The Effect of Sucrose Concentration on Olfactory-Based Associative Learning in *Culex quinquefasciatus* Say (Diptera: Culicidae)

M. R. Sanford · J. K. Olson · W. J. Lewis ·
J. K. Tomberlin

Abstract A classical conditioning approach was used to examine the ability of *Culex quinquefasciatus* Say (Diptera: Culicidae) to associate odor with a sugar-meal of varying quality. The objectives of this study were to investigate the impact of different sucrose concentrations (5 %, 10 %, or 50 %) on positive response to conditioning and to examine sucrose concentration preference following exposure to a 10 % solution. Mosquitoes conditioned in conjunction with all three sucrose concentrations showed evidence of learning; including the concentration of the conditioning stimulus, and the sex of the mosquito. Using colored solutions to determine feeding patterns of experienced mosquitoes indicated male mosquitoes showed no preference but females showed a preference for 10 % over 5 % sucrose but not between 10 % and 50 % sucrose solutions.

Introduction

Sugar foraging behavior is an understudied aspect of mosquito (Diptera: Culicidae) behavior even though it is a life history requirement for almost all mosquito species

M. R. Sanford · J. K. Olson · J. K. Tomberlin
Department of Entomology, Texas A&M University, College Station, TX 77843, USA

W. J. Lewis
Crop Management Research Unit, USDA-ARS, 2747 Davis Road, Tifton, GA 31793, USA

Present Address:
M. R. Sanford (✉)
Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine,
One Shields Ave, University of California, Davis, Davis, CA 95616, USA
e-mail: uranotaenia@gmail.com

and for both sexes (Foster 1995). Mosquito-pollination relationships have been documented (Knab 1907; Thein 1969; Brantjes and Leemans 1976; Gorham 1976), but there has been little recent work in this area (Kevan et al. 1993). Evidence that mosquitoes prefer certain plants has been suggested in the anthropophilic *Anopheles gambiae* s.s. (Manda et al. 2007) which is matched in other species with a broader host preference and potentially more plastic behavior with respect to resource seeking, such as *Aedes albopictus* (Skuse) (Müller et al. 2011). Although there is little documentation of the specifics of the nectar resources that mosquitoes acquire from plants, mosquito attraction to floral odors is well documented and the behavior has been proposed for developing trapping programs (Foster 2008) and exploitation for novel control methods (Müller and Schlein 2006).

The *Culex pipiens* complex consists of several species capable of vectoring multiple viral pathogens as well as nematodes, with a worldwide distribution and a broad host preference. In *Culex pipiens pipiens* biotype *molestus* Forskal, Jhumur et al. (2006) found that a higher percentage of mosquitoes exposed to sugar rewards in conjunction with the odors associated with the flowers of *Silene otites* (L.) Wibel (Caryophyllales: Caryophyllaceae) responded positively to the odors presented without sugar in a wind tunnel experiment. These data showed that experience enhanced the innate attraction of this mosquito species to the floral odors and suggested that the mechanism for this increase was associative learning. In the sister species, *Culex quinquefasciatus* Say, Tomberlin et al. (2006) found evidence that adult female and male mosquitoes are capable of associative learning to novel odors presented in a classical conditioning paradigm with a 10 % sucrose solution and that females associated a novel odor with a blood-meal. Using this same species, Sanford and Tomberlin (2011) confirmed these observations with assay and analytical improvements that evaluated conditioning of male and female *Cx quinquefasciatus* from laboratory and field populations as well as the effect of age on learning.

The observation that mosquitoes are capable of learning about their environment and resources is not a new idea (Ribbands 1949; Charlwood et al. 1988; Renshaw et al. 1994; McCall et al. 2001). It was initially explored as a conceptual idea by McCall and Kelly (2002) and specific examination of this behavior has been attempted in the laboratory for *Aedes aegypti* L. (Alonso et al. 2003), *Cx. pipiens* (Jhumur et al. 2006), *Cx. quinquefasciatus* (Tomberlin et al. 2006; Sanford and Tomberlin 2011), *Anopheles cracens* Sallum & Peyton (Sanford 2010) and most recently with *Anopheles gambiae* s.s. (Chilaka et al. 2012). Despite these many attempts to demonstrate learning in mosquitoes, many technical questions remain about how to best present conditioning stimuli and how the response of conditioned mosquitoes is measured and interpreted (Alonso and Schuck-Paim 2006).

Aside from the innate value of understanding mosquito learning behavior there are ecological and applied interests to understanding this behavior with respect to sugar feeding. In their study of *An. gambiae* in east Africa, Manda et al. (2007) noted that the plants that were most commonly used for perching and feeding were those that were often associated with human habitations. It raises an interesting question of whether placing these plants away from the house might draw mosquitoes away from their human blood source or reduce sugar-feeding and longevity of females, thus reducing local malaria transmission. Another aspect of mosquito control with a sugar-feeding basis is the recent success of Attractive Toxic Sugar Baits (ATSBs) in a

growing array of locations (Müller et al. 2010a, b; Beier et al. 2012). If mosquitoes learn about rewards associated with certain plant species or baited traps (administering sub-lethal doses of active ingredient) the efficacy of these control methods might be enhanced.

In this study, we used the conditioning approach of Sanford and Tomberlin (2011) combined with reciprocal odor pairing, to determine the effect of sucrose concentration on the probability of positive responses following conditioning. The effect of sucrose concentration is both a technical question in the conditioning protocol and a question of ecological relevance, as the expectation was that a mosquito with a broad host preference (*Cx. quinquefasciatus*) would be more likely to learn about odors associated with a high level of reward. In addition, we examined whether the exposure of the mosquitoes to a 10 % sucrose solution before conditioning had any effect on their preference for the different sucrose concentrations used in the study.

Materials and Methods

Conditioning Experiment

Insects Adult *Cx. quinquefasciatus* were obtained from the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA-ARS, Gainesville, FL, USA where they were reared using standardized methods (Gerberg et al. 1994) and delivered by overnight shipment to the Insect Biology and Population Management Research Laboratory (IBPMRL), USDA-ARS, Tifton, GA, USA, where experiments were conducted. Shipments of mosquitoes were required because the IBPMRL was not equipped to provide blood-meals for female mosquitoes for rearing onsite and the shipment procedure was used successfully in the past (Tomberlin et al. 2006). Each shipment of newly emerged mosquitoes represented a separate cohort and occurred on a weekly basis for a total of five cohorts.

Once they arrived at the IBPMRL, the mosquitoes were held at 27–29 °C with 70–85 %RH at 16:8 L:D cycle and were offered a 10 % sucrose solution before being starved for 24 h prior to conditioning (distilled water was provided throughout). One hour prior to conditioning, the mosquitoes were moved to the main laboratory and allowed to acclimate in front of the chemical fume hood at room temperature. Individual mosquitoes were isolated within clean individual 2-dram glass shell vials (40 mm height × 17 mm diameter) as described by Sanford and Tomberlin (2011).

