Four simple stimuli that induce host-seeking and blood-feeding behaviors in two mosquito species, with a clue to DEET's mode of action

Jerome A. Klun[1], Matthew Kramer[2]✉, and Mustapha Debboun[3]

[1]*Retired, USDA, ARS, Potomac, MD 20854, U.S.A.*
[2]*USDA, ARS, Beltsville Agricultural Research Center, Beltsville, MD 20705, U.S.A., matt.kramer@ars.usda.gov*
[3]*U.S. Army Medical Department Center and School, Academy of Health Sciences, Department of Preventive Health Services, Fort Sam Houston, TX 78234, U.S.A.*

Received 1 October 2012; Accepted 23 January 2013

ABSTRACT: Bioassays in a wind tunnel showed that a combination of four stimuli releases intense host-seeking and blood-feeding behavioral responses from females of the Asian tiger mosquito, *Aedes albopictus*, and the yellow fever mosquito, *Aedes aegypti*. The stimuli are carbon dioxide, water vapor, warmth, and adenosine triphosphate (ATP). Mosquitoes responded to this combination with a repertoire of blood-feeding behaviors that included upwind flight, landing, probing, and engorgement. Absence of carbon dioxide, water vapor, or ATP from the combination of stimuli or exposure to temperatures 12° C below or above human-host temperature (38° C) significantly attenuated blood-feeding behavior in both species. Although there is literature documenting the individual importance of each of these stimuli, our work represents the first instance where this combination of stimuli was found sufficient to elicit a complete repertoire of blood-feeding behaviors in these mosquitoes without involvement of any host specific odor. When mosquitoes were exposed to the four stimuli along with N,N-diethyl-3-methylbenzamide (DEET), feeding behavior was greatly suppressed. We hypothesize that a possible mode of action for DEET against these mosquitoes involves interference of warmth and/or water vapor receptors. An electrophysiological study designed to determine if DEET adversely affects the function of these receptors would be illuminating. ***Journal of Vector Ecology* 38 (1): 143-153. 2013.**

Keyword Index: Mosquito repellent, mosquito wind tunnel, ATP, water vapor, carbon dioxide.

INTRODUCTION

Entomologists have long been fascinated by the efficiency with which mosquitoes successfully locate their hosts and obtain blood meals. Unraveling how they accomplish this feat has been a challenge, both to understand the cues themselves and how they must vary from one mosquito species to another, which depends on mosquito host specificity and evolutionary history. The feeding habits of mosquito species range from being generalists that feed upon many hosts to specialist feeders that are highly adapted to a narrow range of hosts.

Extensive literature reviews of mosquito-human host interactions (Brown 1956, Takken and Knols 1999) document the intricate interactions among cues known to involve visual, chemical, and physical stimuli (Bernier et al. 2000, Qiu et al. 2004). Our study focuses on four stimuli (carbon dioxide, water vapor, warmth, and adenosine triphosphate [ATP]) and shows this combination is key to elicitation of vigorous blood feeding behavior by *Aedes aegypti* (Linnaeus) and *Aedes albopictus* Skuse.

Decades ago, host emanations of carbon dioxide, water vapor, or warmth were shown to be involved in elicitation of blood feeding by the anthropophilic mosquito *Ae. aegypti* (Rudolfs 1922, Brown 1956, Wright 1962, Khan and Maibach 1966, Wright 1968, Kellogg 1970, Gillies 1980). More recent studies by Hoel et al. (2009) and Kline (2002) showed that mosquito traps designed to simultaneously produce a combination of carbon dioxide, water vapor, and warmth by burning propane proved very effective in attracting and capturing *Ae. aegypti* and *Ae. albopictus*. The role of ATP in blood engorgement by mosquitoes and other blood-feeding arthropods was discovered by Galun et al. (1963), who demonstrated the importance of taste receptors located on the dorsal wall of the buccal cavity for detecting ATP and stimulating blood engorgement by *Ae. aegypti*. The phenomenon was also studied in other species of flies and Reduviid bugs (Friend 1978, Galun and Kabayo 1988). The earlier works cited above provided direction and background for our study.

We had two objectives. The first was to define a minimal set of stimuli that elicits blood-feeding behavioral responses, including upwind flight, landing, probing, and engorgement in *Ae. aegypti* and *Ae. albopictus*. Defining this set was important inasmuch as we thought that it could stimulate development of new chemicals that target and specifically interfere with mosquito perception of one or more of the stimuli within the set, lead to the design of simpler bioassay systems to study mosquito blood feeding, and inspire discovery of minimal sets of stimuli for other medically important arthropods. Our second objective was to identify, using behavioral measures, which stimuli in this minimal set might be susceptible to sensory interference by N,N-diethyl-3-methylbenzamide (DEET). Despite extensive research efforts over the past five decades to elucidate DEET's mode of action against a wide range of arthropods, no broadly accepted explanation for the compound's biological activity exists (Pellegrino et al. 2011). Our research allows us to offer a hypothesis for how DEET may work against blood-feeding by *Ae. aegyyti* and *Ae. albopictus.*

MATERIALS AND METHODS

Chemicals

Citrate, Phosphate, Dextrose and Adenine (CPDA-1) (AABB 2005) aqueous solution, used as a mosquito ingestion solution, was prepared by dissolving 3.33 g sodium citrate, 0.376 g citric acid, 0.28 g monobasic sodium phosphate, 4.02 g dextrose, and 0.035 g adenine in 126 ml water. This corrects the mangled recipe for CPDA-1 in Klun et al. (2008) (printed as: 33.3 g sodium citrate, 0.376 g monobasic sodium phosphate, 4.02 g dextrose, and 0.35 g adenine in 63 ml water).

Klun et al. (2008) showed that mosquitoes ingest aqueous CPDA-1 containing ATP as avidly as they ingested human red blood cells in CPDA-1. With the exception of one experiment where the concentration of ATP was varied, we always added ATP to CPDA-1 to yield 10^{-3} M ATP. This concentration of ATP was selected for use because Galun et al. (1985) showed that *Ae. aegypti* engorging behavior was concentration dependent over a range of 10^{-3} M to 10^{-6} M ATP, and because Gribble et al. (2000) determined that the ATP physiological sub-membrane concentration in living cells is approximately 10^{-3} M. Solutions were freshly prepared on the day of their use in the bioassays. ATP and CPDA-1 components have low vapor pressures, and as such are not odorants. The CPDA-1 chemical components, ATP and DEET, were purchased from Aldrich Chemical Co., Milwaukee, WI.

Mosquitoes

Aedes aegypti eggs (Liverpool-strain, derived from the 1b12 selection) were obtained from the Walter Reed Army Institute of Research, Silver Spring, MD, and *Ae. albopictus* eggs were obtained from the U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL. The mosquitoes were reared in a walk-in incubator using 12:12 h (light:dark) photoperiod, 30 +/-1° C and 45 +/-5% RH. Larvae were fed ground tropical fish pellets (Hikari Cichlid Gold Fish Food, Kamihata Fish Ind. Ltd., Himeji, Japan). Adults were held in screened, 3.79 liter plastic buckets, and fed sugar using cotton pads moistened with 10% aqueous sucrose solution. Female mosquitoes were five to ten days old and incubated in plastic buckets along with a water-moistened pad without sugar for 24 h before they were used in bioassays.

Wind tunnel

Wind tunnels of various designs have been used in studies of mosquito behavior (Wright 1968, Eiras and Jepson 1991, Sharpington et al. 2000, Cooperband and Cardé 2006). The tunnel we used differed from others used previously in important ways. Thus, the following detailed description of its components and operation is presented.

