RESEARCH REPORT
MOSQUITO RESEARCH PROGRAM

PRINCIPAL INVESTIGATOR : Ring T. Cardé
Department of Entomology, University of California, Riverside CA 92521

PROJECT TITLE:
Adjuvants for Traps Baited with Carbon Dioxide: What is Their Mode of Action?

PROJECT SUMMARY:
The use of adjuvants such as octenol to increase catch of mosquitoes traps baited with carbon dioxide has the potential to increase trap sensitivity and species selectivity. Although many field tests of such adjuvants have been conducted, how they work remains speculative. We propose two hypotheses: they act with carbon dioxide at long range to lower the odor threshold (and thereby increase the odor plume's effective reach) or they act near the trap to increase the time of local search (which increases the likelihood of a mosquito traversing the trap's "suction zone"). Because of several technical barriers to testing these hypotheses in the field, we propose wind-tunnel tests aimed at establishing if one (or both) explanations are correct. As exemplars, we will use *Culex quinquefasciatu*s with nonanal and *Aedes aegypti* with octenol and two kairomone mixtures. Understanding how adjuvants work may lead to new behavioral methods that enable screening for new adjuvants and it may provide a bioassay for testing for natural constituents of host odors. At present we do not lack for candidate compounds—for example, hundreds are known from human skin "head space." Which blend of compounds are actually used, however, generally remains a mystery.

This work was funded in May, 2012 and since then we have completed one study with *Aedes aegypti*. We have found that when carbon dioxide and human foot odor are presented alone or in choice assays, either sequentially along the tunnel or in side-by-side competition at the tunnel's upwind end, females will orient to foot odor over CO_2, both in terms of the proportion locating the source and their propensity to land on it. When both odor stimuli are presented in a small, narrow odor plumes, then females will orient to but not land on the odor source unless carbon dioxide and foot odor are presented together. These and two other completed tests indicate that we have new methods to determine how adjuvants influence orientation.

ADJUVANTS FOR TRAPS BAITED WITH CARBON DIOXIDE: WHAT IS THEIR MODE OF ACTION?

Background

Orientation by female mosquitoes to distant hosts (10 or more meters away) generally seems mediated by CO_2 with other host-emitted odors contributing to orientation at a range of several meters (Cardé and Gibson 2010). These non-CO_2 host odors, from what it known, often are somewhat specific for a given mosquito species and its hosts. CO_2, conversely, seems to be a universal attractant for nearly all mosquito species.

Traps baited with CO_2 are widely used for mosquito surveys and surveillance. Several chemicals have been identified as "synergizing" attractiveness of CO_2, most notably 1-octen-3-ol (hereafter referred to as "octenol"), which is effective for some but not all species (e.g., Kline 1994, Kline et al. 1990). Originally octenol was tested for its possible effect on mosquito response to CO_2 following its identification as a host-released (from bovine breath and urine) attractant for tsetse. Recently nonanal (a common odor released from many kinds of natural sources, including birds and humans) also has been characterized as increasing capture of *Culex quinquefasciatus* over CO_2 alone (Zyed and Leal 2009). Just how these two chemicals modulate mosquito capture in traps baited with CO_2 is unknown.

Early behavioral assays used racemic octenol, but recent tests by Cook et al. (2011) have attempted to resolve whether the pure R and S enantiomers of octenol or some enantomeric mixture have the highest activity. In *Aedes aegypti* and *Cx. quinquefasciatus*, the R-enantiomer produces in the octenol-sensitive sensilla a higher electrophysiological activity than the racemate or the S-enantiomer. The R-form also increased attractiveness to *Ae. aegypti* of CO_2 in a Y-tube, although field tests remain to be conducted. Other field tests generally have supported that the R-form yields a higher catch than racemic octenol (Kline et al. 2007), although no enantiomeric form or mixture of octenol evidently synergizes the capture of *Cx. quinquefasciatus*.

Counterflow and CD-style traps baited with just CO_2 do not capture a high proportion of mosquitoes lured to their immediate vicinity (Cooperband and Cardé 2006a, b). Using a large, walk-in field wind tunnel and 3-D video recording, this study showed that many female *Culex quinquefasciatus and Cx. tarsalis* were lured upwind along the plume of CO_2 to the immediate vicinity of traps where they flew circuitous tracks. Indeed, of those *Cx. quinquefasciatus* lured to the immediate vicinity of an Encephalitis Virus Surveillance trap (widely used in California programs), only 13% actually were captured. Whether or not they were eventually captured seemed contingent on how long they engaged in "localized search" (a meandering to-and fro circuit near the trap). This path on occasion carried the mosquito into the trap's suction zone.