Conditioning Procedure Individual male and female mosquitoes, aged 3–5 days, were conditioned to either pure vanilla extract (McCormick & Co., Inc., Hunt Valley, MD, USA) or 100 % technical grade myrcene (Sigma-Aldrich, Co., St. Louis, MO, USA) in combination with a 5 %, 10 %, or 50 % (w/v) technical grade sucrose (Sigma-Aldrich, Co., St. Louis, MO, USA) solution using the method described in Sanford and Tomberlin (2011). The selection of vanilla was made for comparison to previous work (Tomberlin et al. 2006). Myrcene was selected because it is a compound with which the mosquitoes should not have any innate preference but may be part of an attractive blend of compounds (Allan et al. 2006), and it differs from vanilla in chemical structure.

The conditioning protocol was conducted according to the method described by Sanford and Tomberlin (2011). Briefly this method includes feeding the mosquito a sucrose solution in a 200 L calibrated glass micropipette (Drummond Scientific Company, Broomall, PA, USA) with the distal ~1 cm of the exterior coated with an odor compound. The three treatments consisted of 5 %, 10 % and 50 % sucrose concentrations, which are within the range of flower nectar resources recorded in nature (Baker 1975). Each mosquito was offered the training pipette by lifting the edge of the vial approximately 30° and placing the solution-filled pipette directly onto the mosquito's proboscis and allowing it to feed for 10 s. This procedure was repeated three times with an inter-trial interval of 30 s (Sanford and Tomberlin 2011). All conditioning and testing was conducted under an actively ventilating fume hood under laboratory lighting between 1,100 and 1,800 h (during the mosquito photophase).

Sample sizes for each of the treatments in the experimental design are listed in Table 1. The lower number of mosquitoes conditioned to the 5 % sucrose unconditioned stimulus reflected difficulties in conditioning females to this sugar concentration especially when vanilla was the target. Females were the most difficult to condition at the 5 % sucrose solution, but fewer refused the conditioning protocol at higher concentrations of sucrose (Fig. 1).

Testing Procedure Mosquitoes were conditioned using the above procedure and then allowed to wait in the glass vial in the fume hood for a period of time (13.0±9.0 min) while other mosquitoes were being conditioned prior to testing.

Table 1 Sample sizes (*N*=283) for the categories examined in the conditioning of *Culex quinquefasciatus* to two different target odors, both sexes, and three different concentrations of sucrose solution

Target:	Sex	Concentration	n
Vanilla			140
	Female		59
		5 %	9
		10 %	22
		50 %	28
	Male		81
		5 %	21
		10 %	27
		50 %	33
Myrcene			143
	Female		62
		5 %	17
		10 %	19
		50 %	26
	Male		81
		5 %	25
		10 %	26
		50 %	29

Fig. 1 Sorted male and female *Culex quinquefasciatus* fed on a red or green colored sucrose solution representing of a choice of either 5 % vs. 10 % or 10 % vs. 50 %. The color of the meal indicates their feeding choice

The testing protocol was as described in Sanford and Tomberlin (2011). Each conditioned mosquito was tested with an empty pipette that was either a new unused pipette (blank), a pipette coated with the target odor they were conditioned to (either myrcene or vanilla), or a pipette coated with the non-target odor that they had not had experience. The pipette was placed under the vial at a distance of approximately one-half the diameter of the vial from the mosquito for 15 s to allow for a response.

Responses to the odor stimuli were recorded as described in Sanford and Tomberlin (2011) and consisted of either a positive or a negative response by each mosquito exposed to one of the stimulus options. A positive response was recorded if the mosquito walked toward the pipette and moved the proboscis as if searching for the sucrose solution. A negative response was recorded if the mosquito did not move when exposed to the test pipette or moved away from it.

Sugar Preference Experiment

Insects The mosquitoes used in this experiment were from the laboratory colony maintained at the Mosquito Research Laboratory at Texas A&M University, College Station, TX, USA that was initiated with eggs from the CMAVE as previously described. All rearing and experiments were carried out in a Rheem Environmental walk-in growth chamber (Asheville, NC, USA) at 25–27 °C and approximately 50–70 % RH with a 14:10 L:D cycle.

The mosquitoes were reared at a density of two egg rafts per liter of deionized water in white metal enamel pans and fed a diet consisting of a ground Tetramin® Tropical Flakes (Tetra Holding (US) Inc., Blacksburg, VA, USA) slurry at a ratio of 3 parts ground Tetramin® to 1 part deionized water. Pupae were collected from multiple rearing pans (on the second day of pupation to ensure males and females were collected) and mixed before placing 100 pupae into 100 ml of deionized water in each of four small Plexiglas cages (19.5×19.5×19.5 cm) for each replicate of the

experiment. Each cage was provided with a square piece of clean flat cotton (approximately 3×3 cm) soaked in 10 % sucrose solution and placed in a plastic weigh boat. At 3–5 days of age, the sugar solution was removed from each cage for 24 h to prepare the mosquitoes for the experiment using the same protocol used in the conditioning experiment.

Evaluation The sucrose solutions used in the experiment were 5, 10 and 50 % sucrose by weight created with reagent grade sucrose (Aldrich®, Sigma-Aldrich, Inc., St. Louis, MO, USA) and deionized water as in the conditioning experiment. The solutions were tested by offering the mosquitoes the solutions in pairs on clean flat cotton squares as previously described with each solution dyed with either red (Strawberry Red Color, Royallee Brand, Bangkok, Thailand) or green (Apple Green Color, Royallee Brand, Bangkok, Thailand) food coloring at a concentration of two drops in 5 ml of sucrose solution. Two milliliters of the dyed solution was then soaked into each cotton pad. The colors were alternated between the solutions for each replicate to mitigate any preferences for one food coloring or the other. The experiment was replicated three times for each concentration pairing of 5 % versus 10 % sucrose and 10 % versus 50 % sucrose.

A preliminary run of the study indicated the mosquitoes preferred the red food coloring under the lighting of the growth chamber, so all experiments were conducted with the growth chamber lights turned off which appeared to eliminate the red food coloring bias (Fig. 9). The sucrose soaked cotton was placed in each cage in the dark with the aid of a red filtered flashlight for 2 h during the beginning of the scotophase (18:30 h–20:30 h) to allow the mosquitoes to feed. Upon completion of the 2 h the cages were placed in the freezer (20 °C) overnight to kill and preserve the mosquitoes for determination of their sugar-meal choice. Data were recorded for the number of males and females as well as the meal they chose as indicated by color (Fig. 1). The number of non-feeding mosquitoes and the number of mixed meals were also recorded for each cage. Mixed meals were rare but when they occurred the color of the sugar-meal was brown as observed by Lindh et al. (2006).

