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ORIGINAL ARTICLE

Associative learning of odor with food- or blood-meal by *Culex quinquefasciatus* Say (Diptera: Culicidae)

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Abstract The ability of many insects to learn has been documented. However, a limited number of studies examining associative learning in medically important arthropods has been published. Investigations into the associative learning capabilities of *Culex quinquefasciatus* Say were conducted by adapting methods commonly used in experiments involving Hymenoptera. Male and female mosquitoes were able to learn a conditioned stimulus that consisted of an odor not normally encountered in nature (synthetic strawberry or vanilla extracts) in association with an unconditioned stimulus consisting of either a sugar (males and females) or blood (females) meal. Such information could lead to a better understanding of the ability of mosquitoes to locate and select host and food resources in nature.

Keywords Diptera · *Culex quinquefasciatus* · Associative learning

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Associative learning by insects has received significant attention and has been documented for a number of species (e.g., Lewis and Takasu 1990; Smith and Getz 1994; Gerber and Ullrich 1999; Daly et al. 2001). In many cases, insects are able to associate a conditioned stimulus, such as an odor with an unconditioned stimulus, such as a food or host resource (Lewis and Takasu 1990; Takasu and Lewis 1993, 1995; Daly and Smith 2000). Learning by the parasitic wasp *Microplitis croceipes* Cresson (Hymenoptera: Braconidae) occurs with stimuli other than odor, such as pattern, shape, and color (Wäckers and Lewis 1999). Visual associative learning in honey bees *Apis mellifera* Linnaeus (Hymenoptera: Apidae) also has been documented (e.g., Srinivasan 1994; Zhang et al. 1996; Horridge 1997). Behaviors linked to the expectation of a target resource (food or host) through associative learning for *M. croceipes* are resource specific and are not exhibited in association with other resources such as hosts (Olson et al. 2003). Therefore, these behaviors are an appropriate means for measuring the plasticity of their learning ability in association with a specific resource.

Studies examining associative learning in medically important arthropods are few. Gravid *Culex quinquefasciatus* Say (Diptera: Culicidae) preferred to oviposit in water containing compounds in which they were reared (McCall and Eaton 2001). However, whether emergent mosquitoes were imprinted with the compounds during development or learned in association with the larval habitat at the time of emergence is not known. Charlwood et al. (1988) demonstrated that mosquitoes remembered home range, while Mwandawiro et al. (2000) reported that certain mosquito species return to feed on specific ungulate species on which past blood-feeding was successful. In contrast, Alonso et al. (2003) were unable to record evidence that *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) learned olfactory or visual cues in association with hosts.

C. quinquefasciatus is an important vector with world-wide distribution. It is a significant vector of *Wuchereria bancrofti*, the causative agent of Bancroftian filariasis, in the Old and New World tropics (Foster and Walker 2002). *C. quinquefasciatus* is also an important vector of the West Nile virus (Turell et al. 2001) in North America.

Associative learning by medically important insects, such as mosquitoes, is not fully understood. Demonstrating that mosquitoes can associatively learn odors could lead to a better understanding of the ability of mosquitoes to locate and select host and food resources in nature. In this study, we examined the ability of *C. quinquefasciatus* to associate a conditioned stimulus (odor) with an unconditioned stimulus (blood or sugar meal) and respond with a context specific behavioral response (proboscis probing).

Materials and methods

Compounds examined Compounds used in this study were chosen due to their use in previously conducted associative learning experiments with *M. croceipes* (Lewis and Takasu 1990). Vanilla extract (Adams Extract, Gonzales, TX, USA) and imitation strawberry extract (McCormick and Co., Hunt Valley, MD, USA) were used in this study as target and nontarget odors.

Insect Our study was conducted from February through June 2005. *C. quinquefasciatus* was reared in a laboratory at the Center for Medical, Agricultural, and Veterinary Entomology, USDA-ARS, Gainesville, FL, USA (Gerberg et al. 1994). Adults were maintained in cages that were held at 27–29°C and 70–85% RH under a photoperiod of 16:8 h (L:D). Adults were provided with a 10% sugar solution. For our experiments, each week newly emergent adults were shipped overnight with a 10% sugar solution to the USDA-ARS laboratories in Tifton, GA, USA. Each shipment represented a cohort.