Study Objective: resolve how octenol, nonanal and host-related odors augment capture in CO_2-baited traps by testing two hypotheses:

H_1. Octenol, nonanal, and other host-related odors increase attraction by lowering a mosquito's behavioral threshold to a plume of CO_2, thereby in effect increasing the downwind projection of the active space of CO_2 and so luring more mosquitoes to the trap.

H_2. Octenol, nonanal, and other host-related odors increase attraction by inducing mosquitoes that have been lured to the immediate vicinity of a trap to increase their time of local search near the source of CO_2, thereby increasing the likelihood of a mosquito entering the area where suction pulls the mosquito into the trap.

These behavioral hypotheses are not mutually exclusive, but it is probable that only one of these explains the increase in catch caused by these two compounds.

In the field environmental conditions such as wind flow (speed and turbulence) vary enormously and it is very difficult to record the flight tracks of multiple mosquitoes close to a trap and to know which species are being observed (H_2). It is impossible to do this well downwind of the trap (H_1) and to know if a given mosquito is actually within the odor plume's envelope. Therefore, to understand how host-related odors influence orientation, we have used lab bioassays where we can control precisely a mosquito's physiological state, environmental conditions, and pattern of stimulus presentation and can track resultant orientation responses. In our previous work we have used choice chambers with wind flow (Cooperband et al. 2008), port entry from a large, still-air flight chamber (Dekker et al. 2001, 2002), upwind flight in a wind tunnel (Dekker et al. 2005; Dekker and Cardé 2011, Turner et al. 2011), landing on odor sources in a wind tunnel (Lacey and Cardé 2011) and landing on odor sources in static air (Lacey and Cardé 2012), and orientation to and capture in CO_2 traps in field wind tunnels (Cooperband and Cardé 2006a, b).

Instead to test H_1 and H_2 we propose to use a wind-tunnel paradigm. This approach will allow evaluation of many more treatments than tests in a field wind tunnel, which are very time consuming, logistically difficult, and labor intensive.

Materials and Methods

We will use a 1.5-m-long wind tunnel (Dekker et al. 2005; Dekker and Cardé 2011). Mated females of *Ae. aegypti* or *Cx. quinquefasciatus* will be illuminated by infrared LED lights and their flight recorded in stereo view by two video cameras. Conversion of the video records to x,y,z coordinates is now (thankfully) an automated process using the Noldus computer program Track3D (Lacey and Cardé 2011). Odor delivery will follow several protocols. For CO_2 we will use a glass ring delivery system that creates a 6-cm-wide turbulent plume (Dekker et al. 2005) and for other odors see for example Williams et al. (2006) and Cook et al. (2011).

To test whether octenol (for *Ae. aegypti*) and nonanal (for *Cx. quinquefasciatus*) modulate attraction well downwind of the CO_2 source, we will use plumes of CO_2 varying in strength from 4% (typical of vertebrate exhalations) down to 0.1%, just above the ambient atmospheric concentration of CO_2 (ca. 0.035%). We expect to use 4 concentrations spanning this range. The lowest above ambient concentration (0.1%) is intended to simulate the effect of a mosquito entering a plume well downwind of a CO_2-emitting trap. Each CO_2 concentration will be tested alone, octenol or nonanal will be tested alone, and CO_2 with be paired with either octenol or nonanal. Because the concentration of these two adjuvants also would decrease with distance from the trap, it will be necessary to use lower doses of these two compounds paired with the lower concentrations of CO_2. This of course increases the number of treatments to be tested. We will use pilot tests to select which concentrations to evaluate when stimuli are paired.

The behaviors we will tabulate include percent leaving the release cage (activation), time to departure (latency), percent locking onto the plume and preceding upwind, percent passing through the ring device used to emit CO_2 (a proxy for likelihood of being captured in a suction trap), time to reaching the ring emitter, and time spent near the CO_2 emitter. Mosquitoes orienting "properly" generally pass through the ring. Because we will be recording flight tracks in 3-D, we will measure flight velocity along the track, angular (turning) rate, and a number of other parameters of flight performance that may be useful to interpreting behavioral effects.

