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Long-Range But Not Short-Range Attraction of Male *Aedes aegypti* (Diptera: Culicidae) Mosquitoes to Humans

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Abstract

Female *Aedes aegypti* (Linnaeus) mosquitoes integrate multiple sensory cues to locate human hosts for blood meals. Although male *Ae. aegypti* swarm around and land on humans in nature to mate, direct evidence of attraction to humans is limited. Male mosquito attraction to human host cues is often undetectable in confined laboratory assays, leading to a misconception that male mosquitoes are not attracted to humans. We used semifield experiments to demonstrate robust attraction of male *Ae. aegypti* to humans. Human-baited traps captured up to 25% of released males within 15 min, whereas control traps without humans as bait failed to capture males. Rapid attraction to humans was further demonstrated through videography. Males swarmed around and landed on human subjects, with no activity recorded in paired unbaited controls. Finally, we confirm the lack of discernible male attraction to humans in small laboratory cages. Our experiments demonstrate that both male and female *Ae. aegypti* show attraction to humans, but with clear sex-specific behavioral differences at short-range. Male mosquito attraction to humans is likely to be important for mating success in wild populations and its basis should be further explored. Our results highlight the importance of arena size and assay design for mosquito behavioral research. A better understanding of host cues that attract males could help us to improve mosquito surveillance and control.

Key words: male, mosquito, attraction

Aedes aegypti female mosquitoes are strongly anthropophilic (Harrington et al. 2001, McBride et al. 2014). Human skin odors, exhaled CO_2, body heat, and visual contrast all act as signals for female mosquitoes to find blood meal hosts (Liu and Vosshall 2019). Humans vary in their attractiveness to female mosquitoes and this is influenced by factors including body size (Port et al. 1980), diet (Paskewitz et al. 2018), and variation in skin odors (Logan et al. 2008, Verhulst et al. 2010). Although male mosquitoes do not blood feed, they have sophisticated auditory and olfactory systems (Wheelwright et al. 2021) used to locate female mosquitoes (Cator et al. 2009, Menda et al. 2019), nectar and other sugar sources (Barredo and DeGennaro 2020), and conspecific males (Cabrera and Jaffe 2007, Fawaz et al. 2014, Pitts et al. 2014).

A number of observations point to male *Aedes* mosquitoes being attracted to humans despite their inability to blood feed. Field observations report males swarming around and landing on humans (Banks 1908, Lumsden 1957, McClelland 1960, Yasuno and Tonn 1970, Hartberg 1971, Gubler and Bhattacharya 1972, Trpis et al. 1973, Cator et al. 2011). Furthermore, male *Aedes* capture rates increase when traps are baited with CO_2 and human odor mimics (Pombi et al. 2014, Roiz et al. 2016, Amos et al. 2020, Visser et al. 2020). In a pilot experiment, Lau et al. (2020) demonstrated rapid attraction of males to humans under semifield conditions. While males and females show similar rates of attraction to humans, sex-specific behaviors exist, with males typically swarming around humans and rarely landing. Swarming *Ae. aegypti* males fly in a characteristic figure-of-8 pattern around humans. This behavior is likely to increase their reproductive success as they intercept and mate with host-seeking females (Hartberg 1971, Cabrera and Jaffe 2007, Cator et al. 2011).

Despite the above evidence, some researchers still consider that male mosquitoes lack attraction to humans. In a recent paper, Basrur et al. (2020) claim that only female *Ae. aegypti* mosquitoes host-seek, but removal of the *fruitless* gene in males activates host-seeking behavior in male *Ae. aegypti*. Their conclusions are based on laboratory experiments, which often fail to detect male attraction to human host cues (McMeniman et al. 2014, van Breugel et al. 2015, Peach et al. 2019).

Male *Ae. aegypti* behavior is less well-understood than female behavior and males are often neglected in mosquito laboratory behavioral and physiological research. Consequently, appropriate bioassays for quantifying and determining the nature of male host attraction have not been developed. In the current study, we performed experiments under semifield conditions and demonstrate that male *Ae. aegypti* are attracted to humans, even in the absence of female mosquitoes. Our methods can be applied to further understand the basis of male mosquito attraction to humans.