A Plexiglas® wind tunnel (Raina et al. 1986), 3.0 m long and 61 cm wide with a 60 cm tall horseshoe-shaped cross section, was used in our studies (Figure 1A). The tunnel was housed in a white room, and its floor was covered with white plastic-backed absorbent paper to allow easy visualization of mosquitoes in the tunnel. The supply of air for the tunnel was drawn from above the laboratory building (not building air, which might contain human host cues) into a 12 m^3 air-conditioning chamber, where it was conditioned before entering the tunnel. Air temperature in this chamber was controlled by a window air conditioner and baseboard electric heaters to produce 25 ± 2° C in the tunnel. Relative humidity (RH) in the chamber was maintained at 40-55% RH using a misting humidifier and a fan-forced falling-water humidifier. The upwind end of the tunnel was connected to the air-conditioning chamber by a cowling that housed a 61cm diameter fan driven by a rheostat-controlled Dayton Electric Model 2Z846A motor that pushed conditioned air through a Dayton cartridge 5W921 air filter into the tunnel. The filter was designed to remove 95% of bacteria, smoke, dust, pollen, and chemical contaminants from the air passing through it. Air flow through the tunnel (25 cm /sec) was monitored with an airflow gauge, and flow through the tunnel was laminar according to observations using smoke plumes generated in the tunnel using $TiCl_4$. Temperature and RH within the tunnel were monitored with a wireless thermo-hygrometer (Radio Shack, Model 63-1030) and recorded with a HOBO data logger (Model H08-003-02). The monitoring assured that air velocity, temperature, and RH conditions were consistently maintained over the course of all bioassays conducted in the wind tunnel. Air flowing out from the end of the tunnel entered a fume hood and was positively fan exhausted out of the building through a roof vent. Incandescent white lights over the tunnel provided daylight illumination. Access to the tunnel interior was through three piano-hinged doors along the tunnel's side. Ends of the tunnel were enclosed with aluminum mesh screen. A hole cut in the center and 16 cm above the tunnel floor of the screen at its downwind end was fitted with a 7 cm diameter plastic collar. A 6.9 cm OD x 18.5 cm cylindrical Plexiglas® canister fitted with an upwind butterfly valve opening and screened downwind end inserted into the plastic collar was used to release mosquitoes into the wind tunnel (Figure 1B).

The tunnel was fitted with a mosquito feeding reservoir (Figure 1C) that was positioned centered and at the upwind end of the tunnel perpendicular to tunnel air flow. The reservoir was made of Plexiglas® and was fabricated commercially by Precision Plastics Inc., Beltsville, MD. The 29.7 cm x 7.1 cm x 3.8 cm (L x W x H) reservoir, with six 3 cm x 4.1 cm x 0.7 cm deep wells (Klun et al. 2005), was originally developed and used as a component of a bioassay system designed and successfully used in the discovery of new chemicals having mosquito feeding deterrent activity (Cantrell et al. 2005, Cantrell et al. 2011, Klun et al. 2008, Zhang et al. 2009). The reservoir was placed on a stand made of 0.5 cm thick Plexiglas® with a 33.8 cm x 15.4 cm base and a 29.8 cm x 7.0 cm (L x W) upper shelf with 1.2 cm high edges at each end of the shelf to secure the reservoir on the shelf. Positioned on the shelf, the top surface of the reservoir was 18 cm above the tunnel floor, at the center of the tunnel, and 47 cm away from the upwind tunnel screen. With the exception of the experiments designed to test thermal stimuli on mosquito feeding behavior, the reservoir was always held at a constant temperature (38° C) by water pumped through the reservoir from a water-bath circulator (Lauda E100, Wobser GMBH and Co., Konigshofell, Germany) that was positioned outside and under the tunnel cowling.

Two wells at each end of the reservoir were filled with 6 ml CPDA-1 aqueous solution containing 10^{-3} M ATP, and two wells at its center were usually left empty. Solutions in opposing end

wells of the reservoir were colored with a drop of different food-dye color (Esco Foods Inc., San Francisco, CA), to track where and how many mosquitoes engorged in a test.

The upper surface outer edges of the reservoir was coated with a 0.5 cm wide light coating of high-vacuum silicone grease (Dow Corning Corp., Midland, MI), and then covered with a 7 x 30 cm sheet of Edicol collagen membrane (Devro, Sandy Run, Columbia, SC). The Edicol manufactured-collagen membrane was water permeable, and the type of collagen used commercially in sausage casings and other food products. A very thin coat of silicone grease about 0.5 cm wide was then applied to the collagen membrane edges. A 7 x 30 cm strip of white organdy cloth (G St. Fabrics, Rockville, MD) was placed over the membrane. The organdy cloth and Edicol membrane each adhered to the thin strip of grease between them and were secured to the reservoir. Organdy cloth was used to cover the top of the reservoir because it provided a surface that mosquitoes could readily land upon, penetrate the collagen membrane below, and engorge CPDA-1 containing ATP. The cloth also provided a surface that could be uniformly treated with a solution of any test chemical. Depending upon the experiment, the areas of organdy cloth covering end wells of the reservoir were either left untreated or, when DEET was tested, the cloth covering two adjacent wells at one end of the reservoir was treated with a DEET ethanol stock solution; the control-wells cloth at the other end of the reservoir was treated with 95% ethanol.

A breath-delivery tube (2.5 m x 22 mm ID, Tri-Anim, Sylmar, CA) ran from outside of the upwind end of the tunnel to a breath exhaust position inside the tunnel 20 cm above the tunnel floor, at the tunnel center, and 20 cm from the upwind end of the tunnel. Carbon dioxide was delivered into the tunnel by 10-15 exhalations/min from a human volunteer or by using a breath simulation module (Michigan Instruments, Grand Rapids, MI) that pulsed compressed air containing 5% carbon dioxide (Airgas East, Certified Specialty Gases, Bladensburg, MD) into the tunnel at a rate of 13 exhalations/min. In this way, a train of breath pulses traveled in the tunnel air flow over the reservoir (Figure 1C) and down the center of the tunnel. Carbon dioxide levels (ml CO_2/min, maximum CO_2 at end of breath, and volume of gas/breath) in each human or simulated exhalation, were measured and recorded by using a $NICO_2$, Model 7600, Respironics Detector (Respironics California, Inc., Carlsbad, CA). Use of the infrared-based $NICO_2$ detector assured that the carbon dioxide stimulus, whether from a human source or the breath simulator, was standardized from one bioassay test to another.

The reservoir, outfitted and positioned in the wind tunnel in the plume of carbon dioxide as described, mimicked a host by providing the mosquitoes with a source of water vapor (from the collagen covered wells holding aqueous solution), warmth (warm water pumped through the reservoir from a water bath under the tunnel), a membrane to penetrate, and aqueous CPDA-1 containing ATP in the wells beneath the membrane to engorge (Figure 1C).

Wind tunnel protocol

A test began by inserting a mosquito release canister (Figure 1B) containing 20 female mosquitoes into the collar on the tunnel's downwind screen. After 1 min of conditioning in the tunnel air flow, the butterfly valve on the canister was opened to give mosquitoes access to the tunnel. In the following 10-min