Statistical Analysis

Conditioning Experiment Model selection and implementation of the analysis was carried out as described in Sanford and Tomberlin (2011). Binary logistic regression was employed using the backward stepwise variable selection method based on the change in likelihood ratio (entry at $P=0.05$ and exit at $P=0.10$; SPSS, Inc 2005). For the analysis of those variables with more than two categories, the overall effect was tested first then dummy variables were created through binary coding and a reference variable to which the dummy variables were compared; thus any results obtained from these variables represent the difference between the dummy category and the reference category (DeMaris 1995). The variables included in the model were the categorical variables indicating: cohort membership (coded: 1–5), sex (coded: 0 = male; 1 = female), target to which the mosquito was conditioned (coded: 0 = vanilla; 1 = myrcene), sucrose concentration to which the mosquito was conditioned (coded: 1 = 5 %; 2 = 10 %; 3 = 50 %), and test to which the

mosquito was subjected (coded: 1 = blank; 2 = non-target; 3 = target) with the reference indicated by the last factor coded in each set. All potential interaction terms were also tested in the initial model. The amount of time a mosquito waited to be tested was also included in the initial model but was not found to be a significant predictor of response.

This analysis provided model results in terms of odds ratios, which are presented in Table 2 as well as predicted probabilities for positive outcome with the model, which were plotted for the variables of interest. An independent-samples T-test was conducted on the mean predicted probability for each two-category variable and an analysis of variance with Tukey's HSD post hoc test was conducted for each three-category variable (SPSS, Inc 2005). Statistical significance for all tests was observed at α=0.05.

Sugar Preference Experiment Non-fed individuals were considered non-responders and the possibility that these individuals were dead in the cage prior to the introduction of the sucrose solutions (although they were rarely observed they were not specifically removed) they were not considered in the analysis although their numbers were recorded (Table 3). Mixed meals were relatively rare (Table 3) and were dropped from the analysis, which allowed for a parametric statistical analysis. The data were analyzed separately for each concentration pair (5 % vs. 10 % or 10 % vs.

Table 2 Final binary logistic regression model (model χ^2:81.137, d.f.: 11, P<0.001) based on backward stepwise variable selection for likelihood ratio of associative learning data in *Culex quinquefasciatus* to three different sucrose concentrations. The model is based on the response variable of positive outcome. See text for detailed description of variables and tests

Variable	Log odds (β)	Wald χ^2	d.f.	P	Odds ratio (e^β)
Concentration		5.708	2	0.058	
Concentration: 5 % vs. 50 %	0.771	3.429	1	0.064	0.462
Concentration: 10 % vs. 50 %	0.814	4.436	1	0.035[a]	0.443
Sex: female vs. male	1.801	15.778	1	<0.001[a]	0.165
Test		29.498	2	<0.001[a]	
Test: blank vs. target	2.500	17.127	1	<0.001[a]	0.082
Test: non-target vs. target	3.345	17.712	1	<0.001[a]	0.035
Target odor × test		7.189	2	0.027[a]	
Target odor (vanilla vs. myrcene) × test (blank vs. target)	0.521	0.568	1	0.451	0.594
Target odor (vanilla vs. myrcene) × test (non-target vs. target)	2.133	6.622	1	0.010[a]	8.441
Sex × test		5.076	2	0.790	
Sex (female vs. male) × test (blank vs. target)	1.639	3.941	1	0.047[a]	5.149
Sex (female vs. male) × test (non-target vs. target)	0.744	0.400	1	0.527	0.475
Constant	1.155	9.894	1	0.002[a]	3.172

[a] indicates significant difference at α=0.05 level

Springer

Table 3 The percentage of total mosquitoes selecting the 5, 10, or 50 % sucrose concentrations, mixed meals and non-feeding individuals by sex and color during an experiment on sucrose concentration preference following exposure to 10 % sucrose. For the 5 vs. 10 % pairing experiment $N=1083$ with a ratio of males to females = 610:530 and for the 10 % vs. 50 % pairing experiment $N=1140$ with male to female ratio = 602:481

Pairing	Sex	Color	Concentration	%
5 vs. 10 %	Female	Red	5 %	4.6
	Male	Red	5 %	8.2
	Female	Green	5 %	3.9
	Male	Green	5 %	7.5
	Female	Red	10 %	13.5
	Male	Red	10 %	9.6
	Female	Green	10 %	9.6
	Male	Green	10 %	10.4
	Female	Mixed	N/A	4.6
	Male	Mixed	N/A	1.1
	Female	None	N/A	6.7
	Male	None	N/A	16.8
10 vs. 50 %	Female	Red	10 %	9.5
	Male	Red	10 %	10.2
	Female	Green	10 %	8.6
	Male	Green	10 %	11.3
	Female	Red	50 %	9.6
	Male	Red	50 %	10.6
	Female	Green	50 %	11.7
	Male	Green	50 %	9.3
	Female	Mixed	N/A	2.0
	Male	Mixed	N/A	0.5
	Female	None	N/A	3.0
	Male	None	N/A	13.8

50 %) with a full factorial analysis of variance (ANOVA) using the general linear model (GLM) procedure in SPSS 16.0 (SPSS, Inc 2007). The factors of interest were the sex of the mosquito, the concentration of the sucrose solution, the color of the solution and the interactions between each of these factors.

Results

Conditioning Experiment

The final model for the binary logistic regression analysis is presented in Table 2. Neither cohort membership nor target odor contributed significantly to the prediction of positive response and were the only main effect variables dropped from the model

(variable selection ended at the twelfth step). Among the significant interactions were those between target odor and test as well as between sex and test (Table 2). The final model had a -2log-likelihood of 237.692 and the χ^2 for the final step was 81.137 (d.f.=11, $P<0.001$). The P-values presented in Table 2 are based on the Wald statistic for each individual variable and indicate the ability of each variable to predict a positive response.

In the final model, several variables were significant predictors of a positive response (Table 2). Each of the significant predictors in the model was also plotted as predicted probability for each category for a visual representation of the data that included the results of the post-modeling analyses for each significant set of predictors. Significant main effect variables included sucrose concentration (Fig. 2), with lower probability of response to the 5 % and 10 % sucrose concentrations when compared to positive response to the 50 % sucrose concentration. An ANOVA showed significance among the mean predicted probabilities ($F=6.412$; d.f.=2,282; $P=0.002$) with Tukey's HSD test indicating that the predicted probability of positive response was significantly higher to the 50 % sucrose concentration (Fig. 2). The sex of the mosquito was also a significant main effect predictor with males having 0.165 higher odds of positive response according to the model (Table 2). This relationship is reflected in Fig. 3 where the independent samples T-test indicated an $F=186.2$ (d.f.= 281; $P<0.001$) in the mean predicted probabilities generated by the model. The test that the mosquito was administered was the only other significant main effect predictor of positive response by the mosquitoes. This result is expected to demonstrate learning we expect that there will be higher probability of mosquito response to the target odor and the model does indicate higher odds of positive response to the target odor over the blank (odds ratio = 0.082) and the non-target (odds ratio = 0.035). This result is also illustrated in Fig. 4 where the mean predicted probability of response to the target was significantly higher than to the blank and non-target (ANOVA: $F=155.5$; d.f.=2,282; $P<0.001$). However, the significance of test as a predictor is tempered by strong interaction effects as illustrated by the factorial model.
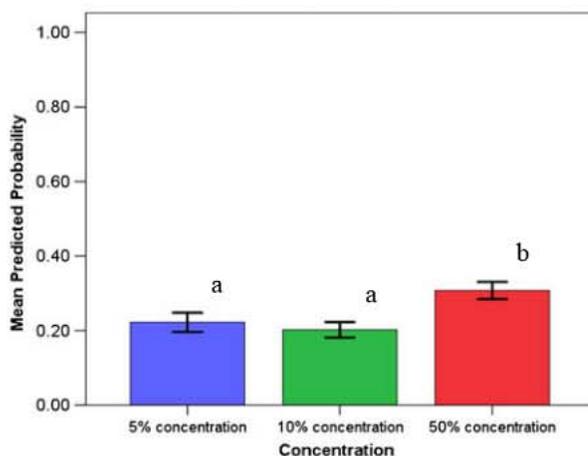