Materials and Methods

Mosquito Strains and Maintenance

Aedes aegypti colonies were established from field collections in Cairns (Queensland, Australia) in January 2021. Mosquitoes used in all experiments ranged from F_1 to F_3. Laboratory colonies were maintained at 27 ± 1°C, 70% RH with 12:12 (L:D) h regime. Adults were provided with a honey/water solution (50:50) and were blood fed using human volunteers (Human ethics approval from James Cook University H4907 and The University of Melbourne 0723847). Eggs were collected and allowed to embryonate for 3 d before being stored in air-tight containers for up to 2 mo. Eggs were hatched in water containing 0.2 g bakers' yeast (Lowan Whole Foods, Glendenning NSW, Australia) per liter. Mosquito larvae were reared on fish food powder (TetraMin Tropical Flakes Fish Food, Tetra, Melle, Germany). Pupae were sexed and transferred to clear plastic containers (300 ml) covered with a white mesh cloth (0.5 mm pore size) with a sponge on top (30 × 40 mm²) soaked with honey/water solution (50:50).

Semifield Experiments

We tested male mosquito attraction to humans under semifield conditions through two approaches. In the first approach, we captured mosquitoes with paired male *Aedes* sound traps (MASTs; Staunton et al. 2021a) that were either unbaited or baited with a human subject sitting near the trap entrance. In the second approach, we used videography to quantify male presence and activities in the vicinity of a human subject, compared to an unbaited control on the other side of the cage. Experiments in semifield cages were conducted during daylight hours in March and April, 2021. The experimental arena measures 17.5 × 8.7 m and is described in detail by Ritchie et al. (2011). Competing visual stimuli in the semifield cage were minimized (e.g., dark-colored objects were covered with lighter-colored materials). Nitrile gloves were worn when handling objects and frequently touched objects (e.g., door handles, MASTS) were regularly wiped with EtOH (80%) throughout the experimental period to minimize human odor interference. Male mosquitoes used in semifield experiments were unmated, between 2- and 7-d post-emergence. All human subjects acting as lures wore light-colored clothing, minimized movement, and did not use perfumed products 24 h before and during the trials.

MASTs use sound frequencies which mimic female mosquito flight tones to capture male *Ae. aegypti* (Staunton et al. 2021a).

MAST trials involved two paired treatments within the same semifield cage (Flight cage B). The black MAST bases (which act as swarm markers) were not used in these trials. Instead, MAST heads (the capture container of the trap system which included the sound lures) were placed on upturned white plastic buckets (3 L) such that they were 15 cm above the ground. MASTs were placed 5.5 m apart outside of a structure built to resemble the downstairs area of a typical house in Queensland (Fig. 1A). A white plastic and metal chair was placed over each trap. A human (subject A in the below experiment) acting as bait sat in one chair, with the other left empty, with the positions of human-baited and unbaited treatments swapping each replicate. Male *Ae. aegypti* were released remotely at a central location in the cage approximately 6 m from the traps. We ran 4 replicate trials with 100 males at sound lure settings of 495 Hz, continuous tone (volume level 1), and 16 replicate trials with 20 males at sound lure settings of 550 Hz, continuous tone (volume level 2). Trials ran for 15 min and mosquitoes captured by the MAST were counted. Numbers of males captured by human-baited and unbaited MASTs were compared using Wilcoxon signed-rank tests. All data were analyzed using SPSS statistics version 24.0 for Windows (SPSS Inc, Chicago, IL).

Videography trials involved two paired treatments on opposite sides of flight cage A (3.2 m apart; Fig. 1A). Two cameras (Professional Series Motorised Bullet 8MP cameras (VIP Vision) were installed at ground level facing upturned plastic white (3 L) buckets in front of white corrugated plastic paneling (600 × 800 × 5 mm; Corex Plastics Australia Pty. Ltd.). In the human-baited treatment, a human subject sat on a white plastic and metal chair and placed their bare feet on the bucket for the duration of each trial. Unbaited treatments were set up identically to human-baited treatments, but on the opposite side of the cage and without a human subject. Two subjects were used, subject A (female, Caucasian, age 32) and subject B (male, Caucasian, age 29), and the position of human-baited and unbaited treatments was swapped each replicate (n = 16 replicate trials per subject). Before trials commenced, 50 male *Ae. aegypti* were released remotely at a central location in the cage once per day. Video footage was used to count the number of visible mosquitoes (both flying and landed in the frame (approximately 1200 × 700 mm field of view, covering the vicinity of the subject's feet and lower legs in the human-baited treatment) every 30 s for 10 min, starting at time zero when the participant placed their feet on the bucket. Numbers of males observed in human-baited and unbaited treatments at 10 min were compared using Wilcoxon signed-rank tests.