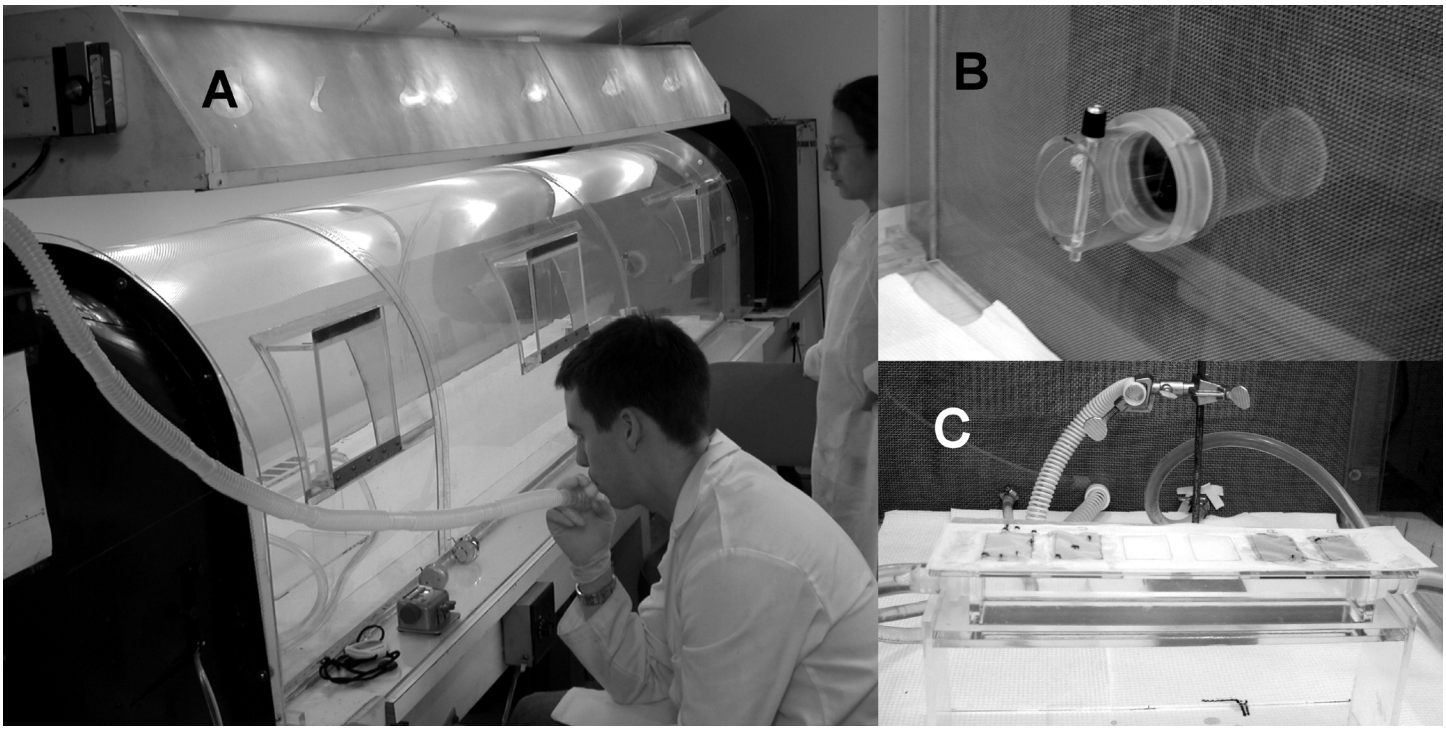

Figure 1. (A) is a photograph of the length of the wind tunnel showing the tunnel's features and a technician delivering breath into the tunnel. (B) shows the release canister at the downwind end of tunnel. (C) shows the complete mosquito feeding reservoir positioned in the tunnel during Experiment 5. Cloth covering the two right wells was treated with 25 n mol DEET/cm^2 cloth, and was avoided by mosquitoes. Cloth covering the two left wells was untreated. The two middle unfilled wells were covered with untreated cloth.

test period, human breath or simulated breath was continuously pulsed into the tunnel. Manual event counters (Forest Suppliers, Jackson, MS), were used to record the number of proboscis insertions (probes) mosquitoes made into the organdy cloth on control and treatment wells.

At the conclusion of a test, the release-canister valve was closed and the canister removed from the collar hole of the downwind screen. The hole was plugged with a paper towel, and the number of mosquitoes that remained in the canister throughout the test was recorded. Mosquitoes within the tunnel were collected using a portable vacuum cleaner with a cloth-covered hose inlet. The number of mosquitoes collected from the reservoir and positions upwind and downwind of it were recorded. The mosquitoes were transferred to a Ziploc® plastic bag containing a sheet of absorbent tissue paper and each mosquito was crushed on the tissue to determine the number of mosquitoes that engorged in the test and upon which dye-colored treatment they fed upon.

Between tests, the used organdy cloth and collagen membranes were removed from the reservoir and discarded. At this time, positions of the colored CPDA-1/ATP solutions in the reservoir wells were refreshed. Although preliminary testing (see below) showed neither a color nor a side-of-tunnel preference by the mosquitoes, these factors were routinely alternated from one test to another.

Nitrile gloves were always worn when handling wind tunnel equipment to avoid contamination with human skin-derived compounds. Before starting a day of bioassay tests, all equipment in the wind tunnel was routinely wiped with ethanol-moistened paper towels to ensure cleanliness of the bioassay equipment.

Preliminary testing results

We provide some data from our initial testing of the wind tunnel, in part to demonstrate normal host-seeking and feeding behaviors of mosquitoes in the apparatus. In initial testing, feeding wells contained expired human blood in half the feeding stations and 10^{-3} M ATP in CPDA-1 in the other half. Human breath was introduced for one minute near the feeding station. Data on the distribution of the mosquitoes in the tunnel, the number of probes, and the number of engorging mosquitoes were taken. Twenty trial runs were made over several days and at various times of day. Summing over the 20 trial runs, the mosquitoes distributed themselves at the end of each 10-min test as follows: 87% left the canister; of those, 17% were closer to the canister end of the tunnel, the remainder nearer to the end containing the feeding reservoir; and 56% of those that left the canister were on top of the feeding station. Of those that left the canister, 46% tested engorged. We recorded 418 probes into the feeding wells (total number of mosquitoes = 20 × 20 = 400; total number of mosquitoes that left the canister = 349).

We conducted some simple statistical testing to determine if there were differences in feeding or probing on the two food sources, also if there was a day or time-of-day effect, using the lme4 package (Bates et al. 2011) in R (R Development Core Team 2012). Details are given below in the Experiment 1 section. We found that mosquitoes preferentially engorged from human blood (105 vs 54, $p < 0.01$) but did not preferentially probe in wells with human blood ($p = 0.89$). The variance estimates for both the day effect and the time-of-day effect were essentially zero.

To achieve our first objective, four experiments were conducted to evaluate the role of carbon dioxide, water vapor, ATP, and thermal stimuli, and to demonstrate the importance of each stimulus in the elicitation of feeding behavior by *Ae. aegypti* and *Ae. albopictus*. A fifth experiment, in pursuit of our second objective, was conducted to evaluate the behavioral impact of DEET when it was presented to the mosquitoes along with a combination of the four stimuli. The five experiments are sequentially described below.

Experiment 1 (carbon dioxide)

Mosquitoes were tested in the wind tunnel against three airflow treatments: (1) human breath (plus air), (2) CO_2 scrubbed human breath (plus air), and (3) air only, with ten trials per treatment using 20 mosquitoes/trial, totaling 600 mosquitoes. In this experiment, human breath acts as a positive control. If the apparatus is working correctly, mosquitoes are expected to exhibit directional flight in the presence of this normal host cue. CO_2-scrubbed human breath is the critical test condition, since it is the same as human breath with only one element, CO_2, lacking. Air only is a control for the apparatus. Mosquitoes should not show directional flight to air only since there are no host cues. If results from CO_2-scrubbed human breath and air only are similar, this demonstrates the importance of the CO_2 component in human breath as a host cue.

In Treatment 1, exhaled breath entered the breathing tube through a breathing-valve mouthpiece that allowed inhalation from room air and exhalation into the tunnel. In treatment 2, the carbon dioxide in exhaled breath was removed by passing it through a 19 cm wide x 21.6 cm tall cylindrical canister filled with medical grade soda lime (Sofnolime 2550, Molecular Products, Inc., Boulder, CO). Breath entered the canister through a breathing tube that was attached to a union near the mid-height of the cylinder. A tube inside the canister carried the breath to the bottom of the soda lime bed, and the scrubbed breath passed through a tube attached to the top of the canister into the tunnel. In Treatment 3, only conditioned air flowed through the tunnel.

For each of the ten trials, the three treatments were tested in random order. The two wells at each end of the reservoir were filled with the same dye-colored solution, the two middle wells were left empty, and all wells were covered with collagen membrane and organdy cloth, as described earlier. Thus, in Experiment 1, mosquitoes were provided with three stimuli they should detect in the tunnel: water vapor, 38° C warmth, and one of the three types of airflow.

Throughout each 10-min test period, we counted the number of mosquitoes that entered the tunnel and the number of times mosquitoes probed the organdy cloth. At the end of the test, mosquitoes were collected from the tunnel, transferred to a plastic bag, and each crushed on absorbent tissue paper to determine its engorgement state. These data (proportion entering, number of probes, proportion engorged) were analyzed in the generalized linear model framework (McCullagh and Nelder 1989). Of those entering the tunnel, we considered the number engorging as a sample from a binomial distribution. The numbers of probes were treated as samples from an over-dispersed Poisson distribution (over-dispersed because individual mosquitoes might probe multiple times, so that probes are not independent). Statistical

models were fit using the lme4 package (Bates et al. 2011) in R (R Development Core Team 2012) including a random trial effect for both species; other random effects (e.g., day effect) had variance estimates close to zero when included in a statistical model. Over-dispersion in the probe data was accounted for by including a random effect for each Poisson sample (for every count). For *Ae. aegypti,* we also included the square root of the number of mosquitoes that entered the tunnel for the probe analysis; of the four models this was the only one where this variable was a significant predictor ($p < 0.02$). A posteriori multiple comparisons of pairs of the three treatments were made using the multcomp R package (Hothorn et al. 2008).