Fig. 2 Mean (+/− S.E.) predicted probability of positive response to conditioning of *Culex quinquefasciatus* to three different sucrose concentrations. Different letters indicate significant differences between groups as determined by Tukey's HSD following analysis of variance at the significance level of $\alpha=0.05$
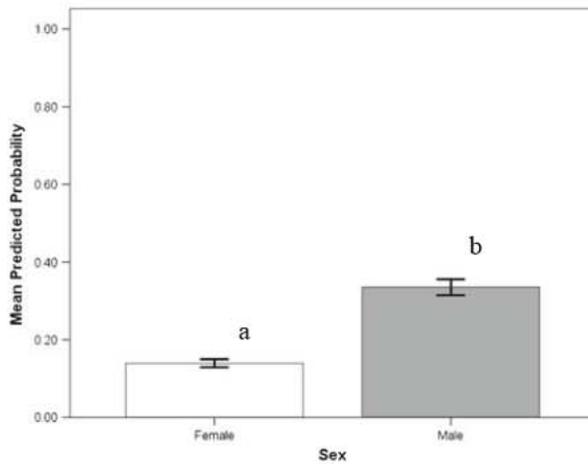
Fig. 3 Mean (+/ S.E.) predicted probability of positive response to conditioning of male and female *Culex quinquefasciatus*. Different letters indicate significant differences between groups as determined by independent samples *T*-test at =0.05

Two interactions were significant in modeling the positive response of the mosquitoes trained in the study. The target odor that the mosquito was conditioned to was not a significant predictor of positive response on its own, but was a significant predictor when considered jointly with the test that each mosquito was administered following conditioning with each target odor (Fig. 5). The mean predicted probabilities illustrate the relationship between target odor and the mosquito's test with a differential response to the three tests depending on which training odor was used (Fig. 5). The predicted probability of those mosquitoes trained with vanilla responding positively to the blank, non-target and target increases incrementally but the predicted probability of positive response to the tests when initially
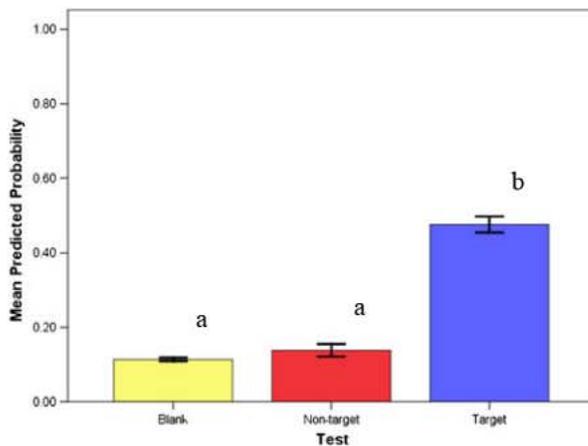

Fig. 4 Mean (+/ S.E.) predicted probability of positive response to conditioning of *Culex quinquefasciatus* when tested to a blank pipette, a pipette coated with an unknown odor or a pipette coated with the target (conditioning) odor. Different letters indicate significant differences between groups as determined by Tukey's HSD following ANOVA with significance observed at =0.05
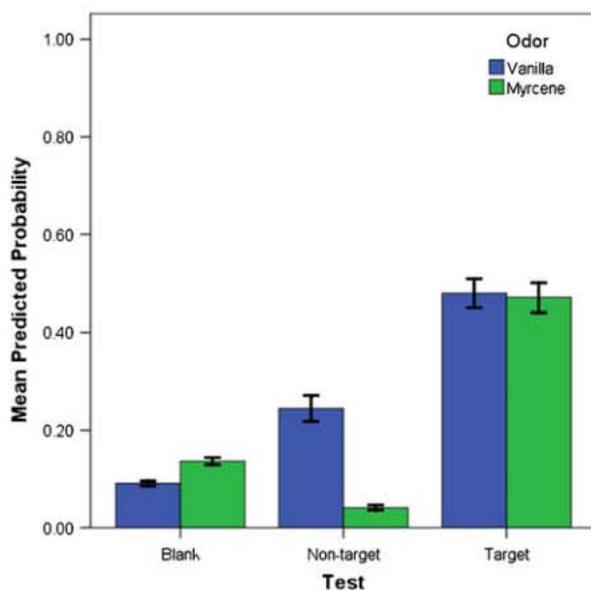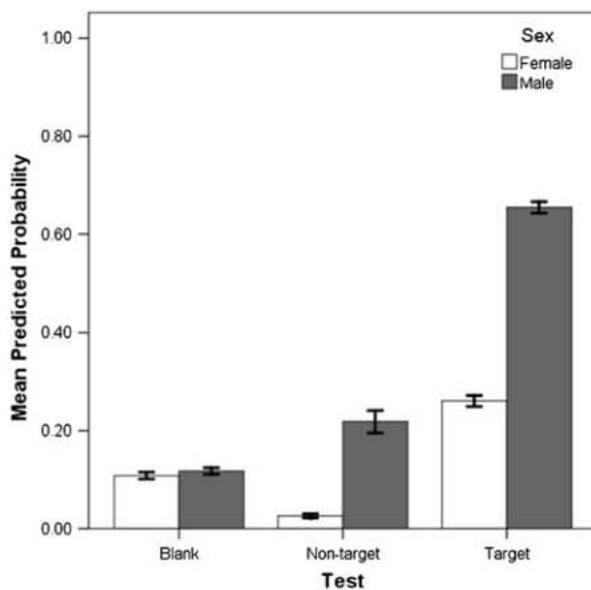
Springer

Fig. 5 Mean (+/ S.E.) predicted probability of positive response to conditioning of *Culex quinquefasciatus* to pure vanilla extract or myrcene by the test to a blank pipette, a pipette coated with an unknown odor or a pipette coated with the target (conditioning) odor each mosquito received to evaluate conditioning

trained with myrcene is significantly different. Mosquitoes initially trained with myrcene had higher mean predicted probability of positive response to the blank over the non-target (Fig. 5). However, the highest probability for both target odor