Laboratory Olfactometer Assays

We compared the attraction of male and female *Ae. aegypti* to a live human host (male, Caucasian, age 31) under confined laboratory conditions. Experiments were performed in a two-port olfactometer (30 × 30 × 30 cm; Fig. 2A) identical to the one used by Ross et al. (2019), except that the stimulus ports were removed. We performed three treatments: males only, females only, and females + males, with each treatment replicated eight times. Mosquitoes of both sexes were 6-7 d post-emergence, allowed to mate, and sugar-starved for approximately 24 h. In a separate experiment, we performed an additional treatment with unmated, nonstarved, 2- to 3-d-old males to match the physiological state of males used in the semifield experiments. In each treatment, approximately 20 adults per sex were released into the cage and left to acclimate for 1 min. A box fan placed at the opposite end of the cage drew air (~0.2 m/s) through two traps into the cage. The hand of the human subject was placed 1 cm in front of one of the traps, with the other blank. Sides were alternated
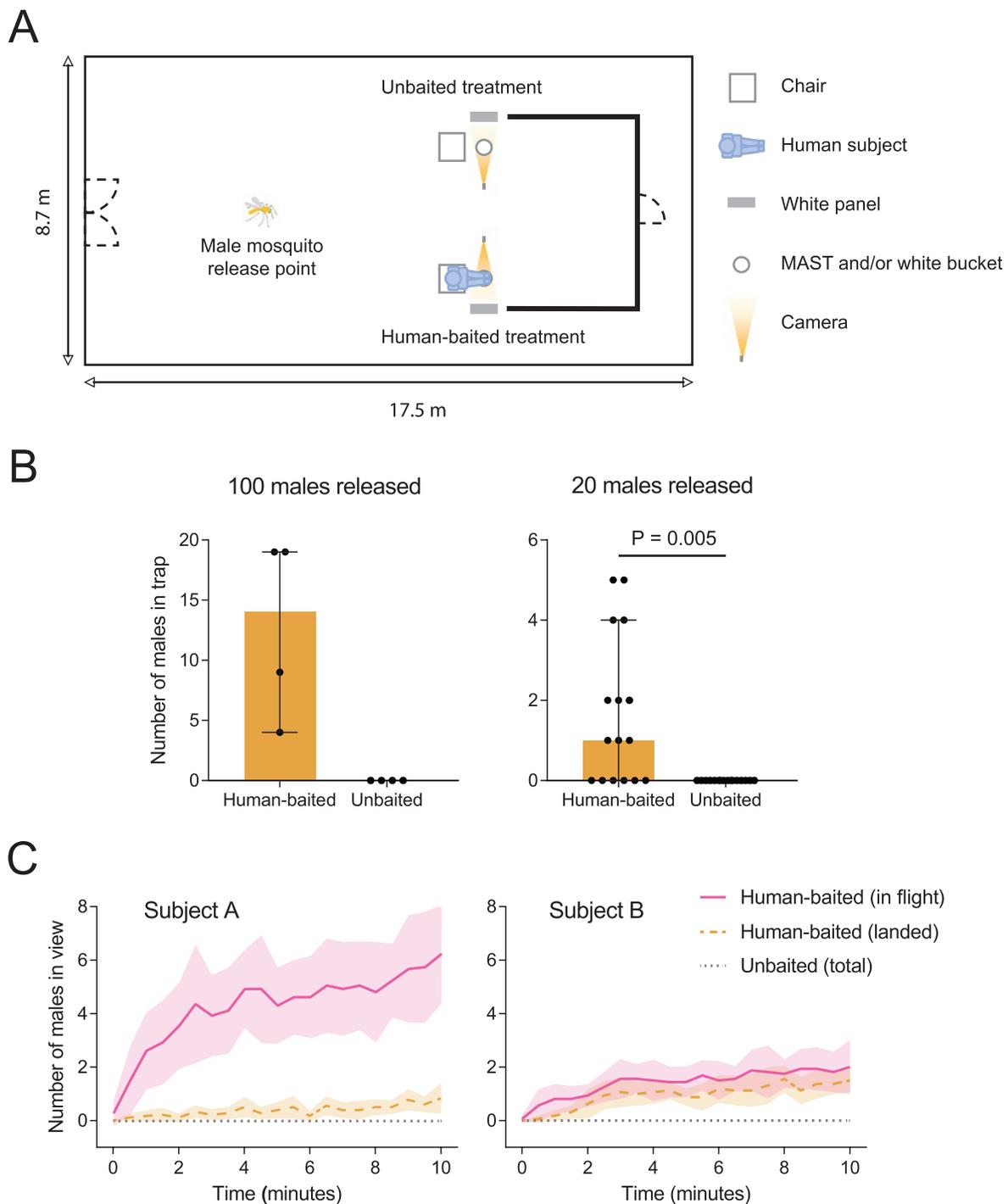
Fig. 1. Male *Aedes aegypti* mosquitoes locate, swarm around, and land on human subjects. (A) Layout of semi-field enclosure, showing the locations of humanbaited and unbaited treatments for MAST (B) and videography (C) trials. (B) Number of males caught by human-baited and unbaited MASTs in 15 min when either 100 or 20 mosquitoes were released into the semifield cage. Bars represent medians with dots showing data from individual replicate trials. Error bars are 95% confidence intervals. (C) Males in view of cameras in human-baited (solid and dashed lines) and unbaited (dotted lines) treatments at 30-s intervals. Experiments were performed with two human subjects (*n* = 16 replicate trials per subject). Solid lines represent males in flight while dashed lines show males that had landed on the human subject or the footrest. Means are shown with shaded regions representing 95% confidence intervals.