If H_1 is correct, we expect the proportion of mosquitoes activated and proceeding upwind to be increased by octenol and/or nonanal at the lowest concentrations of CO_2. If H_2 is correct, we expect the time spent near the CO_2 source to increase, and, possibly, for mosquitoes to fly more torturous tracks (repeatedly transecting the area near the CO_2 emitter with an increased angular velocity).

We also will examine H_1w and H_2 with *Ae. aegypti* using the same protocols but with combinations of human skin odors that are somewhat attractive alone (i.e., without CO_2) or but synergistically so with CO_2. There are a number of possible compounds to use, but we will follow up on field trapping reported by Williams et al. (2006). Lures comprised of either acetone + lactic acid + dimethyl disulfide or lactic acid + caproic acid + ammonia induced trap capture in the field without CO_2. We will use these mixtures dispensed as in Williams et al. (2006). In *Ae. aegypti*, initial exposure to a whiff of just CO_2 instantaneously lowers the odor threshold for subsequent orientation to human skin odor (Dekker at al. 2005, Dekker and Cardé 2011), but we have not examined the premises that skin odor modulates (H_1) the threshold of response to CO_2 or that it modulates (H_2) time of localized search near a source of CO_2.

Results

We have to date conducted four wind tunnel experiments with *Ae. aegypti*. The overall objective of these tests has been to tease apart the interactions of CO_2 and human skin odor in the modulation of upwind flight, close-range orientation, and landing. (Human skin odor is presented as foot rubbings on glass beads.) As mentioned previously, our previous work demonstrated that CO_2 sensitized (i.e, lowered the olfactory threshold) of the *Aedes aegypti* mosquito to human skin odor and that homogenous plumes of skin odor and turbulent plumes of CO_2 evoked higher levels of successful orientation than the corresponding stimuli of turbulent plumes of skin odor and homogeneous plumes of CO_2 (Dekker et al. 2005, Dekker and Cardé 2011).

We now have found with *Ae. aegypti* that when these two kinds of odors are presented alone or in choice assays, either sequentially along the tunnel or in side-by-side competition at the tunnel's upwind end, females will orient to foot odor over CO_2, both in terms of the proportion locating the source and their propensity to land on it. Moreover, even though females orient upwind along a plume of CO_2 presented alone, when a foot odor plume is added, they always choose the foot odor source. These and the earlier experiments (Dekker et al. 2005, Dekker and Cardé 2011) suggest that CO_2 is the long-range olfactory cue and that it sensitizes the mosquito to human skin odors. The latter cues then seem to come into play when the mosquito arrives in the vicinity of a human host where they induce final orientation to the host and landing.

Our first objective has been to devise new wind tunnel assays that define the orientation cues that operate at close range. We have used plumes from a source of 4% CO_2 and from a dish of glass beads or cartridge coated with human foot odor. Both of these sources evoke plume following in *Ae. aegypti*, but, given a choice, these female mosquitoes clearly "prefer" the skin odor over CO_2 (see Figs. 1 & 2).