Fig. 6 Mean (+/ S.E.) predicted probability of positive response to conditioning of male and female *Culex quinquefasciatus* tested with either a blank pipette, a pipette coated with a non-target (unknown) odor or a pipette coated with the target (conditioning) odor
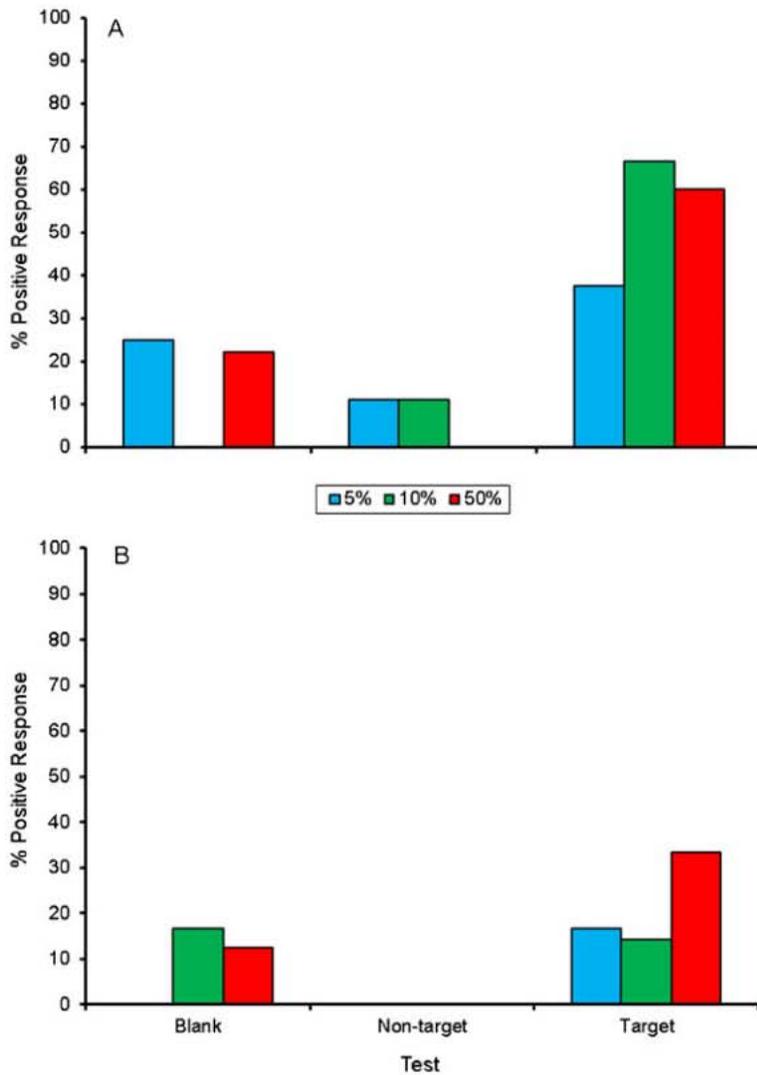
Fig. 7 Percent positive response of mosquitoes trained to three different sucrose concentrations when the target odor was myrcene for males (panel **a**) and females (panel **b**). Each mosquito was trained to the sucrose-odor combination and subjected to only one test

groups was to the target odor, which is consistent with conditioning. The other significant interaction of sex with test, illustrated in Fig. 6, suggests a similar pattern with the predicted probability of male mosquito response to the three tests increasing incrementally from blank to non-target to target, similarly as with the mosquito response after conditioning to vanilla. The predicted probability of female response to each test (Fig. 6) is also visually similar to that of myrcene-conditioned mosquitoes (Fig. 5).

The raw data illustrates trends in the percentage of mosquitoes responding positively to a test when they were conditioned to the different sucrose concentrations. A higher percentage of female mosquitoes responded to the target when conditioned to
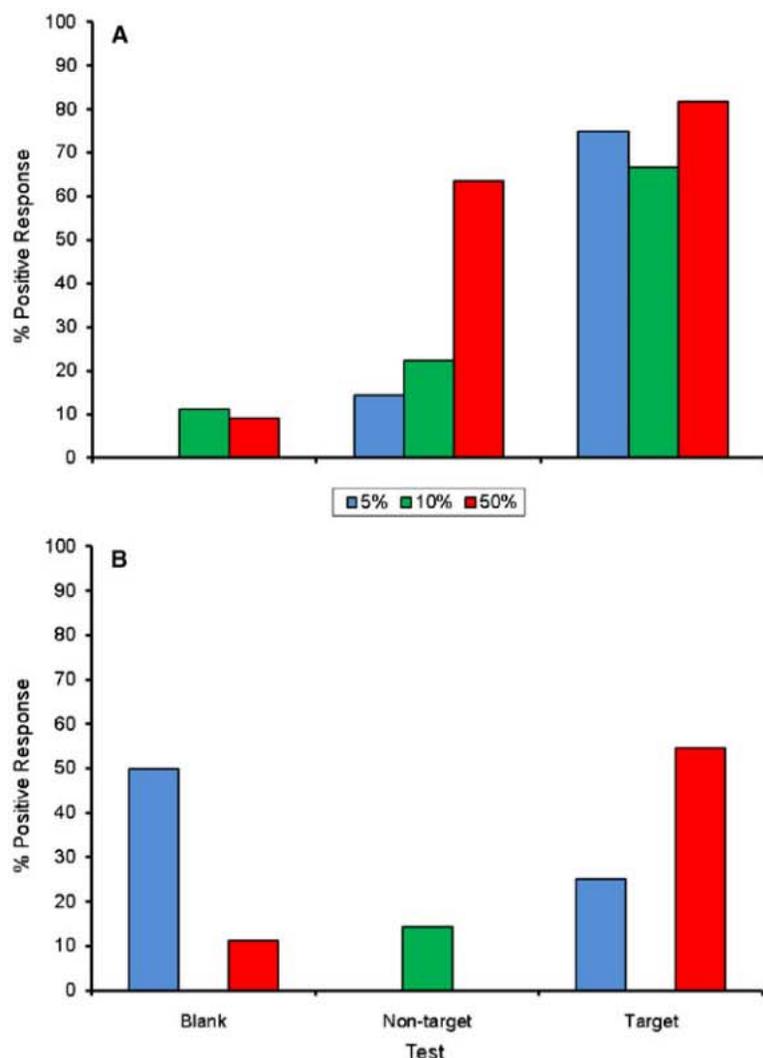
Fig. 8 Percent positive response of mosquitoes trained to three different sucrose concentrations when the target odor was vanilla extract for males (panel **a**) and females (panel **b**). Each mosquito was trained to the sucrose-odor combination and subjected to only one test

the 50 % sucrose than when conditioned to the other sucrose concentrations regard-less of the target odor (Figs. 7b and 8b). The percentage of males responding positively to the target odor was similar across sucrose concentrations and target odors with the exception of those trained to vanilla extract as the target with 5 % sucrose (Figs. 7a and 8a). Acceptance of the conditioning assay does not infer that these mosquitoes were successfully trained only that they accepted feeding for the three trials constituting the conditioning assay. In general, there was a higher per-centage of positive responses by male mosquitoes than females regardless of target odor was determined (Figs. 7 and 8).

