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Citronella is not a Repellent to Africanized Honey Bees *Apis mellifera* L. (Hymenoptera: Apidae)

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Citronela não é Repelente para Abelhas Africanizadas *Apis mellifera* L. (Hymenoptera: Apidae)

RESUMO – Experimentos foram realizados a fim de se investigar a repelência de citronela (*Cymbopogon winterianus* Jowitt) a abelhas africanizadas *Apis mellifera* (L.) (Hymenoptera: Apidae) no Brasil. Os resultados indicaram que citronela não foi repelente. As abelhas aprenderam facilmente a associação pavloviana entre citronela e aprendizagem. No segundo experimento a supressão condicionada foi usada para se avaliar o efeito da citronela sobre a liberação da probóscida a partir da aprendizagem e pelo estímulo utilizando sucrose. A resposta foi indistinguível quando se ofereceu às abelhas um odor diferente do que havia sido oferecido anteriormente. Os experimentos de laboratório foram confirmados em campo quando a citronela foi aplicada diretamente às abelhas que sobrevoavam as flores da área. As abelhas que receberam o odor de citronela permaneceram nas flores. O valor do potencial de avaliação do repelente usando a aprendizagem é discutido.

PALAVRAS-CHAVE – *Cymbopogon winterianus*, aprendizagem, condicionamento pavloviano

ABSTRACT – Experiments were performed investigating citronella (*Cymbopogon winterianus* Jowitt) as a repellent to Africanized honey bees *Apis mellifera* (L.) (Hymenoptera: Apidae) in Brazil. Results indicated that citronella is not a repellent. Bees exposed to a 100% concentration of citronella easily learned a Pavlovian association between citronella and feeding. In a second experiment, conditioned suppression was used to evaluate the effect of citronella on a proboscis extended by learning and by sucrose stimulation. Performance was indistinguishable from the application of a novel control odor. The laboratory experiments were confirmed in a field experiment in which citronella was applied directly to individuals foraging on a flower patch. Bees did not fly off flowers when the odor of citronella was applied directly to them relative to a control odor. The value of evaluating potential repellents using learning paradigms is discussed.

KEYWORDS – *Cymbopogon winterianus*, learning, Pavlovian conditioning

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In the course of an on-going investigation cataloging the learning of Africanized honey bees *Apis mellifera* (L.) (Hymenoptera: Apidae) (Abramson *et al.* 1999a, Abramson & Aquino 2002a, 2002b, Aquino *et al*. 2004), we came upon a Brazilian report suggesting that exposure to citronella odor repels Africanized honey bees (Malerbo-Souza & Nogueira-Couto 2004). Considerable effort has been directed at finding a honey bee repellent because of public safety issues (Abramson *et al*. 1997a), the possibility of providing researchers interested in the comparative analysis of behavior with another training stimulus to complement rewarding stimuli such as sucrose (Abramson 1994), and reducing the effects of harmful agrochemicals (Atkins, Jr. *et al.*1975a, 1975b; Mayer 1997).

There are several studies in the literature suggesting, for example, that *n*, octyl.acetate, benzyl acetate, iso-pentil-acetate, and 2-heptanone are repellent to honey bees (Blum *et al.*1978, Free 1987, Free *et al.* 1989). All these studies base their conclusions primarily on field tests. Malerbo-Souza & Nogueira-Couto (2004), for example, sprayed a diaper with citronella and observed a temporary decrease in the number of bees visiting a test site.

There is a fundamental limitation in a field test design because the potential effect of a repellent cannot be separated from a stimulus novelty effect (Harpaz & Lensky 1959, Atkins, Jr. *et al*. 1975a, 1975b). It has been known for sometime that honey bees learn to associate a floral scent with nectar and that this olfactory memory is quite persistent and can develop within a single pairing of scent and nectar (Menzel *et al*. 1993). It is entirely likely that the odor of the test repellent conflicts with the olfactory memory of the honey bee and provides data that looks like a repellent effect but is actually a stimulus novelty effect in which the new stimulus situation temporary confuses the honey bee.

An effect of novelty is readily seen in free-flying experiments in which honey bees are trained to fly to the laboratory on their own accord (Abramson *et al*. 1996). It is common practice in such experiments to first train an individual honey bee to land on an unscented gray target. When the animal visits the target on its own, the target is switched to those used in training. For example, when training a honey bee to discriminate two targets differing in odor, the single gray target is replaced with the two training targets. It is not uncommon for the honey bee to take 10 min or more to eventually land on one of the targets. When observing such a situation, are the new targets "repellent?" Most likely, they are not. What is producing the change in the animal's behavior is that it is confronted with a novel stimulus situation.