each replicate. After 5 min, the entrances to both traps were closed and the number of males and/or females in each trap as well as the cage was counted. Mosquitoes that were damaged before or during the experiment were excluded. Proportions of males and females collected in stimulus and blank traps after 5 min were compared using Wilcoxon signed-rank tests.

Results

Semifield Experiments

In the first experiment, we released 100 males and recaptured them using MASTs (Staunton et al. 2021a). After 15 min, we found that MASTs baited with a human subject sitting over the trap captured
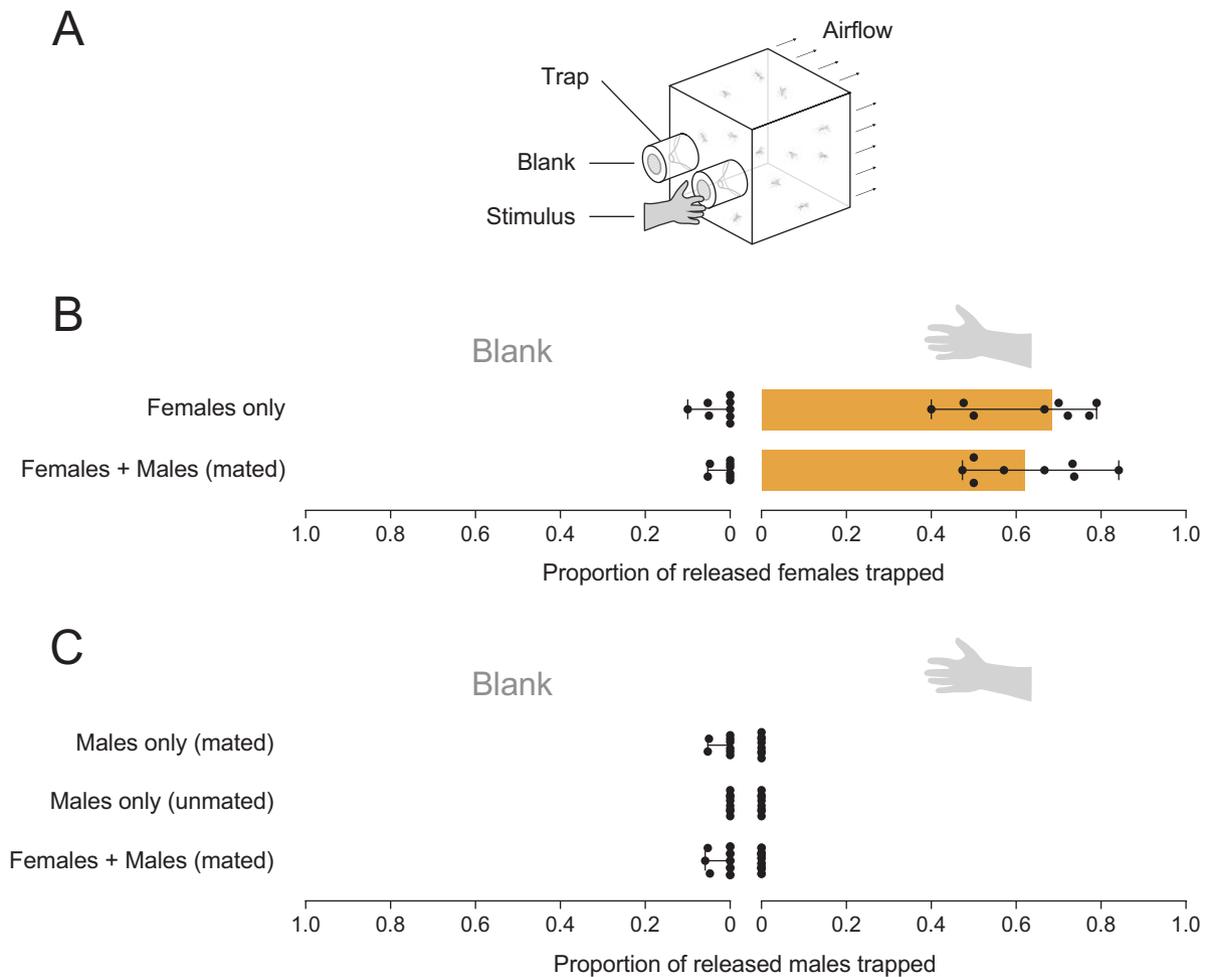
Fig. 2. Male *Aedes aegypti* mosquitoes are not attracted to humans under confined laboratory conditions. (A) Diagram of two-port olfactometer. Mosquitoes were released into the cage and collected by one of two traps after 5 min. Traps were either unbaited (blank) or baited with the palm of a human subject (stimulus). (B and C) Proportions of released (B) females and (C) males attracted to a human hand or unbaited trap [n = 8 replicate trials each for females only, males only (mated), males only (unmated), and females + males (mated)]. Bars represent median trap proportions with dots showing proportions from individual replicate experiments. Error bars are 95% confidence intervals.