🖄 Springer

Sugar Preference Experiment

Table 3 displays the percentage of total mosquitoes selecting each of the color–concentration combinations by sex for each of the pairing experiments. A higher percentage of males did not feed as opposed to females. In the 5 % vs. 10 %, pairing experiment there was a higher percentage of males and females with mixed meals compared to the 10 % vs. 50 % concentration experiment. The full factorial ANOVA did not indicate any significant effects in the comparison between the 10 % and 50 % sucrose concentrations for any of the factors of interest (Table 4). Figure 9a illustrates the mean (+/ SE) number of males and females with either red or green sugar-meals by concentration for the 10 % versus 50 % comparison. There is no obvious trend in the data, males and females chose both solutions and colors in approximately equivalent numbers (Fig. 9a).

Data for both of the comparisons between sucrose concentration pairings met the assumptions of ANOVA for homogeneity of variance and normality of residuals. The results of the full factorial ANOVA for the comparison between 5 % and 10 % sucrose solutions indicated a significant difference for the factors of color, concentration and the interaction between concentration and sex (Table 4). Overall significantly more males and females chose the higher 10 % sucrose solution regardless of color (Fig. 9b). From Fig. 9b, it can also be seen that significantly fewer females chose the green solution when compared with the number choosing red within each concentration. There does not appear to be a difference in the mean number of males selecting green or red within each sucrose concentration (Fig. 9b). The indication of a significant interaction between sucrose concentration and sex appears to be within the response of females. Significantly more females chose 10 % sucrose regardless of color and this effect does not appear to be as pronounced in males (Fig. 9b).

Discussion

In this study, we expanded the technical knowledge of mosquito conditioning response to different sucrose concentrations and implemented reciprocal odor pairing to demonstrate conditioning. The use of a 50 % sucrose concentration resulted in higher

Table 4 ANOVA results for the factors of interest in the comparison of mosquito sucrose concentration choice between 10 % vs. 50 % and 5 % vs. 50 % sucrose using dyed solutions following access to 10 % sucrose. The model included the color of the sucrose solution, concentration of the solution, sex of the mosquito, and the interactions among significant factors

[a]indicates significance observed at the =0.05 level

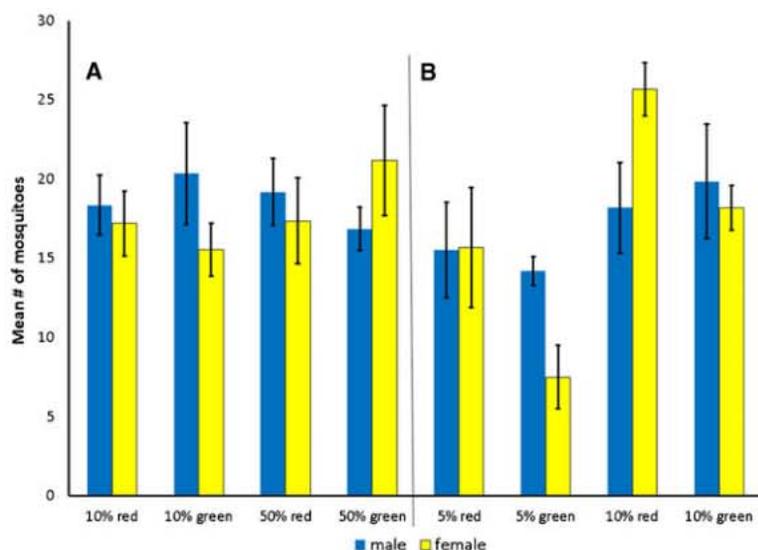| Factor	d.f.	F statistic	P-value
10 % vs. 50 %			
Color	1	0.072	0.789
Concentration	1	0.214	0.645
Sex	1	0.262	0.611
5 % vs. 10 %			
Color	1	4.351	0.043[a]
Concentration	1	15.564	<0.001[a]
Sex	1	0.008	0.928
Color × sex	1	4.738	0.035[a]

Springer

Fig. 9 Mean (+/ SE) number of male (*blue*) and female (*yellow*) mosquitoes selecting 10 % or 50 % (**a**) and 5 % or 10 % sucrose solutions (**b**)

odds of a positive response to olfactory conditioning, which was accompanied by the apparent readiness of mosquitoes to feed on the higher sucrose concentration reward (Table 1). In cage trials following exposure to a 10 % sucrose solution, mosquitoes showed little preference for a high sucrose concentration sugar-meal (50 %) over a 10 % sucrose sugar meal but showed a more clear preference for the 10 % sugar-meal over a low sucrose concentration meal (5 %).

Conditioning Experiment

Sucrose concentration was a significant predictor of positive response in the binary logistic regression model. Wäckers et al. (2006) found that the solitary parasitoid, *Microplitis croceipes* (Cresson), (Hymenoptera: Braconidae) demonstrated no difference in learning when trained with 0.25 M or 1 M concentrations of sucrose solution. They found that the higher sucrose concentration was a better feeding stimulant but did not result in higher learning rates despite what has been observed in the bumblebee, *Bombus terrestris* L. (Hymenoptera: Apidae) (Laloi et al. 1999) and the honeybee, *Apis mellifera* L. (Hymenoptera: Apidae) (Bitterman et al. 1983). The data presented here suggest that the mosquitoes have a modestly higher probability of positive response to a 50 % sucrose training solution and will readily feed from a high sucrose concentration solution. The ecological data we have available with respect to mosquito plant feeding preference for *An. gambiae* (Manda et al. 2007) suggest that mosquitoes are selective but the study was not able to relate particular plants with the resource quality and quantity that they provide. The field data also suggest an association in a mosquito that is highly host specific. The mosquito examined in the current study, *Cx. quinquefasciatus*, accepts a wide array of hosts (e.g. Molaei et al. 2007), suggesting a plastic behavioral response to hosts and the potential for

🍂 Springer

developing associations to reduce search time and maximize resource acquisition effort.

Many of the female mosquitoes refused the pipette containing 5 % sucrose after one or two training trials; consequently, our sample size was lower for this category (Table 1 and Fig. 2). While this low sucrose concentration was not as readily accepted by females, it is often used in colony maintenance (e.g.: Awono-Ambene et al. 2001; Nasci et al. 2001; Fradin and Day 2002; Kim et al. 2003). The colony used in this experiment was maintained on a 10 % sucrose concentration and was exposed to this concentration prior to conditioning. It has been shown that exposure to the unconditioned stimulus prior to condition can have an effect of depressing positive responses to conditioning (e.g. pre-exposure effect; Randich and LoLordo 1979). Thus, the mosquitoes' responsiveness to the 10 % sucrose solution at training may have been affected by previous exposure. A lower mean predicted probability for 10 % sucrose concentration was observed (Fig. 5), suggesting that the significant difference observed between the mean predicted probabilities of 10 % and 50 % sucrose concentrations should be considered carefully.