Our belief in the fundamental limitation of field tests when applied to such an intelligent insect as the honey bee gave us the opportunity to determine whether the Pavlovian conditioning of the proboscis extension reflex can be used to screen for potential repellents. Originally developed by Kuwabara (1957), proboscis extensions are elicited by olfactory stimuli signaling food. Pavlovian conditioning of proboscis extension is used to study a variety of phenomena in honey bees including exposure to pesticides and to catalog learning (Decourtye & Pham-Delègue 2002; Decourtye *et al.* 2005). We have used the proboscis conditioning paradigm in Brazil for a number of years to catalog learning (Abramson *et al.* 1997b), assess the impact of pesticides (Abramson *et al.* 1999b, Abramson *et al*. 2006), and as a bioassay to screen for adulterated honey (Silva *et al.* 2001).

In this report, the ability of citronella odor to support Pavlovian learning in harnessed foragers was tested. In addition, a "conditioned suppression" paradigm was utilized to determine whether proboscis extension could be suppressed by exposure to citronella odor. In the final experiment a field test was conducted in which the citronella odor was directly sprayed onto foraging honey bees in the field. We believe this approach can serve as a model for testing repellents in honey bees because of the greater control of training variables and better experimental designs.

Materials and Methods

Harnessing and Training. The methods used were identical to our previous work in Brazil (Abramson 1990). Foraging honey bees (*Apis mellifera* L.) were captured from laboratory hives in glass vials, placed in an ice water bath, and while unconscious harnessed in metal tubes constructed from .32 caliber shells. Upon regaining consciousness, bees were fed 1.8 M sucrose until satiated and set aside for use approximately 24 h later.

Citronella was collected and turned into an essential oil in the laboratory using a steam distillation process. It was applied neat (approximately 3 µl) each day at 100% concentration on a 1 cm^2 piece of filter paper (Whatman n. 4) attached to a 20 ml plastic syringe to create an odor cartridge. To apply the odor to a honey bee, the plunger of the syringe was pulled back to the 20 ml mark and depressed. This method, although not automated, is highly effective and inexpensive. In a study directly comparing this method with an automated proboscis conditioning situation, no significant differences in conditioning were detected (Abramson & Boyd 2001). It must also be noted that in rural areas of Brazil such as in the state of Paraíba where these experiments were conducted, automation is often difficult to obtain and not practical. Experiments were conducted during the months of June, July, and August of 2005, which is considered the winter or "rainy season" in the northeast of Brazil. Animals from all experiments were run simultaneously to control for calendar variables and fluctuating hive conditions.

The odors of cinnamon (Gilbertie's, Easton, CT) and fennel (*Foeniculum vulgare*) were used in addition to cinnamon. The cinnamon odor and fennel odor cartridges were prepared in the same way as the citronella cartridge. Cinnamon was used to provide a training odor that has been shown to be effective in our previous honey bee experiments (Abramson *et al.* 2004). Without including such a training stimulus, it would be difficult to interpret the results of the experiments if exposure to citronella retarded learning. The odors of cinnamon and of citronella were used as conditioned stimuli (CS). The unconditioned stimulus (US) was a 1 µl droplet of 1.8 M sucrose solution applied with a Hamilton microsyringe. Fennel odor was included to provide a novel olfactory stimulus for the conditioned suppression and field studies described in Experiments 3 and 4. It was steamed distilled in the laboratory and was not diluted.

Pavlovian Conditioning. To assess whether the odor of citronella could support Pavlovian conditioning, 60 honey bees were selected from a group of approximately 150 harnessed the previous day. All animals were given a pretest 10 min before the experiment began to ensure that motivation to feed was high. The test involved stimulating an antenna with 1.8 M sucrose and if the proboscis vigorously extended, the animal was used.

The 60 animals were randomly divided into 3 groups of 20 bees each. Group 1 received 12 paired presentations of a citronella CS with a 1 µl droplet of 1.8 M. sucrose US. The CS duration was 2 s and the US duration approximately 1 s (the time needed to consume the US). The time between the end of the US and the next CS (known in the conditioning literature as the intertrial interval or ITI) was 10 min. Following the 12 paired trials, each animal received 12 additional trials in which the US was omitted. The rationale behind using these "extinction" trials was to determine whether the repellent influenced not only the acquisition of a learned response but also its persistence when the US was no longer presented. The ITI was again 10 min. Group 2 was treated exactly as the citronella group with the exception that the CS was the odor of cinnamon.