Experiment 2 (water vapor)

Mosquitoes were given a choice of landing upon and probing surfaces on reservoir wells emitting different levels of water vapor. Two wells at each reservoir end were filled with CPDA-1/ATP solution, colored with different food dyes, and the length of the reservoir was covered with the water-permeable collagen membrane. Wells at the center of the reservoir were empty. Wells at one end of the reservoir were capped with plastic food wrap (Reynolds Clear Plastic Wrap, Alcoa Consumer Products) to suppress water vapor emission from the wells, and then organdy cloth was placed over the entire upper surface of the reservoir covering all wells. Carbon dioxide from the breath of a human subject and 38° C warmth from heated water circulated through the entire reservoir. Each trial consisted of 20 mosquitoes; 800 mosquitoes total with both species.

For each of 20 trials, the side-to-side location of control or plastic-capped wells in the tunnel was alternated from one trial to another. Data were collected and recorded in the same way as in Experiment 1. This assay was a choice test, and the dye color of the crushed mosquitoes revealed where they fed.

We recorded the number of mosquitoes on each reservoir well at the ten-min mark of each test. A chi-square test was used to determine if the distributions of mosquitoes across the three well types deviated from an even distribution (the null hypothesis). We analyzed probing but not engorgement behavior because mosquitoes were not able to penetrate the plastic wrap to engorge, even though they actively probed capped wells. Probes were treated as over-dispersed samples from a Poisson distribution, as in Experiment 1. The square root of the number of mosquitoes that left the canister was not a useful predictor for *Ae. aegypti* and it was dropped from the model, but it was retained for *Ae. albopictus*. Statistical analyses and means separations were performed using the R software, described in Experiment 1. Wells with zero counts (thus a variance equal to zero; i.e., most empty wells) were not included in the analyses.

Experiment 3 (ATP)

To evaluate the influence of ATP upon engorgement, a range of CPDA-1 solutions containing 0, 10^{-4}, 10^{-3}, to 10^{-2} M ATP were prepared as follows: A stock solution of ATP x 10^{-2} M was prepared by dissolving 0.138 g ATP in 25 ml CPDA-1, serial dilutions were then made by adding 3 ml of that stock solution to 27 ml of CPDA-1 solution (ATP x 10^{-3} M), and then 3 ml of that solution to 27 ml of CPDA-1 solution (ATP x 10^{-4} M). CPDA-1 without ATP was the control.

For each of ten trials, the wells at each end of the six-celled reservoir were left empty, and the four adjacent middle wells were filled in random order with 0, 10^{-4}, 10^{-3}, or 10^{-2} M ATP in CPDA-1, and each was treated with a different color of food dye to determine in which each mosquito engorged. The four wells were covered with the collagen membrane and organdy cloth as described previously.

Carbon dioxide from the breath of a human subject, water vapor evaporating from the CPDA-1/ATP solutions, and warmth from the heated water circulating through the reservoir were all present; wells contained either water or one of the ATP concentrations. Data were collected and recorded as described earlier, using 200 mosquitoes total for each species, in ten trials.

The number of mosquitoes that engorged at the various ATP concentrations were considered a sample from a multinomial distribution with five categories (comprised of the counts at the four concentrations; the fifth containing the count of those that did not feed). Because the objective of this experiment was to determine whether there were engorging preferences, mosquitoes that did not engorge were eliminated from the analysis and the four remaining categories tested for equality (the probability that a process where engorgement was equally likely for each solution would generate the numbers observed) using a chi-square goodness-of-fit test. We found no day or time of day effects for either species. Analysis of the probe data was conducted as described earlier. We assumed an over-dispersed Poisson distribution for the number of probes on each solution, and allowed for a random trial effect.

Experiment 4 (thermal)

A Plexiglas® reservoir of the same dimensions as the previously described six-well reservoir was constructed for use in the thermal study. The new reservoir had two wells at each end, and the middle portion of the reservoir, where two center wells previously existed, was solid and insulated with a block of polystyrene. The four-well reservoir was positioned in the tunnel as described for the original six-well reservoir. Each set of two wells at each end of the reservoir had separate input and output water ports that were connected to water pumps using Tygon tubing. Tests of the role of warmth in mosquito-feeding behavior were conducted by offering mosquitoes a choice between two wells held at 38˚ C at one end of the reservoir and two wells at the other end of the reservoir held at a choice temperature of 26, 32, 44, or 50˚ C. A Lauda E100 constant temperature water heating circulator pump supplied 38° C water to one end of the reservoir, and a Julabo F25 variable water temperature circulator (Julabo USA, Inc., Kutztown, PA) delivered water to the other end of the reservoir at 26, 32, 38, 44, or 50° C.

The left to right position of the 38° C end of the reservoir and the opposing choice temperature was alternated from one trial to another. Alternating the left to right temperatures of the reservoir was accomplished by using a combination of T-valves and tubing connections to direct thermally controlled water flow from the respective circulators to the desired reservoir end. The dye colors of the CPDA-1/ATP solutions used for test temperatures were also alternated during testing of ten trials. Similar to the other experiments, wells were covered with collagen membrane and organdy cloth. Using a handheld remote infrared thermometer (Fluke, Model 561, Everett, WA), the surface temperature of the

organdy cloth overlying each feeding cell was confirmed to be at the programmed water temperature set points. Carbon dioxide coming from the breath of a human volunteer and water vapor evaporating from the CPDA-1/ATP solution, were all present and reservoir surface temperatures were varied.

Data were collected and recorded as previously described. Because this bioassay was set up as a paired-choice test between two surface temperatures, the color of the squashed mosquitoes identified the surface where each mosquito engorged.

In these series of experiments, mosquitoes had access to feeding wells at two temperatures. We assumed that engorging mosquitoes were samples from a binomial distribution, thus we tested whether the number of engorging mosquitoes at the wells of different temperatures was the same using a chi-square test, as in Experiment 2. The number of probes was analyzed as described in Experiment 1.

Experiment 5 (DEET)

In this experiment (ten trials), two wells at each end of the reservoir were filled with CPDA-1/ATP solution dyed with different food colorings and the reservoir was covered with collagen membrane. Four rectangular areas (3 x 4.1 cm) of a 7 x 30 cm length of organdy cloth that were destined to cover two wells at each end of the reservoir were marked with a graphite pencil. Two adjacent 3 x 4.1 cm cloth areas at one randomly selected end of the cloth were each treated with 100 µL of a 3.07 nmol DEET/µl 95% ethanol stock solution to yield 25 nmol DEET/cm^2 on the cloth. As a control, areas at the other end of the 30 cm length of cloth were treated with 95% ethanol. The cloth was then attached to the collagen membrane on the reservoir as previously described.

Sets of 20 mosquitoes were tested separately in the wind tunnel against the DEET-treated reservoir using three airflow conditions: (1) human breath (plus air), (2) CO_2 + air (simulated human breath using compressed breathing air containing 5% CO_2, as described earlier), and (3) air only. With air-only test conditions, only conditioned air flowed down the tunnel. Experiment 5 airflow conditions (1) and (3) were the same as airflow conditions (1) and (3) of Experiment 1. Thus, Experiment 5 trials were similar to Experiment 1, except that mosquitoes had a choice of feeding through untreated organdy cloth or through DEET-treated cloth. Since we had already determined that CO_2-scrubbed human breath was not more effective than ordinary air in eliciting upwind flight (Experiment 1), we instead used simulated human breath as the third condition. If results using human breath + air and CO_2 + air were similar, this would again demonstrate the importance of CO_2 as a host cue to mosquitoes. If there were differences between these two treatments, either on the DEET treated or control wells, human odorants could be implicated as an explanatory factor.

To fit these multinomial data (fed at DEET treated well, fed at control well, did not feed) in a generalized linear models framework (allowing for over-dispersion), we used the MCMCglmm R package (Hadfield 2010). Since this package uses Bayesian methodology, results reported reflect sampling from the parameters' posterior distributions (credible intervals are reported instead of confidence intervals). Probes were analyzed as described in Experiment 1, and models for both species included the number of mosquitoes entering the tunnel as a covariate.