14% (median) of males, while no mosquitoes were captured by unbaited MASTs with no human present (Fig. 1B). In the second experiment using 20 males, human-baited traps captured up to 25% (median 5%) of the released males (Fig. 1B). In contrast, no mosquitoes were captured by unbaited MASTs in all 16 replicates (Fig. 1B). Differences in capture rates between human-baited and unbaited traps were significant according to a Wilcoxon signed-rank test ($Z = 2.821$, $P = 0.005$). Capture rates were higher when larger numbers of males were released, although we could not perform direct comparisons between trials due to different sound settings which could plausibly influence capture rates. We observed many males swarming near the human subject that were not captured, suggesting that trap numbers underrepresent attraction.

We performed an additional experiment using videography to quantify male *Ae. aegypti* attraction to humans without traps (Supp Videos S1–S4 [online only]). Male mosquitoes began swarming almost immediately and occasionally landed on the subjects (Fig. 1C), consistent with observations of male swarming in nature (Hartberg 1971, Cator et al. 2011). The number of males observed in flight or landing increased over time, exceeding 10% after 10 min for subject A; fewer males were viewed in flight around subject B. The number of males observed in human-baited treatments after 10 min

was significantly higher than in unbaited treatments for both human subjects [Wilcoxon signed-rank test: Subject A (in flight): $Z = 3.535$, $P < 0.001$; Subject A (landed): $Z = 2.751$, $P = 0.006$; Subject B (in flight): $Z = 3.077$, $P = 0.002$; Subject B (landed): $Z = 3.482$, $P < 0.001$]. Attraction rates are likely underestimated since some males within the vicinity of subjects were outside the field of view of the camera. Mosquitoes were not observed in the unbaited treatments for either subject (Fig. 1C).

Laboratory Olfactometer Assays

We tested whether male *Ae. aegypti* show attraction to humans under laboratory conditions using a two-port olfactometer (30 × 30 × 30 cm; Fig. 2A). Females showed strong attraction to humans, with >60% being collected in human-baited traps after 5 min (Fig. 2B). The number of females caught in human-baited traps was higher than in unbaited controls (Wilcoxon signed-rank test: females only: $Z = 2.521$, $P = 0.012$, females + males: $Z = 2.524$, $P = 0.012$). Rates of attraction were similar regardless of whether males were present in the same cage. In contrast to females, no males were captured in human-baited traps in any treatment, regardless of whether they were mated or unmated (Fig. 2C). Few mosquitoes were attracted to blank ports across all treatments (Fig. 2B and C).