A conditioning trial began by picking up a bee and placing it in front of a ventilation fan. The rationale behind the use of a fan was to remove training scents from the experimental area. Several seconds after being placed in front of the fan, the appropriate stimulus was introduced. After application of the stimuli, the animal was returned to a holding area and a second animal was run. A trace conditioning procedure was used where the CS was presented first followed by the US. The CS and US presentations did not overlap. If the animal extended its proboscis during the CS but before the US a "1" was recorded. If the proboscis did not extend to the CS a "0" was recorded. Responses were recorded visually.

To ensure that any learning observed in the paired citronella group was actually the result of CS-US pairings and not sensitization, animals in Group 3 received explicitly unpaired citronella/sucrose pairings. Three successive sequences of ABBABAAB were used where A was the CS and B the US. The durations of the CS and US were the same as those in Groups 1 and 2 however, the ITI was 5 min rather than 10. The reason for the change was that the pseudorandom sequence ensures that the time between CS presentations is approximately 10 min – the same ITI used in the paired group. If a 10 min ITI was used for the unpaired animals, the time between CS presentations would be 20 min and any experimental-control differences could readily be accounted for by differences in ITI. There was not an unpaired control group for animals that received cinnamon because we have employed such a group in previous research and showed that animals learned to associate the odor of cinnamon with a sucrose feeding (Abramson *et al.* 2004).

Proboscis Extension Suppression. To determine whether the odor of citronella would suppress an extended proboscis when the proboscis was extended by learning or reflex stimulation, a variation of the conditioned suppression technique originally developed by Estes & Skinner (1941) was utilized. To estimate the impact of emotional responses produced by classical conditioning on behavior controlled by its consequences, we wished to determine whether exposure to the odor of citronella would suppress an already extended proboscis. Previous research we have conducted over a number of years has repeatedly shown that honey bees readily learn to retract their proboscis while drinking high molarity sucrose solutions in response to aversive events (Abramson 1986, Abramson & Bitterman 1986a, 1986b, Smith *et al*. 1991). A preliminary experiment was conducted on 10 animals trained to discriminate cinnamon (CS+) from citronella (CS-). Following training, each animal received a single test trial in which proboscis extension was elicited by cinnamon and while extended, the CS- odor was presented. Five of 10 animals retracted their proboscis during presentation of the CS- odor. Our previous research and the results of this preliminary experiment suggest that the conditioned suppression procedure is sensitive enough to detect repellent effects.

Two groups of 20 animals were used. Those in Group 1 were harnessed and maintained as in the previous experiments. Group 1 assessed whether exposure to citronella would lead to a retraction of the proboscis when the proboscis was elicited by a reflex. To elicit proboscis extension, an antenna was stimulated for 1 s by touching it with a Hamilton microsyringe containing 1.8 M sucrose. When the proboscis extended, the animal was allowed to drink a 1 µl droplet. This continued for 5 trials. Following the 5th trial, animals received eight test trials, 4 with the odor of citronella and 4 with the odor of fennel. The duration of citronella and fennel odors was 2 s.

Fennel was included as a control stimulus to provide an assessment of proboscis contraction to a novel stimulus. If such a control stimulus was not included it would be impossible to determine whether any contraction observed to citronella was the result of a repellent effect or the result of novelty. A test trial began by placing an animal in front of the exhaust fan, the proboscis reflex elicited by application of sucrose to the antenna, and with the proboscis extended, applying one of the two test odors. The presentations of citronella and fennel was pseudorandom following the order ABBABAAB with A being citronella and B fennel. The ITI both during the 5 sucrose only trials and the 8 test trials was 10 min. The animals were not allowed to feed during any of the 8 test trials.

The selection process for animals in Group 2 was more complicated. All of our previous research on Africanized honey bees in Brazil has consistently shown lower levels of learning than is typically reported with European honey bees (Abramson & Aquino 2002a). Therefore, to get a sample of 20 bees, we used the superior learners in Experiment 1 ($n = 8$) and trained an additional population of 40 bees. The bees used from Experiment 1 were re-conditioned following the extinction phase. By using some of the bees in Experiment 1 combined with the superior learners in the new population of 40 bees ($n = 12$), we were able to acquire a sample of 20 bees that always responded to the CS of cinnamon odor.

The basic experimental design for Group 2 animals was conceptually similar to those of Group 1. Prior to receiving 8 test trials, all animals received 5 CS-US trials with cinnamon odor as the CS and a 1 µl droplet of 1.8 M sucrose as the US. The duration of the CS was 2 s during these 5 training trials. Animals were allowed to feed on the US droplet; this was why animals in Group 1 were permitted to feed on the 5 sucrose-only trials prior to receiving their 8 test trials. We needed to equate the effect of sucrose stimulation in the two groups prior to receiving the subsequent test trials.