RESULTS AND DISCUSSION

Experiment 1 (carbon dioxide)

This experiment demonstrated that carbon dioxide is an essential stimulus in the elicitation of upwind mosquito flight, probing, and engorging. The number of mosquitoes probing and engorging was dependent upon whether or not they flew up to the mosquito feeding reservoir, and these behaviors were significantly attenuated in both species when carbon dioxide was not a component in the air flowing down the tunnel (treatments 2 and 3). Parameter estimates (means and standard deviations) provide two kinds of information (Figure 2A). The mean number of probes is shown with points and one standard deviation error bars; gray lines connecting gray dots show the mean number of mosquitoes that left the release canister and flew upwind. The number of mosquitoes entering the tunnel explains much of the difference in the observed number of probes and engorgements. Results for the number of probes by both species were similar (both $p < 0.01$); there were far more probes in the presence of human breath. The number of probes did not differ significantly for the other two breath types. Mosquitoes flew up the wind tunnel in higher numbers in the presence of human breath than when only air or when CO_2-scrubbed human breath flowed through the tunnel. For both species, significantly more mosquitoes left the release chamber with human breath (both $p < 0.001$), the other two breath types did not differ significantly. Panel A also shows that *Ae. albopictus* was less stimulated to leave the release chamber than *Ae. aegypti* when carbon dioxide was not present in tunnel air. Panel B shows the mean proportion of mosquitoes that engorged and one standard deviation error bars for the three breath types for those mosquitoes that flew upwind. Engorging was significantly higher in the presence of human breath ($p < 0.001$ for both species), with no significant differences between the other treatments. These results demonstrate that both mosquito species require the presence of carbon dioxide for normal upwind flight and subsequent probing and feeding behaviors.

Experiment 2 (water vapor)

This experiment demonstrated that detection of water vapor is essential to the orientation of mosquitoes to a surface and stimulation of probing. Figure 3A shows the mean number of probes on each well type as points with one standard deviation error bars. For both species, there were significantly more probes on wells without caps (both $p < 0.001$). No mosquitoes probed the empty center wells, however, they did probe the cloth covering the capped wells filled with CPDA-1/ATP solution. This indicates that the plastic covering these wells did not totally prevent water evaporation from the wells; the leakage was probably responsible for the probing elicited at these wells.

Panel B shows the mean number of mosquitoes at the tenth min of the experiment located on each well type with one standard deviation error bar (only the upper is given to avoid the bars extending below zero). For both species, there were significantly more probes on wells without caps (both $p < 0.001$, $\chi^2 = 215.67$ for *Ae. aegypti;* $\chi^2 = 33.16$ for *Ae. albopictus*). Only one *Ae. aegypti* mosquito landed on one of the empty wells and did not probe it. While far fewer mosquitoes landed on the capped wells than on uncapped ones, those that landed on capped wells probed at

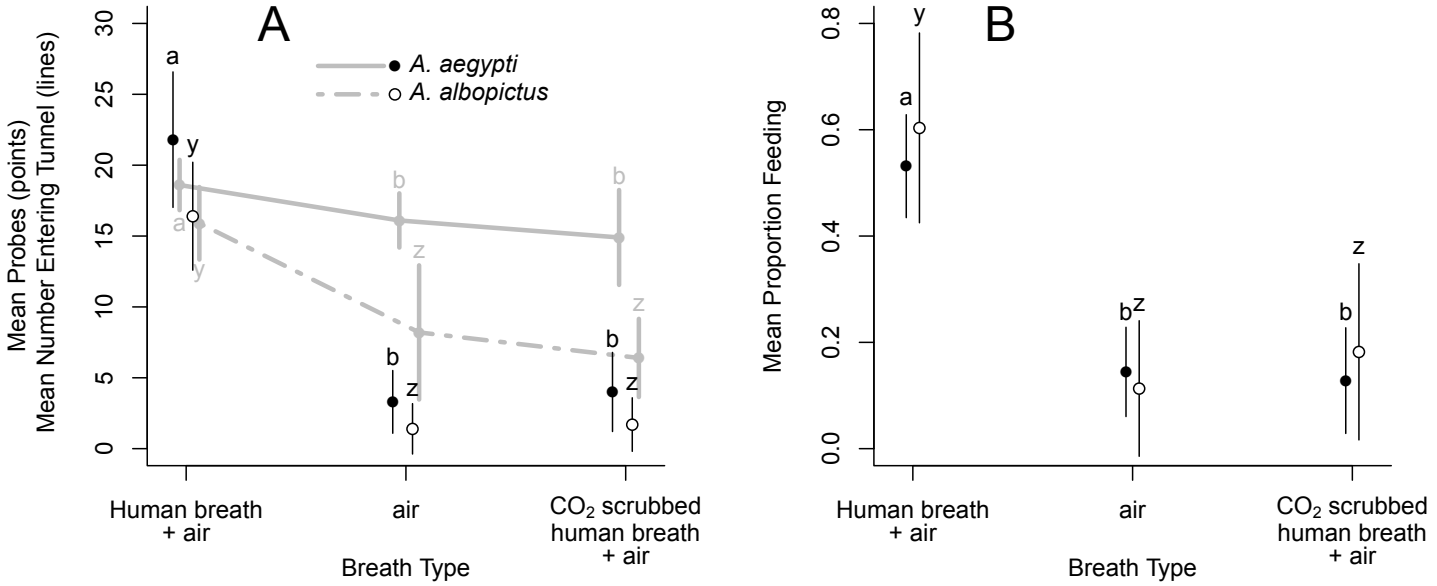

Figure 2. (A) shows the mean number of probes (black points, vertical lines show ± 1 SD) for *Ae. aegypti* and *Ae. albopictus* for three breath types (human breath + air, air only, and CO_2-scrubbed human breath + air) tested in a wind tunnel. The gray points connected by gray lines give the mean number of mosquitoes that entered the wind tunnel (vertical bars show 1 SD). (B) shows the mean proportion feeding (± 1 SD) for the three breath types. Means that do not differ significantly are adorned with the same letter ([a, b] for *Ae. aegypti*, [y, z] for *Ae. albopictus*).

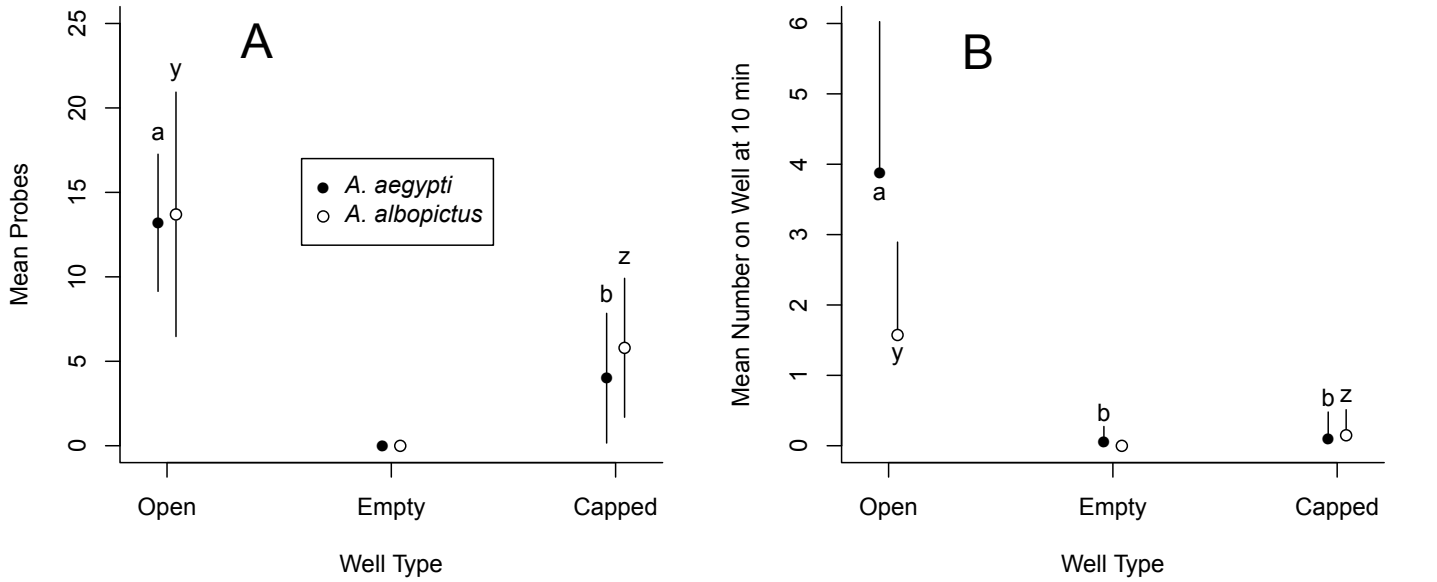

Figure 3. (A) shows the mean number of probes (± 1 SD) for the three well types (releasing differing amounts of water vapor) for the mosquitoes, *Ae. aegypti* and *Ae. albopictus*, tested in a wind tunnel. (B) gives the mean number resting on each well type at 10 min (vertical line gives + 1 SD). Means that do not differ significantly are adorned with the same letter ([a, b] for *Ae. aegypti*, [y, z] for *Ae. albopictus*). Wells with zero counts (and zero variance) are not included in the means separation lettering.