Mosquitoes were transferred to a 12.7 (*L*)×12.7 (*W*)×7.6 (*H*) cm Plexiglas® cage, provided only water, and starved for 24 h after arriving in Tifton, GA, USA to increase their search motivation (Wäckers and Lewis 1999). A total of 77 male mosquitoes from 12 cohorts were conditioned to vanilla and 83 mosquitoes from 13 cohorts were conditioned to strawberry in association with a sugar-meal, respectively. In regards to females, 49 mosquitoes from eight cohorts were conditioned to either vanilla or strawberry, respectively, in association with a blood-meal. The same number of female mosquitoes was conditioned to either odor in association with a sugar-meal. Sexes were not mixed during each experiment. Males were examined only with males and similarly with females. Voucher specimens were placed in the Georgia Museum of Natural History at the University of Georgia, Athens, GA, USA.

Conditioning of mosquitoes Mosquitoes were conditioned to an odor through either sugar- (male and female) or blood-meal (female) association. Sugar-meal association was defined as mosquitoes learning to recognize and respond to a target odor (conditioned stimulus) in association with a sugar-meal (unconditioned stimulus). Blood-meal association was defined as female mosquitoes learning to recognize and respond to a target odor (conditioned stimulus) in association with a blood-meal (unconditioned stimulus).

Mosquitoes were conditioned to a target odor in association with a sugar- or blood-meal using methods adapted from Olson et al. (2003). Mosquitoes were starved for 24 h before their use in an experiment. Individual 5-day-old mosquitoes were held in a sterile two dram shell glass vial (Bioquip® Products, Rancho Dominguez, CA, USA) placed on a single piece of white paper (Tejas Office Products, Houston, TX, USA) located under a chemical fume hood with constant ventilation. Individual mosquitoes were conditioned by tilting the glass vial approximately 30 to 45° to present the mosquito with the tip of a 200 µl calibrated glass pipette (Drummond Scientific Company, Broomall, PA, USA) containing a 10% sugar solution (sugar-meal) and with approximately one cm of the pipette tip coated with the target compound. The mosquito was allowed to feed on the sugar-meal (Fig. 1) for 10 s during three feeding intervals with each interval separated by a minimum of 30 s.

Methods previously described for conditioning mosquitoes to an odor in association with a sugar-meal were used to condition female mosquitoes to an odor in association with a blood-meal except the pipette contained defibrinated

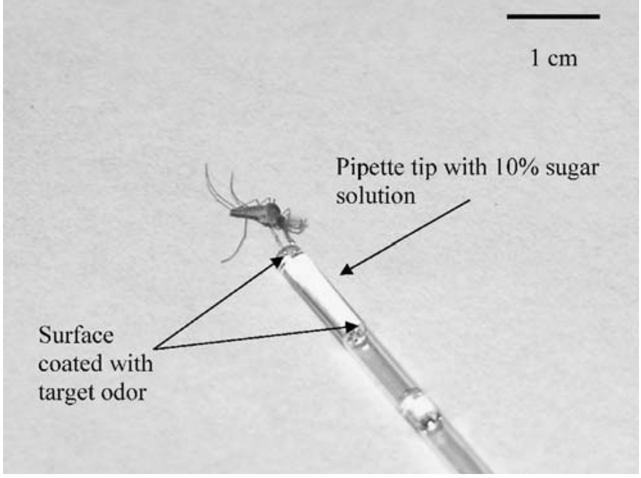


Fig. 1 Male *C. quinquefasciatus* mosquito feeding on sugar solution in pipette treated with target odor

bovine blood at room temperature instead of sugar water. Bovine blood was refrigerated <14 days before use. Individual female mosquitoes were provided a sugar-meal via a sterile pipette for 10 s and then allowed 30 s to acclimate in the two dram vial before being conditioned to an odor in association with a blood-meal.

