

One priority area for application of this or similar lures would be
for malaria control, where there is currently an over-reliance on
insecticide-based and intra-domiciliary methods, specifically ITNs
and IRS. Mathematical simulations using adaptations of estab-
lished transmission models [29], suggest that appropriate trap
devices baited with these lures could reduce exposure to malaria,
and effectively complement ITNs in areas of highly intense
malaria transmission in Africa (Okumu et al., Unpublished).

The fact that this synthetic blend greatly increased mosquito
densities inside huts, yet humans sleeping in those huts remained
equally or more attractive to the mosquitoes, necessitates careful
consideration of safety and ethics whenever this or similar long-
range attractants are used. Trap devices containing these blends
should not be positioned in or near human habitations because
this can increase exposure to mosquito bites and mosquito-borne
pathogens. Our observations, combined with measurements of the
attractive range of mammalian hosts to mosquitoes [30], imply
that such devices must be located at least 30 meters away from
human houses, a concept which can actually be exploited by

Figure 3. Short range performance of the synthetic blend.
Addition of the synthetic blend into huts occupied by human
volunteers significantly increased the number of mosquitoes caught
in the huts compared to huts with only a human volunteer (A).
However, once the mosquitoes were inside the huts, preferences for
either bait type were similar for An. funestus (P = 0.179) and the other
unidentified Anopheles mosquitoes (P = 0.82) but humans remained
significantly more attractive to An. gambiae s.l. (P < 0.001). Culex
(P = 0.045) and Mansonia mosquitoes (P < 0.001) (B), by General Linear
Model using SPSS version 15 (SPSS Inc.). The error bars represent 95%
confidence intervals.
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Table 1. Proportions of various mosquito taxa making up the total mosquito catches in experimental huts baited with the blend,
its variant (without 3-methylbutanoic acid) or humans.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Blend variant</th>
<th>Blend</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anopheles gambiae</td>
<td>30% (1921)</td>
<td>28% (991)</td>
<td>28% (991)</td>
</tr>
<tr>
<td>Anopheles funestus</td>
<td>2% (125)</td>
<td>2% (78)</td>
<td>2% (78)</td>
</tr>
<tr>
<td>Other Anopheles</td>
<td>11% (715)</td>
<td>8% (281)</td>
<td>8% (281)</td>
</tr>
<tr>
<td>Mansonia species</td>
<td>26% (1998)</td>
<td>34% (1015)</td>
<td>34% (1015)</td>
</tr>
<tr>
<td>Culex species</td>
<td>33% (1576)</td>
<td>33% (1576)</td>
<td>33% (1576)</td>
</tr>
<tr>
<td>Totals</td>
<td>100% (6668)</td>
<td>100% (6492)</td>
<td>100% (3565)</td>
</tr>
</tbody>
</table>

Numbers in parentheses refer to the total number of mosquitoes caught during the entire field study.
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mosquito control programs to target specific areas where mosquitoes are most abundant, notably the periphery of human settlements, thus enhancing impacts of such programs [31,32]. The observation that the blend acts at medium-to-long range also suggests that optimization of behaviorally active formulations in even larger semi-field systems, followed by both short-range and long-range choice tests in the field, could accelerate and enhance the development of even more potent synthetic lures.

**Conclusion**

We conclude that this synthetic odor blend has potential to be used for reproducible and exposure-free sampling of human-biting mosquitoes and also might be developed into high-impact, environmentally-friendly interventions against mosquitoes and diseases that they transmit. Added advantages of such technologies would include possibilities to target mosquitoes that bite outdoors or early in the evening before people go to bed and even diurnal vectors such as *Aedes aegypti* that often attack their victims far away from their houses [33]. Finally, because of the intimate dependence of mosquitoes upon their specialized host-seeking behaviors to obtain blood and survive, it is unlikely that odor-based technologies would be affected by physiological resistance among mosquitoes, as is common with insecticide based methods [34].

**Supporting Information**

File S1  Supporting file for materials and methods section. This file contains additional information on candidate odor compounds, the trapping devices used, description of odor dispensing methods and statistical analysis used. Found at: doi:10.1371/journal.pone.0008951.s001 (0.03 MB DOC)

Figure S1  Picture (A) and Drawing (B) of the MM-X® trap. © American Biophysics Corporation. Found at: doi:10.1371/journal.pone.0008951.s002 (1.08 MB TIF)

**References**