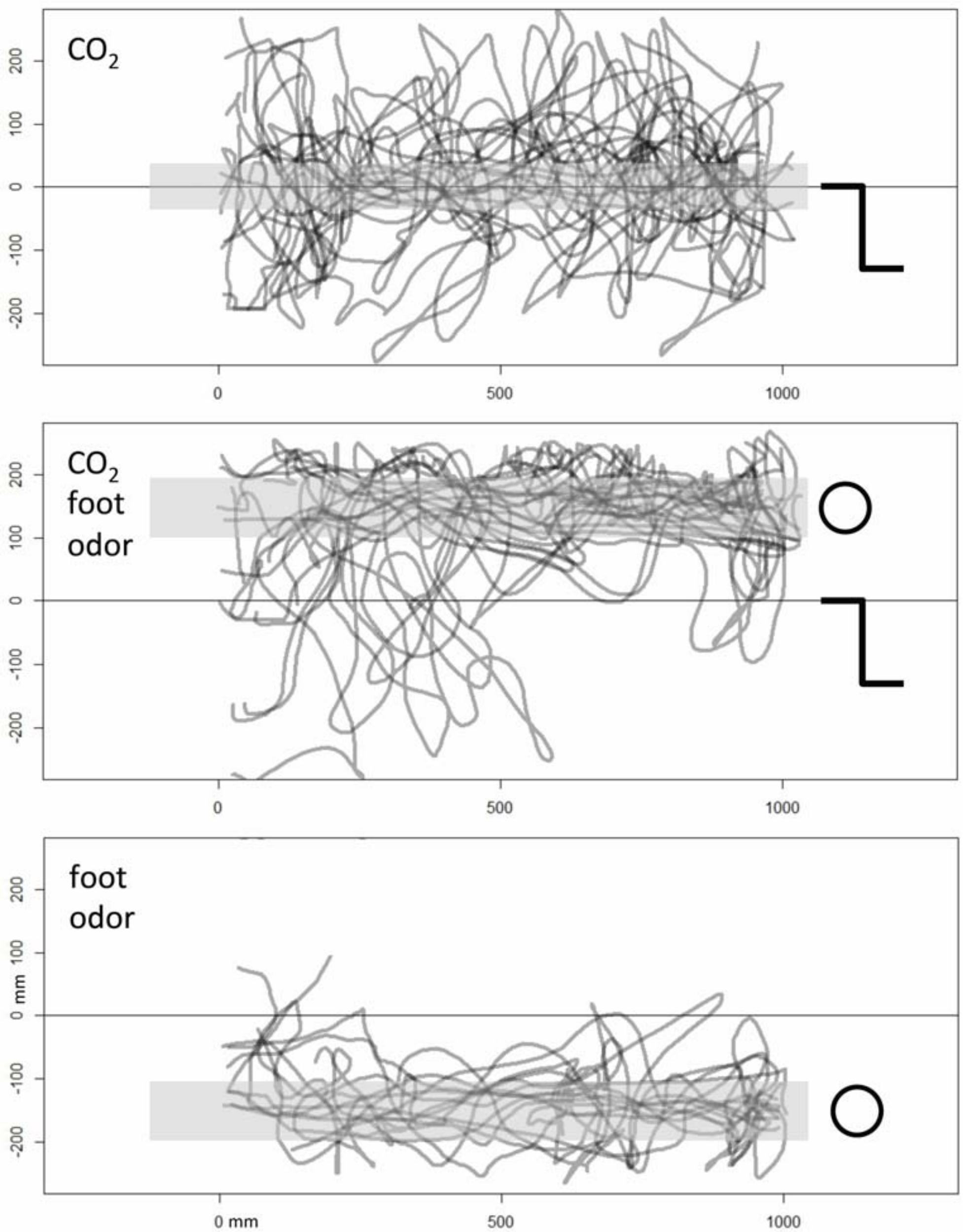

Figure 1. Overlaid 2-D tracks of multiple orientation flights by female *Aedes aegypti* in response to odor plumes in the wind tunnel. Tracks illustrate flight along odor plumes for different odor treatments. Odors were: 4% carbon dioxide (CO_2) (top; n = 20 tracks), CO_2 and foot odor on beads (middle; n = 21 tracks), and foot odor alone (bottom; n = 13 tracks). Circles represent Petri dishes containing beads with foot odor. Bent line represents source of CO_2. The grey shaded areas represent the time-averaged position of the odor plumes. The x-axis is the long axis of the wind tunnel with the scale (in mm) representing the space between the release cage and the odor treatments (the "arena" in which Ethovision could track flights). Wind flow of 20 cm sec^{-1} is from right to left. Along the y-axis are the widths of the wind tunnel (in mm). The

CO_2 plume was along the midline (0 mm) of the wind tunnel and foot odor was 150 mm to the sides. Location of the foot odor was switched between trials. For purpose of illustration, locations of the mosquito on the y-axis in some flights to foot odor were multiplied by -1 so that the foot odor source and the associated flight tracks are always depicted in the as being on one side of the tunnel. Landing and close range orientation to the odor sources are not shown and are analyzed separately.