Methods used to test mosquitoes were modified from the conditioning procedures. Instead of exposing the conditioned mosquito to a pipette possibly swabbed with an odor and containing a sugar solution, it was exposed to a sterile pipette (blank), a pipette swabbed with the nontarget odor, and then a pipette swabbed with the target odor, in this sequence. The time from training to testing was approximately 2 min. Total time to test one conditioned mosquito was less than 5 min. The pipette was placed approximately 1.5 cm directly in front of the proboscis of the mosquito. Glassware was used once. Used glassware was triple rinsed with acetone and placed in a convection oven at 55°C for 24 h to remove any remnants of odors that might be present.

Behavioral response to target and nontarget compounds A positive behavioral response by conditioned mosquitoes was defined as the mosquito walking toward the odor source and probing the outside or within the tip of the pipette with its proboscis within a 15-s period of exposure to the treatment. A negative response was defined as the conditioned mosquito moving away from the odor source or remaining stationary for more than 15 s while exposed to the treatment.

Statistical analysis A PROC GLM was used to analyze the data recorded for percentage of conditioned mosquitoes exhibiting a behavioral response to the blank, target, and alternate compound (SAS Institute 1998). Least significant difference (LSD) test was used after a significant F test ($P<0.05$) to separate means (SAS Institute 1998). The procedure was used to examine the response of conditioned mosquitoes within sex (i.e., male vs male) as well as across sex (i.e., male vs female) to the blank, target, and alternate compound. Data presented as percent of response were arcsine square root transformed for normalization before analysis (SAS Institute 1998).

Results

Based on analysis of the data with PROC GLM, adult *C. quinquefasciatus* females (F=99.00; df=5, 42; P<0.0001) and males (F=79.96; df=5, 70; P<0.0001) sugar-starved 24 h learned and recognized a conditioned stimulus odor in association with the unconditioned stimulus sugar-meal source (Fig. 2a,b). Additionally, our data indicate that *C. quinquefasciatus* females can learn odors in association with blood-meal (F=14.69; df=5, 42; P<0.0001) (Fig. 2c).

Both males and females, when trained to vanilla, exhibited a >90% response to vanilla and a <20% response to the nontarget odor strawberry. However, when the reverse experiment was conducted, males exhibited >90% response to the target (strawberry) and <60% response to the nontarget (vanilla), while the females exhibited a >90% response level to the target odor only. Significantly, more males responded to the nontarget vanilla than females (F=101.42; df=5, 59; P<0.0001).