A test trial began by presenting the CS odor for 2 s and with the proboscis extended, applying either the odor of citronella or fennel based on the same pseudorandom schedule used for animals in Group 1. The ITI was 10 min. The durations of the citronella and fennel test stimuli were 2 s – the same duration used in Group 1.

Field Study. To provide a complete evaluation of citronella as a repellent, a field test was conducted to examine whether the odor of citronella repelled bees from flower petals and while drinking nectar. The field test utilized differed from the more traditional approach of placing a potential repellent in some type of container and assessing its effect on a group of bees in that the suspected repellent was applied directly to individual bees. Moreover, controls were employed to rule out the effect of novelty *per se.*

Thirty-two bees foraging near the laboratory on several patches of sulphur cosmos (*Cosmos sulphureus,* Asteraceae) were studied. When a bee landed on a flower it received a 2 s presentation of either the odor of citronella or cinnamon. The odors were applied within 4 cm of a bee and directed at the head. In some cases, the bee was on a petal and in others it was feeding on nectar. The dependent variable was whether the behavior of the honey bee was disrupted in response to the two odors. Disruption was defined as flying off the flower, although any behavior of interest was noted. Each animal received a minimum of two stimulus presentations (one each of citronella and cinnamon) and most received at least 4 stimulus presentations. Individual bees were marked with different colors so that each bee could be tracked and multiple observations were obtained from each bee. Sixteen of the bees were first presented with the odor of citronella and the remaining bees with the odor of cinnamon. The experiment on an individual bee was terminated when it returned to the hive and the same bee was not used on successive visits.

Data Analysis. SPSS for Windows (2002) was utilized to perform analyses for all experiments. For the Pavlovian conditioning experiment, the General Linear Model (GLM) for Univariate Analyses of Variance was utilized and post hoc analyses were conducted using Tukey's HSD test. For the Proboscis conditioning experiment, within-groups responses were analyzed using the GLM for Repeated Measures, and for between groups responses, the GLM for Univariate Analyses of Variance was employed. Finally, for the field test, responses were analyzed using the GLM for Repeated Measures. Raw data were transformed into mean number of responses across trials for all experiments except the Conditioned Suppression experiment, in which each trial was also tested separately. Alpha was set at 0.05 for all experiments, unless heterogeneity of variances was present, in which case α was set at 0.01.

Results and Discussion

The results of these experiments show that exposure to the odor of citronella was not repellent to Africanized honey bees in the northeast of Brazil. Harnessed honey bees readily learned to associate the odor of citronella with a sucrose solution. Moreover, in a conditioned suppression experiment, an extended proboscis did not contract in response to citronella odor. It did not make a difference whether the proboscis extended because of a prior learned association or by direct contact with sucrose. Finally, a field test in which citronella odor was directly applied to honey bees failed to disrupt their behavior.

Fig. 1 shows the performance of paired and unpaired animals (see Table 1 for group means and standard deviations) that received a CS of citronella and cinnamon in both acquisition and extinction. The response to the unconditioned stimulus in animals that received a CS of citronella is also shown. Consistent with all other proboscis conditioning experiments with honey bees, performance improved as the number of training trials increased during acquisition and decreased during extinction. Analysis confirmed significant differences between groups ($F = 16.05$, df = 2, 56, $P < 0.001$, $\eta = 0.36$). Tukey's HSD post hoc revealed that animals in the paired groups did not differ from each other but responded significantly more often than did those in the unpaired group. It is also interesting to note that, once again, asymptotic performance in this sample of Africanized honey bees was lower than that seen in European honey bees where asymptotic performance may reach 90% (Abramson & Boyd 2001).

Note the pattern of consistent responding to the US in animals that received the citronella CS. If citronella was a repellent it should be expected that exposure to the odor would disrupt subsequent feeding. Clearly this was not the case. Statistical analysis revealed no differences in feeding responses in paired or unpaired animals that received citronella, nor between paired animals that received a CS or cinnamon and those that received a CS or citronella ($F = 2.96$, df = 2, 56, $P > 0.05$, $\eta = 0.10$).

Table 1. Mean responses in harnessed bees (*Apis mellifera*), Pavlovian conditioning.

Group	Response type	Mean	Standard deviation
citronella, paired	CS	0.50	0.34
cinnamon, paired		0.51	0.35
citronella, unpaired		0.06	0.12
citronella, paired	US	0.99	0.03
cinnamon, paired		1.00	0.00
citronella, unpaired		0.90	0.23

[a] *n* = 59, twenty subjects per group (one subject in the paired cinnamon group died and did not complete the experiment).