The significant interaction observed between target odor and test predicted probabilities suggest that the olfactory properties of the odor compounds may have affected mosquito receptivity to conditioning. The positive linear response of mosquitoes to the blank, non-target and target odors respectively (Fig. 5) suggests that the odor of vanilla might have some innate attractive properties or some overlap with myrcene. The mosquitoes trained with myrcene and tested with vanilla (non-target) do not suggest the same probability of positive response, further suggesting that vanilla may contain molecules with similar structure to myrcene. Vanilla is an odor blend derived from natural plant sources that probably has many similarities with other plant-derived compounds and thus may have some innate attractive properties. This explanation is suggested by the results of Tomberlin et al. (2006) where more male mosquitoes responded positively to a vanilla target odor after training to strawberry extract, which may suggest some innate attraction to vanilla or an overlap of compounds between the two odor blends. Thus, conditioning may have increased innate attraction, similar to the results of Jhumur et al. (2006) where exposure to weakly attractive flower odors was significantly increased when the odor was coupled with a sugar reward, or it may have been accomplished but our ability to detect it clearly was hampered by overlap between a chemical blend and a structurally similar constituent. The ability of mosquitoes to discriminate very close chemical structures has not yet been evaluated in the context of learning.

The significance of the interaction between mosquito sex and test can potentially be explained by the different life history demands of the sexes. Males have long been associated with sugar seeking and do not have the same demands of locating a host and oviposition site that female odor processing requires. Strong evolutionary pressure has shaped the olfactory system of female mosquitoes for these primary goals and their ability to detect and acquire sugar may be through generalized olfactory detection rather than a specific association between plant-derived chemicals and sugar. There are several known mosquito host attractants that are also emitted by plants including carbon dioxide, which is emitted by some flowering plants and relates very closely with nectar availability (Thom et al. 2004). Thus, the olfactory system and the mosquito's ability to associate odors with a resource might be

applicable to all resource types rather than restricted to sugar and plants, which also has potential implications for disease transmission (McCall and Kelly 2002). The ability of both sexes for conditioning also suggests that control mechanisms that can take advantage of repeated attractive visits, for example, those that draw mosquitoes away from hosts and pathogen reservoirs might be highly effective.

The significantly lower odds for the positive response of female mosquitoes are similar to those observed by Sanford and Tomberlin (2011). In the previous study, 3–5 day old colony derived females demonstrated memory for only 5 min after conditioning. It was suggested that selection for colony conditions and a well-defined gonotrophic cycle may have had an impact on the physiological state and receptivity of the females to sugar-feeding (Sanford and Tomberlin 2011). In this study, the mosquitoes were also aged 3–5 days and held on average 13 min until testing, which suggests that similar factors may have affected female receptivity to sugar and hence to conditioning.

Sucrose Preference Experiment

The results observed for male and female *Cx. quinquefasciatus* sucrose preference for 5 %, 10 % or 50 % sucrose after exposure to 10 % sucrose were analogous to our ability to condition male and female mosquitoes at these concentrations. When females were presented with a 5 % vs. 10 % sucrose concentration they preferred the 10 % sucrose concentration, which was the same response they presented when attempting to condition individual females to this concentration (sample size data presented in Table 1). Often females presented with a second or third conditioning trial of 5 % sucrose simply refused to feed by pulling the proboscis from the pipette despite repeated attempts to feed them. In the conditioning experiment, the probability of positive conditioned response was associated with the highest sugar reward with little observed difference between the 5 % and 10 % sucrose concentrations. However, mosquitoes offered a choice between 10 % and 50 % sucrose concentrations in a group setting did not choose to feed on one more than the other (Fig. 9a). The lack of mixed meals from these experimental groups suggests that sampling of the sucrose solutions, if it occurred, was rapid (Table 3).

In honeybees, restricted feeding on low sucrose concentration solutions lowered their sucrose response threshold compared to bees fed higher sucrose concentrations (concentrations: 10 %, 30 % and 50 % sucrose; Pankiw et al. 2001). Experience might have altered the bee's sucrose response threshold. In the current study, the mosquitoes had an equivalent response to a concentration higher than what they experienced prior and a lower response to a concentration lower than the one they had previously experienced. There is an inherent difficulty in comparing bees to mosquitoes because bees clearly sample multiple habitats and must remember their location and quality to report back to the hive and communicate with nest mates. It is not clear if mosquitoes sample multiple sugar sources and make decisions about feeding but it could be suggested that the mosquitoes had a similar response and were able to sample and distinguish between the low 5 % concentration and the higher 10 % concentration sucrose within the confines of the test cage. In host seeking females, defensive hosts present a potential problem that may be overcome by persistent attraction to host associated odors and perhaps through conditioning to those odors

for a short period of time, a time frame hypothetically similar to the one examined in the present study. Thus short-term conditioning may increase efficiency for acquiring a resource that is costly to obtain and potentially rare and randomly dispersed in the environment. In a small scale test of the mosquitoes' lower limit of sucrose concentration detection of sucrose (using a simple proboscis probing observation), the lower limit of response for this colony following 10 % sucrose concentration exposure was somewhere between 4.3 % and 8.6 % sucrose was determined (Sanford unpublished data). Thus, the 5 % sucrose solution was potentially near the lower end of their response limit. Observational studies of the mosquito sugar-feeding patch selection process might help to determine whether sampling occurs and at what sucrose concentration mosquitoes will stop and feed.

Conclusions

Many of the model organisms of olfactory learning behavior display more elaborate displays of communication than are attributed to mosquitoes (e.g. honeybees, bumble bees, wasps). Yet mosquitoes appear to be capable of olfactory conditioning and reward discrimination as suggested by the current study. Many technical questions remain with respect to mosquito learning and sugar-feeding behavior in the laboratory and whether it persists under field conditions. As suggested by the data presented here, the implications of odor selection can have unexpected effects on conditioning and the determination of the capabilities of this mosquito to individual odorants, behavioral responses to different molecular structures and odor blends have yet to be discovered. The ability of mosquitoes to learn about odors associated with sugar rewards may easily transfer over to their capabilities for learning about host-associated odors and, as suggested by McCall and Kelly (2002), if mosquitoes can learn about their hosts it may drastically change the way that we think about disease transmission.