Discussion

Our work highlights that wild-type (Cairns, Australia) male *Ae. aegypti* are attracted to humans in open spaces, but not under confined conditions when using a port of entry assay. These findings show how assays performed at different scales can lead to opposing conclusions, likely because they are measuring different aspects of mosquito behaviors (e.g., McMeniman et al. [2014]).

While both MAST and videography trials showed unambiguous attraction of male mosquitoes to human subjects, they have limitations. MASTs were unable to capture many of the swarming males and the sound lures within the MASTs could plausibly influence their attraction to humans. Video recording eliminated the need for quantification through trapping, but this approach may also underrepresent attraction given the limited field of view. Observed attraction may be influenced by several factors, including the number of mosquitoes used in experiments. MAST capture rates were higher when larger numbers of males were released, suggesting a potential male conspecific effect. While different MAST settings were used between trials, the two sound frequencies used did not differ in their capture rates in international field trials (Staunton et al. 2021b). This conspecific effect is consistent with evidence for an aggregation pheromone in this species (Cabrera and Jaffe 2007, Fawaz et al. 2014). However, while swarming activity may increase with the number of males present, the location of males is clearly influenced by the location of the human subject. In the videography trials, we also observed differential attractiveness between two human subjects, consistent with observations on female mosquitoes (Martinez et al. 2021).

Although we did not perform direct comparisons between males and females in the semifield experiments, we suspect that rates of attraction between males and females are similar. In previous work in the same semifield enclosure, females started to land on human hosts within seconds to minutes (McMeniman et al. 2014, Lau et al. 2020). In our study, we observed swarming almost immediately (at the first time point after the trial commenced) and up to 20% of males in some trials were in the camera's field of view at any given time. However, direct comparisons between the sexes are challenging given that males tend to swarm around the human subject (while females land), making it difficult to determine the proportion of total males that demonstrate attraction at each point during the experimental period.

Our experiments under confined laboratory conditions confirm clear sexually dimorphic attraction to and interactions with humans at short range. Female *Ae. aegypti* use separate sets of cues at different distances (Lacey et al. 2014, van Breugel et al. 2015). Given the lack of requirement from males to detect short range or landing cues used by females to alight and feed, it is plausible that males lack attraction to specific short-range cues. It could also be that a lack of space for normal male flight and swarming inhibits olfactory and visual navigation to a host. Future work examining individual host cues (e.g., CO_2, odor, and heat) under different conditions (e.g., confined, unconfined, and long- and short- range) will help us to determine the basis of these sex-specific differences, as well as the specific host cues which attract males at long distances.

Our findings are consistent with those of Basrur et al. (2020) in showing a lack of attraction at close range. However given that long-range attraction was not measured by Basrur et al. (2020), their inferences about male attraction are limited to short range behaviour. Furthermore, because we show that males are attracted to humans in the absence of female *Ae. aegypti*, we disagree with their hypothesis that male mosquitoes in nature are attracted to host-seeking females near humans rather than humans themselves. While the *fruitless* gene studied by Basrur et al. (2020) appears to contribute to sex-specific host-seeking behaviors at close range, our work shows that attraction to humans is already a characteristic of wild-type males. It will be interesting to explore the impact of *fruitless* in larger arenas where *fruitless* mutant males may land on humans more frequently than wild-type males under semi-field conditions.

Male attraction to humans has important implications for mosquito control, particularly for mass-releases of males for mosquito population suppression (Carvalho et al. 2015, Crawford et al. 2020), where released male mosquitoes are likely to be regarded as a nuisance by residents in intervention areas (https://www.todayonline.com/voices/project-wolbachia-residents-are-killing-helpful-mosquitoes-which-can-be-nuisance). Human host odors show potential as lures for traps used as vital monitoring tools in these programs, particularly in combination with sound lures (Staunton et al. 2021a). Measurements of these effects under laboratory conditions but aimed at extrapolating to field scenarios should ensure that a full range of long- and short-distance cues can be used by male *Ae. aegypti*.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Acknowledgments

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