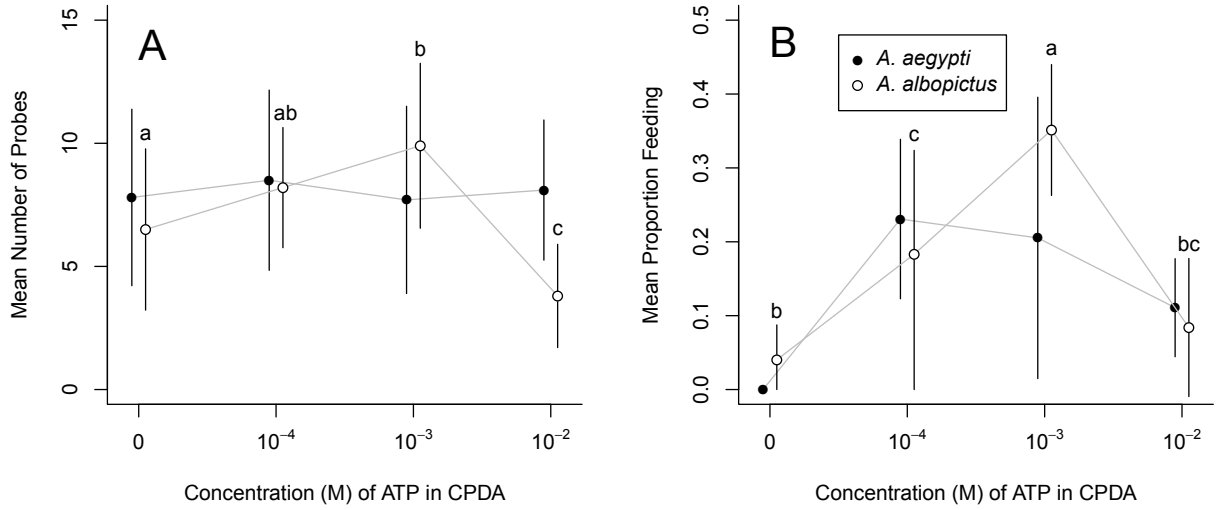

Figure 4. (A) shows the mean number of probes (± 1 SD) for four M concentrations of ATP in CPDA by *Ae. aegypti* and *Ae. albopictus* tested in the wind tunnel. (B) shows the mean proportion feeding (± 1 SD) for these concentrations. Means that do not differ significantly are adorned with the same letter ([a, b, c] for *Ae. albopictus*, no differences for *Ae. aegypti* [0 M concentration not included as no *Ae. aegypti* mosquitoes fed there]).

approximately the same rate as those that landed on open wells, demonstrating not only their sensitivity to the minute amounts of water vapor escaping from these capped wells (empty wells were ignored), but that it is the detection of water vapor, not its quantity, that triggers probing.

Experiment 3 (ATP)

The results from this experiment in Figures 4A and 4B show the means with one standard deviation error bars for probing and engorging responses at different ATP concentrations. For both species, there were strong engorging preferences ($\chi^2 < 0.01$, 3 d.f.), with no *Ae. aegypti* or few *Ae. albopictus* engorging from wells without ATP, and fewer engorging from the 10^{-2} M ATP solution than the other two. However, while *Ae. aegypti* probed equally in all solutions ($p > 0.95$, $\chi^2 = 0.355$), *Ae. albopictus* showed a preference to probe in the 10^{-4} and 10^{-3} ATP solutions ($p < 0.01$, $\chi^2 = 23.061$), with 65, 82, 99, and 38 total probes in 0, 10^{-4}, 10^{-3}, and 10^{-2} M ATP solutions, respectively. Probing thus appears to be largely governed by stimuli other than ATP (e.g., presence of water vapor), though higher concentrations of ATP stimulated more probing in *Ae. albopictus*. However, it is clear that engorging is dependent upon detection of ATP following probing. Engorgement is initiated when a mosquito detects the presence of ATP at concentrations that may span several orders of magnitude, which is in agreement with the findings of Galun et al. (1985).

Experiment 4 (thermal)

Results of mosquito thermal sensitivity from this experiment for both species were similar. Figure 5A shows the number of probes by mosquitoes, while panel B shows the number of mosquitoes engorging (unlike previous figures, we give the results for each trial to better visualize the trial-to-trial variability, much of which is expected due to sampling error, i.e., the usual amount of variability native to a binomial or Poisson distribution with that sample size). In both species, mosquitoes preferred least to probe and engorge on wells with a surface temperature of 26 or 50° C. In these paired tests, *Ae. aegypti* engorged equivalently at 32, 38, and 44° C; however, this species probed significantly less at 32° C. *Aedes albopictus* mosquitoes fed and probed less than they did at 38° C for all temperatures except 44° C. Thus, of the two species, *Ae. albopictus* appeared to be more temperature selective. *Aedes aegypti* is known to accept amphibian hosts (Christophers 1960), while *Ae. albopictus* more strongly favors human hosts (Ponlawat and Harrington 2005). The results on probes and subsequent engorgements are consistent with the accepted knowledge that *Ae. albopictus* prefer hosts with temperatures in the range typical of mammals and birds.

Overall, Experiments 1-4 demonstrated that a combination of carbon dioxide, water vapor, ATP, and warmth alone was sufficient to elicit robust feeding behavior in *Ae. aegypti* and *Ae. albopictus*. These experimental results fulfilled the first objective of our research to define a minimal set of stimuli that elicits blood-feeding behavioral responses, including upwind flight, landing, probing, and engorgement in these species.

The idea that feeding behaviors of many species are released with the right combination of stimuli was developed years ago (Tinbergen 1951), and identifying these stimuli was an early goal. For blood-feeding arthropods attacking humans, identifying these stimuli has important implications for developing means to reduce attacks, by turning (from the feeder's perspective) a potential host into a non-host either during host-seeking or after making physical contact with the host. By identifying the combination of stimuli necessary for a parasite to ultimately feed, researchers are given several avenues for discovery of means to reduce attacks. For *Ae. aegypti* and *Ae. albopictus*, four stimuli appear necessary to elicit host-seeking and feeding, and we have demonstrated that interfering with any of them significantly reduces blood-feeding attacks. Our work gives researchers specific kinds of receptors in *Ae. aegypti* and *Ae. albopictus* to target for interference. The idea of targeting specific receptors for interference was recently