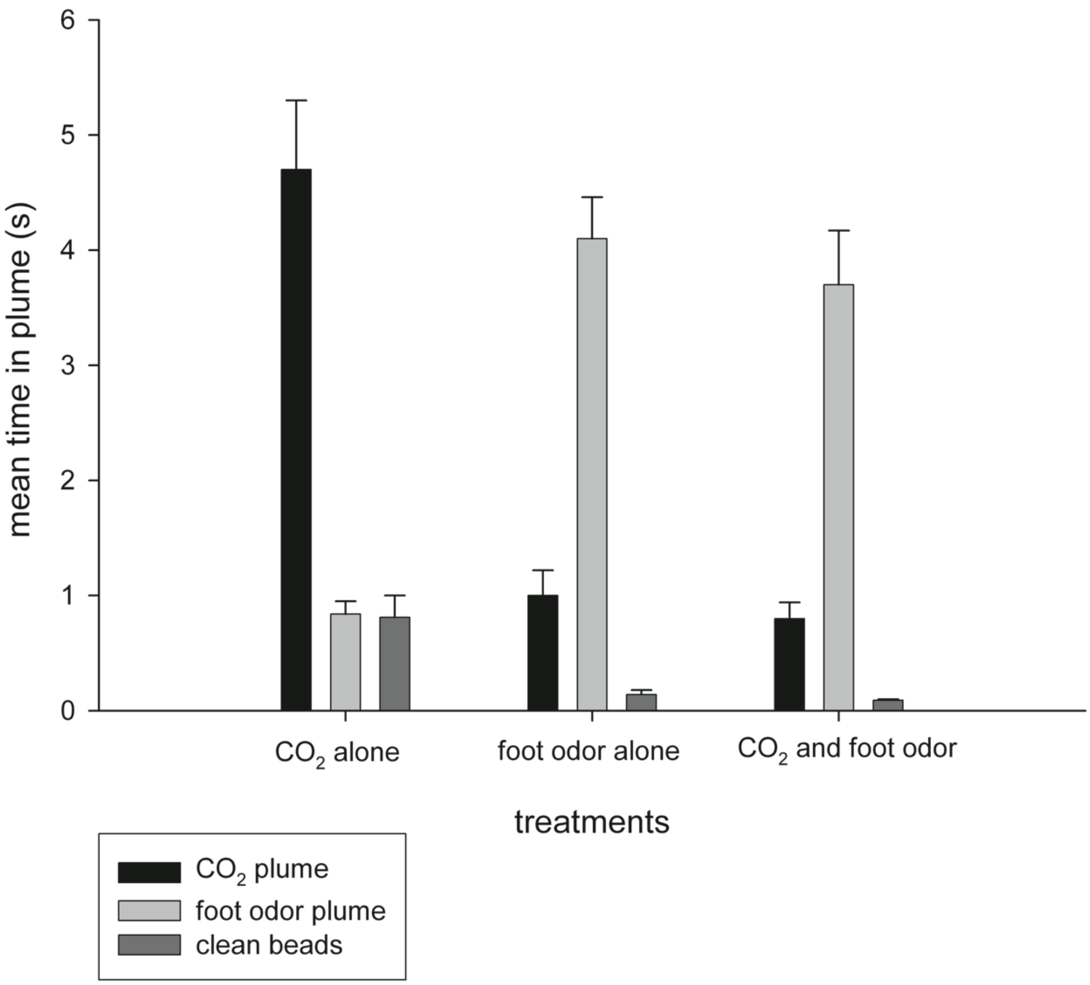

Figure 2. Mean time in seconds spent in the odor plume or the position these plumes would occupy if the odor was present. Treatments are those described in Figure 1.

One of the other four experiments has shown that when 4% CO_2 is emitted from a small point source, females will orient near the source but only rarely land on it. Adding foot odor to the point source greatly increases landing on the odor source. Foot odor alone, however, rarely evokes orientation and landing, although we are yet not sure why. One explanation is that the concentration of foot odor is lowered with this delivery system; another is that the simultaneous presence of CO_2 with the foot odor evokes close-range orientation and landing because of sensitization by CO_2 (both explanations are consistent with Dekker et al. 2005). Our success with this assay means that this method now can be applied to the testing of adjuvants which are expected to increase close-range orientation.

This new testing protocol of a point source with CO_2 alone, one with just human-associated odors (octenol or acetone + lactic acid + dimethyl disulfide or lactic acid + caproic acid + ammonia) that are putative adjuvants for CO_2, and a third treatment combining the CO_2 source with the putative adjuvants should help us understand how adjuvants "work." We will pursue this approach initially with *Ae. aedes*.

Significance beyond the immediate objectives: The proposed study is intended provide a behavioral explanation (in terms of navigational maneuvers) of how adjuvants increase trap capture. This assay method may enable laboratory screening for new adjuvants (e.g., based on neurophysiological and molecular biology leads—see Turner et al. 2011) and it could provide a new diagnostic method for establishing which of the many host-released odors actually influence natural orientation to a host in the field and therefore increase trap catch. Localized search near a source of CO_2 may not be an artifact but an expected behavioral prelude to landing on a host (see Lacey and Cardé 2011, 2012). The new and completed study mentioned above is being prepared for publication and we will credit the Mosquito Research Program for its generous support.

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