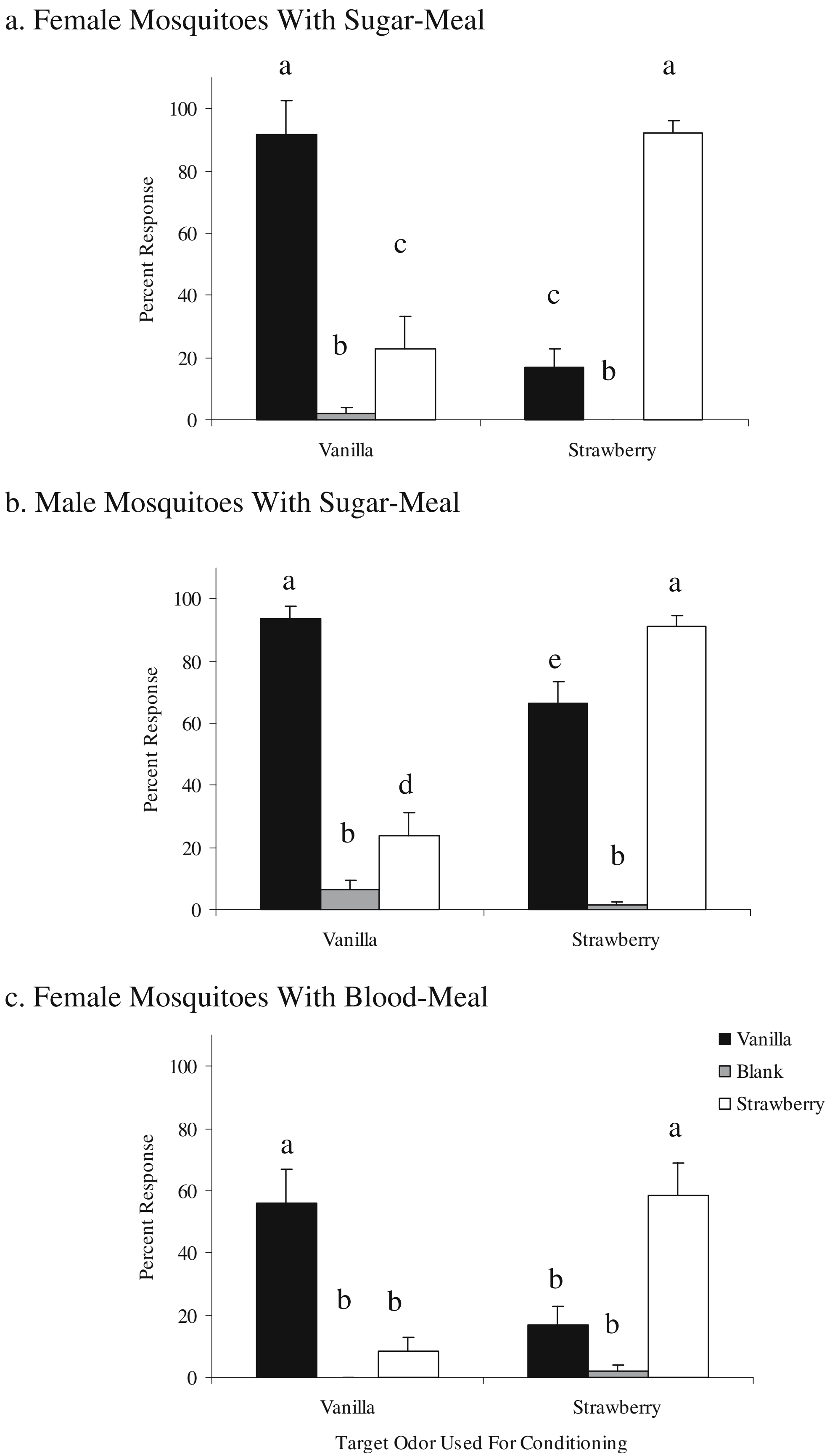
Discussion

Learning can be defined as a modification in behavior due to experience (McCall and Kelly 2002). Presently, limited information has been recorded illustrating the ability of mosquitoes to learn. We demonstrated that *C. quinquefasciatus* adults can learn and associate a conditioned stimulus, such as odor, with an unconditioned stimulus, such as food or blood resource (Fig. 2a–c). The associative learning demonstrated in this study was measured by a context-specific behavioral expression of proboscis probing around or within the tip of the pipette to the conditioned stimulus (odor). Additionally, the behavioral expression differences exhibited by conditioned mosquitoes were used to measure their ability to distinguish between the odor used as the conditioned stimulus and an arbitrary odor.

The ability of *C. quinquefasciatus* to learn was expected due to the number of published accounts demonstrating associative learning by other insect species. The parasitoid wasp *M. croceipes* (Lewis and Takasu 1990), sphingid moth *Manduca sexta* (Linnaeus) (Lepidoptera: Sphingidae) (Daly and Smith 2000) and honey bee (Bhagavan and Smith 1997) are three examples in which associative learning has been recorded. In each study, the investigators were able to identify and measure context-specific behaviors and demonstrate that the insect species being studied could learn a target odor in association with a food resource. Furthermore, once conditioned, the insect could distinguish the target odor from other nontarget odors.