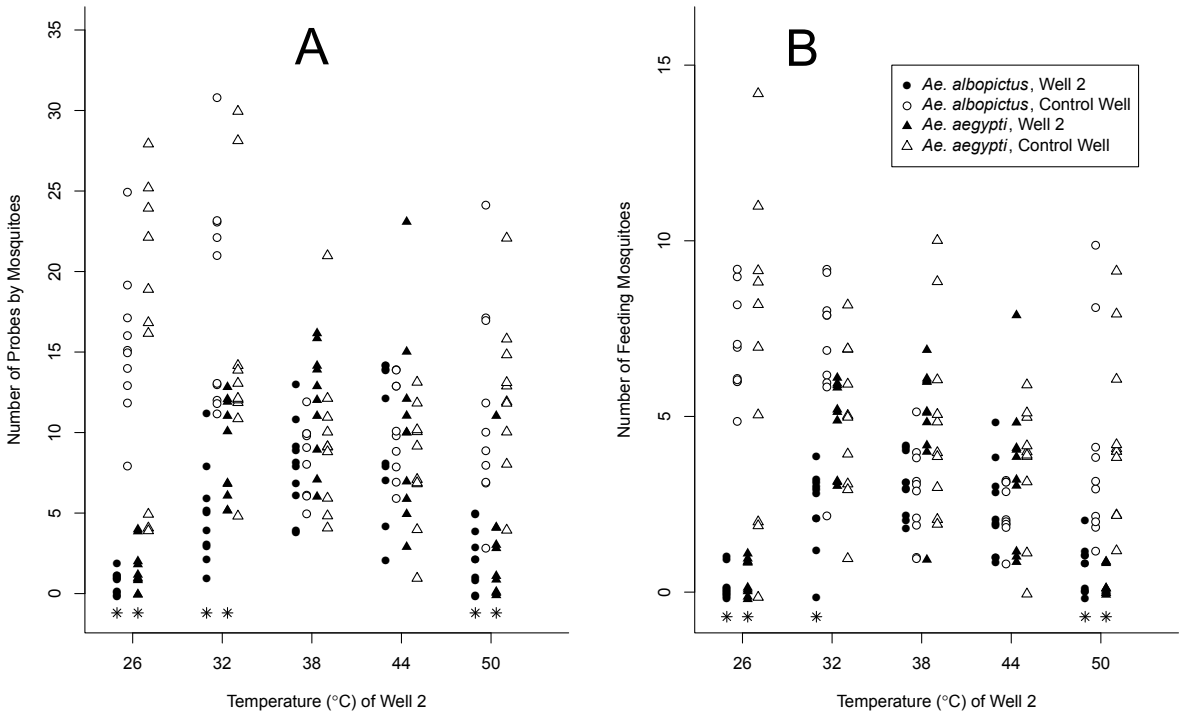

Figure 5. (A) shows the number of probes in each of ten trials at various temperatures, when paired with a control well at 38° C. Results from *t*-tests for each temperature are depicted by placement of an asterisk below the lowest count of the test temperature if differences with controls were significant at $p \leq 0.01$. Panel B gives the number of fed (engorged) mosquitoes in each of ten trials at various temperatures, when paired with a control well at 38° C. Results from chi-square tests (1 d.f.) for equal cell numbers for each temperature are depicted by the placement of an asterisk below the lowest count of the test temperature if differences with controls were significant at $p \leq 0.01$.

proven valid when Turner et al. (2011) focused upon carbon dioxide-detecting neurons in three disease-vectoring mosquito species. This approach led to the identification of three classes of volatile chemicals that specifically interfered with carbon dioxide perception in the mosquitoes and diminished their ability to locate carbon dioxide-emitting hosts.

That both *Ae. aegypti* and *Ae. albopictus* did not require host specific odors to elicit blood-feeding behavior in this study is of interest because it suggests a lack of host specificity. This is not to say that skin odorants and other volatiles are ignored; some appear to have repellent properties, others are attractive (Weldon and Carroll 2007). While our research shows that the four stimuli we used are sufficient to elicit host-finding and feeding, this does not preclude other combinations with some subset of the four plus additional stimuli from being effective releasers of behavior and that may be responsible for the expression of host specificity. However, by reducing the combinations of stimuli to the most elemental sets, a better and deeper understanding of what makes potential hosts attractive should lead to developing new chemicals that more effectively disrupt pest arthropod feeding behavior.

Experiment 5 (DEET)

For our second objective, Experiment 5 evaluated the behavioral impact of DEET when it was presented to the mosquitoes along with a combination of the four stimuli. Figure 1C demonstrates DEET's dramatic impact on mosquito feeding as it shows almost all mosquitoes feeding on reservoir wells without DEET. For both species, there was a large difference between the number of engorging from wells with control vs DEET treatments for both the human breath + air and the CO_2 + air conditions (no overlap of credible intervals). DEET suppressed engorgement behavior for all breath types to essentially zero. However, the rates were also very low for air only in both the control and DEET conditions. The means for proportion of mosquitoes engorging and approximate 67% credible intervals for engorging (corresponding to mean ± 1 SE, back-transformed to the original scale) are shown in Figure 6B. Results for probes were similar (all $p < 0.01$), and are shown in Figure 6A, with a pattern almost identical to that for proportion engorging. As in Figure 2A, Figure 6A (gray lines) also shows the mean number entering the wind tunnel from the release canister for each of the different breath types. Fewer mosquitoes of both species left the release canister under the air only condition. This result confirmed Experiment 1 findings that carbon dioxide plays a critical role in eliciting upwind flight in both mosquito species. Note that human breath + air and CO_2 + air elicited similar entrances into the tunnel, probing and engorgements (Figure 6). These results, along with those observed in Experiment 1, indicate that no chemical constituent of HB other than carbon dioxide stimulated mosquito upwind flight.

Experiment 5 provides evidence that if DEET interacts with receptors, it affects water vapor and/or warmth receptors. Note that the number of *Ae. aegypti* and *Ae. albopictus* engorging on control wells in Figure 6B (Experiment 5) and in Figure 2B (Experiment 1) under the human breath + air conditions are approximately the same. Thus, the DEET adjacent to the control wells and its presence in the atmosphere of the wind tunnel in Experiment 5 did not interfere with carbon dioxide perception and probing upon the control wells. Deet in the tunnel air also had

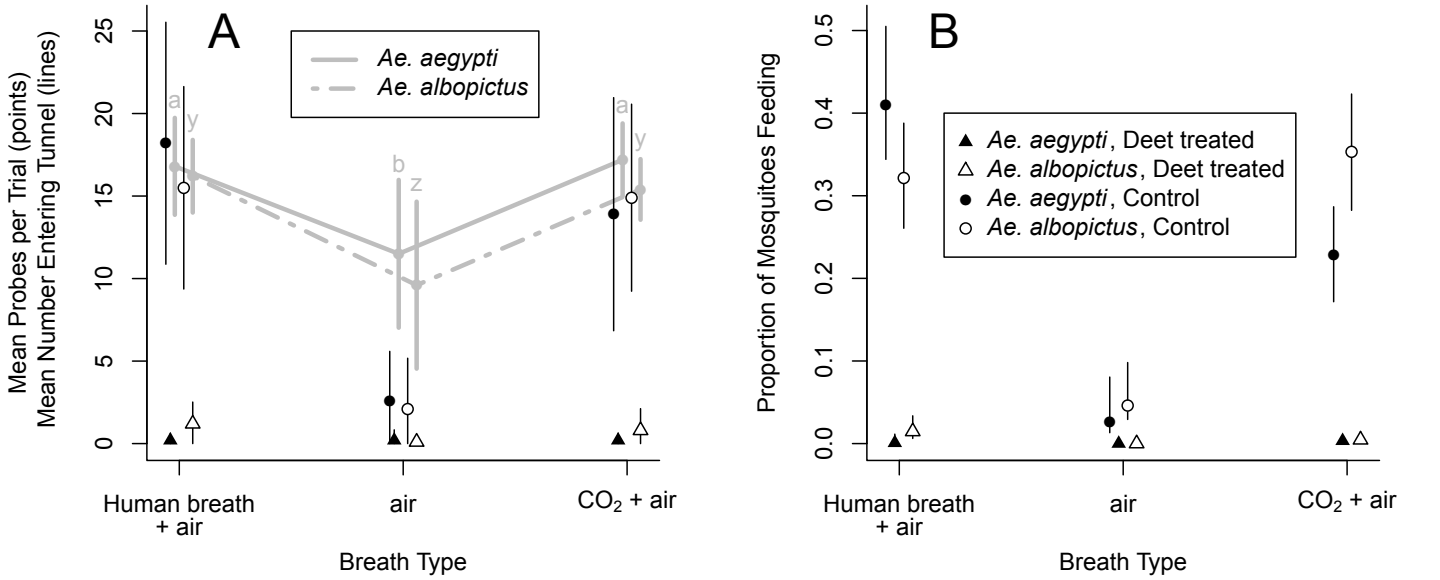

Figure 6. (A) and (B) show information similar to that presented in Figure 2. In these trials, half the wells were covered with DEET treated cloth. The three breath types were human breath + air, air only, and CO_2 + air. In panel A, gray points with gray connecting lines show the mean number in each trial entering the wind tunnel for the two mosquito species (with vertical bars giving 1 SD). The mean number of probes (with vertical bars giving 1 SD) in each air treatment is given by open or closed black symbols. Vertical bars in panel B represent a model based on a 67% credible interval (approximately 1 SD) back-transformed to the proportion scale. Means of the number entering the tunnel that do not differ significantly are adorned with the same letter ([a, b] for *Ae. aegypti*, [y, z] for *Ae. albopictus*). Deet-treated cloth (triangles) were avoided; probing and feeding from these wells were zero or close to zero (significantly different from controls) for both species in the three breath types.

no influence upon ATP-triggered engorgement at control wells in Experiment 5. ATP is not naturally detected in the vapor phase, but instead by taste receptors located on the mosquitoes' buccal cavity which sense ATP in aqueous solution. DEET appears to disrupt mosquito feeding behavior before probing and engorging takes place. Thus, if DEET primarily interferes with receptors (rather than working as a repellent or via some other mode), the only remaining candidate stimuli for DEET interference are water vapor and warmth. Warmth and water vapor receptors have been electrophysiologically studied in *Ae. aegypti* (Kellogg 1970, Davis and Sokolove 1975, Gingl et al. 2005), but, to our knowledge, no one yet has determined if DEET has any affect upon their performance in *Ae. aegypti* or *Ae. albopictus*. We believe that such a study would be illuminating and could provide additional insight into a probable mode of action for DEET, including interference of warmth and/or water vapor receptors functions.