References

Allan SA, Bernier UR, Kline DL (2006) Laboratory evaluation of avian odors for mosquito (Diptera: Culicidae) attraction. J Med Entomol 43:225–231

Alonso WJ, Schuck-Paim C (2006) The 'ghosts' that pester studies on learning in mosquitoes: guidelines to chase them off. Med Vet Entomol 20:157–165

Alonso WJ, Wyatt TD, Kelly DW (2003) Are vectors able to learn about their hosts? a case study with *Aedes aegypti* mosquitoes. Mem Inst Oswaldo Cruz 98:665–672

Awono-Ambene HP, Diawara L, Robert V (2001) Comparison of direct and membrane feeding methods to infect *Anopheles arabiensis* with *Plasmodium falciparum*. AmJTrop Med Hyg 64:32–34

Baker HG (1975) Sugar concentrations in nectars from hummingbird flowers. Biotropia 7:37–41

Beier J, Müller G, Gu W, Arheart K, Schlein Y (2012) Attractive toxic sugar bait (ATSB) methods decimate populations of Anopheles malaria vectors in arid environments regardless of the local availability of favoured sugar-source blossoms. Malar J 11:31

Bitterman ME, Menzel R, Fietz A, Schafer S (1983) Classical conditioning of proboscis extension reflex in honeybees (Apis mellifera). J Comp Psychol 97:107–119

Brantjes NBM, Leemans JAAM (1976) Silene otites (Caryophyllaceae) pollinated by nocturnal Lepidoptera and mosquitoes. Acta Biol Neerl 25:281–295

Charlwood JD, Graves PM, Marshall TFDC (1988) Evidence for a 'memorized' home range in Anopheles farauti females in Papua New Guinea. Med Vet Entomol 2:101–108

Chilaka N, Perkins E, Tripet F (2012) Visual and olfactory associative learning in the malaria vector Anopheles gambiae sensu stricto. Malar J 11:27

DeMaris A (1995) A tutorial in logistic regression. J Marriage Fam 57:956–968

Foster WA (1995) Mosquito sugar feeding and reproductive energetics. Annu Rev Entomol 40:443–474

Foster WA (2008) Phytochemicals as population sampling lures. J Am Mosq Control Assoc 24:138–146

Fradin MS, Day JF (2002) Comparative efficacy of insect repellents against mosquito bites. N Engl J Med 347:13–18

Gerberg EJ, Barnard DR, Ward RA (1994) Manual for mosquito rearing and experimental techniques. Am Mosq Control Assoc Bull 5:102

Gorham JR (1976) Orchid pollination by Aedes mosquitoes in Alaska. Am Midl Nat 95:208–210

Jhumur US, Dotterl S, Jurgens A (2006) Naive and conditioned responses of Culex pipiens pipiens biotype molestus (Diptera: Culicidae) to flower odors. J Med Entomol 43:1164–1170

Kevan PG, Tikhmenev EA, Usui M (1993) Insects and plants in the pollination ecology of the boreal zone. Ecol Res 8:247–267

Kim S, Choochote W, Jitpakdi A, Junkum A, Park S, Min G (2003) Establishment of a self-mating mosquito colony of Anopheles sinensis from Korea. Korean J Entomol 33:267–271

Knab F (1907) Mosquitoes as flower visitors. J N Y Entomol Soc 15:215–219

Laloi D, Sandoz JC, Picard-Nizou AL, Marchesi A, Pouvreau A, Tasei JN, Poppy G, Pham-Delegue MH (1999) Olfactory conditioning of the proboscis extension in bumble bees. Ent Exp Appl 90:123–129

Lindh JM, Terenius O, Eriksson-Gonzales K, Knolls BGJ, Faye I (2006) Re-introducing bacteria in mosquitoes—a method for determination of mosquito feeding preferences based on coloured sugar solutions. Acta Trop 99:173–183

Manda H, Gouagna LC, Nyandat E, Kabiru EW, Jackson RR, Foster WA, Githure JI, Beier JC, Hassanali A (2007) Discriminative feeding behaviour of Anopheles gambiae s.s. on endemic plants in western Kenya. Med Vet Entomol 21:103–111

McCall PJ, Kelly DW (2002) Learning and memory in disease vectors. Trends Parasitol 18:429–433

McCall PJ, Mosha FW, Njunwa KJ, Sherlock K (2001) Evidence for memorized site-fidelity in Anopheles arabiensis. Trans R Soc Trop Med Hyg 95:587–590

Molaei G, Andreadis TG, Armstrong PM, Bueno R Jr, Dennett JA, Real SV, Sargent C, Bala A, Randle Y, Guzman H, Travassos da Rosa A, Wuithiranyagool T, Tesh RB (2007) Host feeding pattern of Culex quinquefasciatus (Diptera: Culicidae) and its role in transmission of West Nile Virus in Harris County. Tex Am J Trop Med Hyg 77:73–81

Müller G, Schlein Y (2006) Sugar questing mosquitoes in arid areas gather on scarce blossoms that can be used for control. Int J Parasitol 36:1077–1080

Müller G, Beier J, Traore S, Toure M, Traore M, Bah S, Doumbia S, Schlein Y (2010a) Successful field trial of attractive toxic sugar bait (ATSB) plant-spraying methods against malaria vectors in the Anopheles gambiae complex in Mali, West Africa. Malar J 9:210

Müller GC, Junnila A, Qualls W, Revay EE, Kline DL, Allan S, Schlein Y, Xue RD (2010b) Control of Culex quinquefasciatus in a storm drain system in Florida using attractive toxic sugar baits. Med Vet Entomol 24:346–351

Müller GC, Xue R-D, Schlein Y (2011) Differential attraction of Aedes albopictus in the field to flowers, fruits and honeydew. Acta Trop 118:45–49

Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey K, Lanciotti RS (2001) West Nile Virus in overwintering Culex mosquitoes, New York City, 2000. Emerg Infect Dis 7:1–3

Pankiw T, Waddington KD, Page RE Jr (2001) Modulation of sucrose response thresholds in honey bees (Apis mellifera L.): influence of genotype, feeding, and foraging experience. J Comp Physiol A 187:293–301

Randich A, LoLordo VM (1979) Associative and nonassociative theories of the UCS preexposure phenomenon: implications for Pavlovian conditioning. Psychol Bull 86:523–548

Renshaw M, Service MW, Birley MH (1994) Host finding, feeding patterns and evidence for a memorized home range of the mosquito *Aedes cantans*. Med Vet Entomol 8:187–193

Ribbands CR (1949) Studies on the attractiveness of human populations to Anophelines. Bull Entomol Res 40:227–238

Sanford MR (2010) Associative learning capabilities of adult *Culex quinquefasciatus* Say and other mosquitoes. Ph.D., Texas A&M University College Station

Sanford MR, Tomberlin JK (2011) Conditioning individual mosquitoes to an odor: sex, source and time. PLoS One 6:e24218

SPSS, Inc. (2005) SPSS for windows, release 14.0.1 computer program, version 14.0. Chicago, IL, USA

SPSS, Inc. (2007) SPSS graduate pack 16.0 for windows computer program, version 16.0.1. Chicago, IL, USA

Thein LB (1969) Mosquito pollination of *Habenaria obtusata* (Orchidaceae). Am J Bot 56:232–237

Thom C, Guerenstein PG, Mechaber WL, Hildebrand JG (2004) Floral CO2 reveals flower profitability to moths. J Chem Ecol 30:1285–1288

Tomberlin JK, Rains GC, Allan SA, Sanford MR, Lewis WJ (2006) Associative learning of odor with food- or blood-meal by *Culex quinquefasciatus* Say (Diptera: Culicidae). Naturwissenschaften 93:551–556

Wäckers F, Bonifay C, Vet LEM, Lewis J (2006) Gustatory response and appetitive learning in *Microplitis croceipes* in relation to sugar type and concentration. Anim Biol 56:193–203