Adult *C. quinquefasciatus* females and males sugar-starved 24 h learned and recognized a conditioned stimulus odor in association with the unconditioned stimulus sugar-meal source. Association of odor with food source would improve the foraging efficiency of newly emerged males and females when searching for a sugar source, such as floral nectar before locating mates or hosts (Smith and Gadawski 1994). Consequently, associative learning would be an adaptive trait that improves mosquito

Fig. 2 **a** Mean percent+SE female mosquitoes (n=8) responding to target and nontarget odors when conditioned with a sugar meal, **b** mean percent+SE male mosquitoes responding to target (vanilla as target odor, n=12; strawberry as target, n=13) and nontarget odors when conditioned with a sugar meal, and **c** mean percent+SE female mosquitoes (n=8) responding to target and nontarget odors when conditioned with a blood meal. Within each figure, *columns with different letters* are significantly different (LSD, P<0.05; analysis based on arcsine square root transformed data, SAS Institute 1998)



survival probability. Therefore, *C. quinquefasciatus* adults that are able to associatively learn and remember, much like the parasitoid wasp *M. croceipes* (Wäckers and Lewis 1999), are able to narrow their search to select targets and conserve energy and greatly enhance their resource-locating efficiency.

Prior studies have demonstrated that volatiles associated with bovine blood elicit flight and landing of *C. quinquefasciatus* females (Allan et al. 2006). The ability to learn and recognize odors associated with hosts has major implications for mosquito and associated pathogen control (McCall and Kelly 2002). Attempts to feed on hosts where interrupted or incomplete blood meals are taken would reduce the likelihood of pathogen transmission, ovary development, as well as result in greater selection for drug resistance by the pathogens (McCall and Kelly 2002). Therefore, learning and remembering hosts where successful meals were taken would be in the best interest of the mosquito.

We initially hypothesized that males and females would have similar abilities to recognize and distinguish target and nontarget odors in association with a sugar-meal. This assumption was correct when using vanilla as the target compound. However, when using strawberry as the target odor, males exhibited high response levels to both the target (strawberry) and nontarget (vanilla) compounds. Females responded primarily to the target odor only. Why significantly more males responded to the nontarget vanilla than females is unclear. However, we suspect that males either might be more or less sensitive to odor characteristics than females when associating with a food resource. In this study, vanilla is a pure extract, while the strawberry flavoring is a proprietary mixture of compounds that includes vanilla extract. In future studies, pure compounds should be used to test the previously mentioned hypothesis.

A clear understanding of the parameters being measured along with an understanding of responses in regards to conditioned and unconditioned stimuli is needed. While we demonstrated that *C. quinquefasciatus* can learn, others did not formulate the same conclusions with different mosquito species (Alonso et al. 2003). Alonso et al. (2003) used an olfactometer to examine the ability of *A. aegypti* mosquitoes to learn single odors (conditioned stimulus) in association with a resource (unconditioned stimulus). Based on their results, they were unable to support their hypothesis that *A. aegypti* is able to learn. Although we did not examine *A. aegypti*, we contend that the difference was not with their interpretation of the data, but that they were correct in suggesting that improper methods were employed. As part of their study, Alonso et al. (2003) used human breath as the unconditioned stimulus in association with an odor as the conditioned stimulus, which was either carvone or citral. Human breath may not be suitable as an unconditioned stimulus to elicit a truly Pavlovian response to a conditioned stimulus as it can vary in the qualitative and quantitative composition. Future studies are needed to (1) optimize the physiological state of the mosquitoes (i.e., hunger or in need of a blood-meal) for learning, and (2) determine a suitable unconditioned stimulus, such as a reward (i.e., sugar- or blood-meal).

Truly, conditioning mosquitoes only can be accomplished through reinforcement of the conditioned stimulus in association with the unconditioned stimulus. In the case of Alonso et al. (2003), two 70-min exposures may not be appropriate for eliciting associative learning in *A. aegypti*. The ability of *M. croceipes* to learn, recognize, and identify an odor increased significantly with reinforcements up to three repetitions (Tertuliano et al. 2004).

In conclusion, we demonstrated that male and female *C. quinquefasciatus* can learn an unconditioned stimulus in association with a conditioned stimulus. However, additional research examining the parameters that affect the ability of mosquitoes to learn is warranted. Deciphering the ability of mosquitoes to learn in association with host and food odors could result in novel methods for their control. Methods could be devised that manipulate host preference and thereby reduce the probability that mosquitoes feed on humans or certain animal hosts (McCall and Kelly 2002). Accordingly, by understanding and using the ability of mosquitoes to learn, developing systems that serve as alternate feeding sites that incorporate sterilization agents might be possible.

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