Acknowledgments

We thank John C. Davis, Entomologist, and Sandra Lara, Biological Science Technician formerly with the USDA ARS Beltsville Agricultural Research Center, for conducting mosquito rearing and wind tunnel bioassays. Thanks to Matt Aubuchon of the USDA, ARS, Center for Medical and Veterinary Entomology, Jittawadee Murphy of the Walter Reed Army Institute of Research for supplying mosquito eggs, and Harriet L. Klun for reviewing and proofreading this manuscript.

REFERENCES CITED

AABB [American Association of Blood Banks]. 2005. Technical Manual, 15th edition. Bethesda, MD.

Bates, D., M. Maechler, and B. Bolker. 2011. lme4: Linear mixed-effects models using S4 classes. R package version 0.999375-42.

Bernier, U.R., D.L. Kline, D.R. Barnard, C.E. Schreck, and R.A. Yost. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). Anal. Chem. 72: 747-756.

Brown, A.W.A. 1956. Factors which attract *Aedes* mosquitoes to humans. Proceedings Tenth International Congress. Vol. 3: 757-764.

Cantrell, C.L., J.A. Klun, C.T.Bryson, M. Kobaisy, and S.O. Duke. 2005. Isolation and identification of mosquito bite deterrent terpenoids from leaves of American (*Callicarpa Americana*) and Japanese (*Callicarpa japonica)* Beautyberry. J. Agric. Food Chem. 53: 5948-5953.

Cantrell, C.L., A. Ali, S.O. Duke, and I. Khan. 2011. Identification of mosquito biting deterrent constituents from the Indian folk remedy plant *Jatropha curcas.* J. Med. Entomol. 48: 836-885.

Christophers, S.R. 1960. *Aedes aegypti* (L.), *The Yellow Fever Mosquito: Its Life History, Bionomics and Structure*. Cambridge Univ. Press, Cambridge.

Cooperband, M.F. and R.T. Cardé. 2006. Comparison of plume structures of carbon dioxide emitted from different mosquito traps. Med. Vet. Entomol. 20: 1-10.

Davis, E.E. and P.G. Sokolove. 1975. Temperature responses of antennal receptors of the mosquito, *Aedes aegypti*. J. Comp. Physiol. 96: 223-236.

Eiras, A.E. and P.C. Jepson. 1991. Host location by *Aedes aegypti* (Diptera: Culicidae): a wind tunnel study of chemical cues. Bull. Entomol. Res. 81: 151-160.

Friend, W.G. 1978. Physical factors affecting the feeding responses of *Culiseta inornata* to ATP, sucrose, and blood. Ann. Entomol. Soc. Am. 71: 935-940.

Galun, R., Y. Avi-dor, and M. Bar-Zeev. 1963. Feeding response in *Aedes aegypti*: stimulation by adenosine triphosphate. Science 142: 1647-1675.

Galun, R., L.C. Koontz, R.W. Gwadz, and J.M.C. Ribeiro. 1985. Effect of ATP analogues of the gorging response of *Aedes aegypti*. Physiol. Entomol. 10: 275-281.

Galun, R. and J.P. Kabayo. 1988. Gorging response of *Glossina palpalis palpalis* to ATP analogues. Physiol. Entomol. 13: 419-423.

Gingl, E., A. Hinterwirth, and H. Tichy. 2005. Sensory representation of temperature in mosquito warm and cold cells. J. Neurophysiol. 9: 176-185.

Gribble, F.M., L. Gildas, S.J. Tucker, C. Zhao, C.G. Nichols, and F.M. Ashcroft. 2000. A novel method for measurement of submembrane ATP concentration. J. Biol. Chem. 275: 30046-30049.

Gillies, M.T. 1980. The role of carbon dioxide in host-finding by mosquitoes: A review. Bull. Entomol. Res. 70: 525-532.

Hadfield, J.D. 2010. MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R Package. Journal of Statistical Software, 33: 1-22. URL http://www.jstatsoft.org/v33/i02/.

Hoel, D.L., D.L. Kline, and S.A. Allan. 2009. Evaluation of six mosquito traps for collection of *Aedes albopictus* and associated mosquito species in a suburban setting in north central Florida. J. Am. Mosq. Contr. Assoc. 25: 47-57.

Hothorn, T., F. Bretz, and P. Westfall. 2008. Simultaneous inference in general parametric models. Biometr. J. 50: 346-363.

Kellogg, F.E. 1970. Water vapor and carbon dioxide receptors in *Aedes aegypti.* J. Insect Physiol. 16: 99-108.

Khan, A.A. and H.I. Maibach. 1966. Quantitation of several stimuli on landing and probing by *Aedes aegypti*. J. Econ. Entomol. 59: 902-905.

Kline, D.L. 2002. Evaluation of various models of propane-powered mosquito traps. J. Vector Ecol. 27: 1-7.

Klun, J.A., M. Kramer, and M. Debboun. 2005. A new in vitro bioassay system for discovery of novel human-use mosquito repellents. J. Am. Mosq. Contr. Assoc. 21: 64-70.

Klun, J.A., M. Kramer, A. Zhang, S. Wang, and M. Debboun. 2008. A quantitative in vitro assay for mosquito deterrent activity without human blood cells. J. Am. Mosq. Contr. Assoc. 24: 508-512.

McCullagh, P. and J.A. Nelder. 1989. *Generalized Linear Models*, 2nd ed. Chapman and Hall/CRC, New York.

Pellegrino, M., N. Steinbach, M.C. Stensmyr, B.S. Hansson, and L.B. Vosshall. 2011. A natural polymorphism alters odour and Deet sensitivity in an insect odorant receptor. Nature 478: 511-515.

Ponlawat, A. and L.C. Harrington. 2005. Blood feeding patterns of *Aedes aegypti* and *Aedes albopictus* in Thailand. J. Med. Entomol. 42: 821-825.

Qiu, Y.Y., R.C. Smallegange, S. Hoppe, J.J.A. Van Loon, E.J. Bakker, and W. Takken. 2004. Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae* Giles *sensu strict* (Diptera: Culicidae) to human emanations. Med. Vet. Entomol. 18: 429-438.

Raina, A.K., J.A. Klun, M. Schwarz, A. Day, B.A. Leonhardt, and L.W. Douglas. 1986. Female sex pheromone of the melonworm, *Diaphania hylinata* (Lepidoptera: Pyralidae) and analysis of male responses to pheromone in a flight tunnel. J. Chem. Ecol. 12: 229-237.

R Development Core Team. 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org/.

Rudolfs, W. 1922. Chemotropism of mosquitoes. Bull. New Jersey Agr. Exp. Sta. No. 327. 23 pp.

Sharpington, P.J., T.P. Healy, and M.J.W. Copland. 2000. A wind tunnel bioassay system for screening mosquito repellents. J. Am. Mosq. Contr. Assoc. 16: 234-240.

Takken, W. and B.G.J. Knols. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. Annu. Rev. Entomol. 44: 131-157.

Tinbergen, N. 1951. *The Study of Instinct*. Clarendon Press, Oxford.

Turner, S.L., Nan Li, T. Guda, J. Githure, R.T. Cardé, and A. Ray. 2011. Ultra-prolonged activation of CO_2-sensing neurons disorients mosquitoes. Nature 474: 87-91.

Weldon, P.J. and J.F. Carroll. 2007. Vertebrate chemical defense: secreted and topically acquired deterrents of arthropods. In: M. Debboun, S.P. Frances, and D. Strickman (eds.). *Insect Repellents: Principles, Methods, and Uses*. pp. 47-75. CRC, Boca Raton.

Wright, R.H. 1962. The attraction and repulsion of mosquitoes. Wld. Rev. Pest Contr. 1: 1-12.

Wright, R.H. 1968. Tunes to which mosquitoes dance. New Sci. 28 March: 94-97.

Zhang, A., J.A. Klun, S. Wang, J.F. Carroll, and M. Debboun. 2009. Isolongifolenone: A novel sesquiterpene repellent of ticks and mosquitoes. J. Med. Entomol. 46: 100-106.