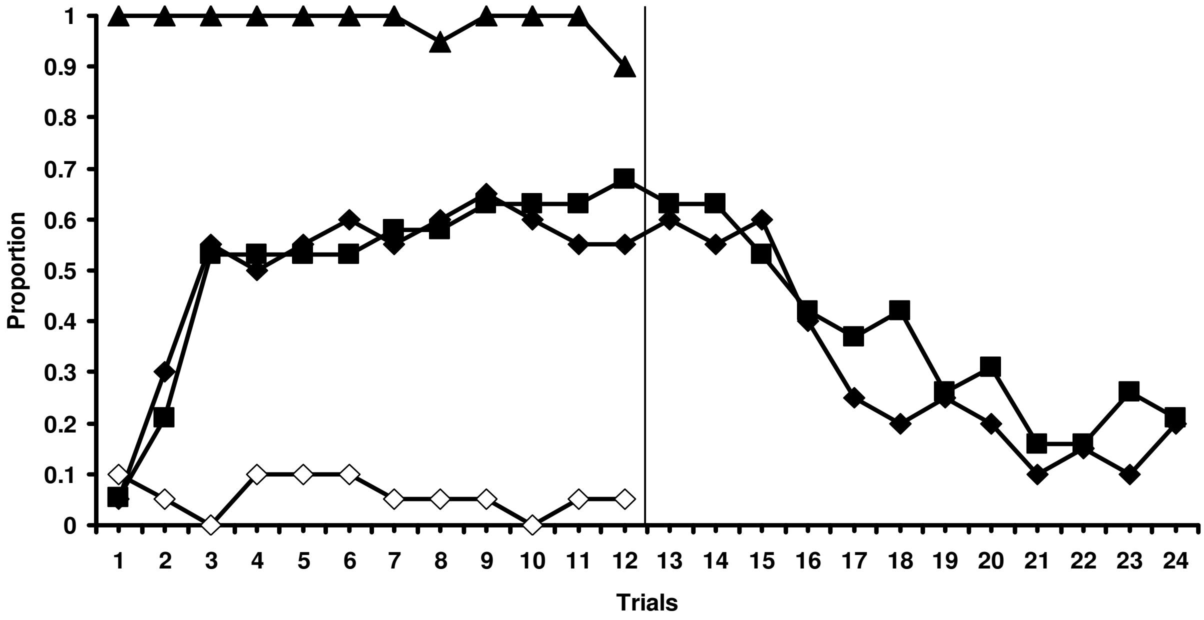

Figure 1. Proportion of honey bees (*Apis mellifera*) receiving paired presentations of citronella or cinnamon odor with sucrose during acquisition. The unconditioned responses to sucrose in citronella animals are shown as is the performance of animals receiving unpaired presentations of citronella and sucrose. Extinction begins on trial 13. Filled diamond – citronella paired; open diamond – citronella unpaired; filled square – cinnamon paired; filled triangle – citronella US response

Fig. 2 shows the conditioned suppression results. When citronella was applied to an extended proboscis, the proboscis did not retract compared to a control odor of fennel ($F = 0.32$, df = 1, 19, $P > 0.05$, $\eta = 0.02$). The effect of applying citronella was negligible. There was no effect across subsequent exposures ($F = 0.704$, df = 1, 19, $P > 0.05$, $\eta = 0.04$) and there were no significant differences between groups whether proboscis extension was elicited by learning or by sucrose stimulation ($F = 0.06$, df = 1, 38, $P > 0.05$, $\eta = 0.02$; M = 0.90, SD = 0.17 for citronella group, M = 0.89, SD = 0.15 for reflex group, $n = 20$ per group).

The results of the conditioned suppression experiments were confirmed by the field test. Of 65 total applications of citronella applied to honey bees feeding on the nectar of sulphur cosmos only 11 flew away. This compares favorably with the 62 total applications of cinnamon odor applied to the same 32 bees where only 10 flew away. ($F = 0.20$, df = 1, 31, $P > 0.05$, $\eta = 0.06$; M = 1.69, SD = 1.12, M = 1.63, SD = 1.24 for the citronella and cinnamon groups respectively, $n = 32$).

The data on the application of citronella and cinnamon to honey bees on the petals of sulphur cosmos supports the feeding results. Of 19 applications of citronella odor only 1 bee flew off a petal. This compares favorably with the 21 applications of cinnamon odor where only 3 bees flew off a petal. Statistical analysis revealed no statistical differences between groups ($F = 0$, df = 1, 31, $P > 0.05$, $\eta = 0$; M = 0.56, SD = 0.88 for both groups, $n = 32$.

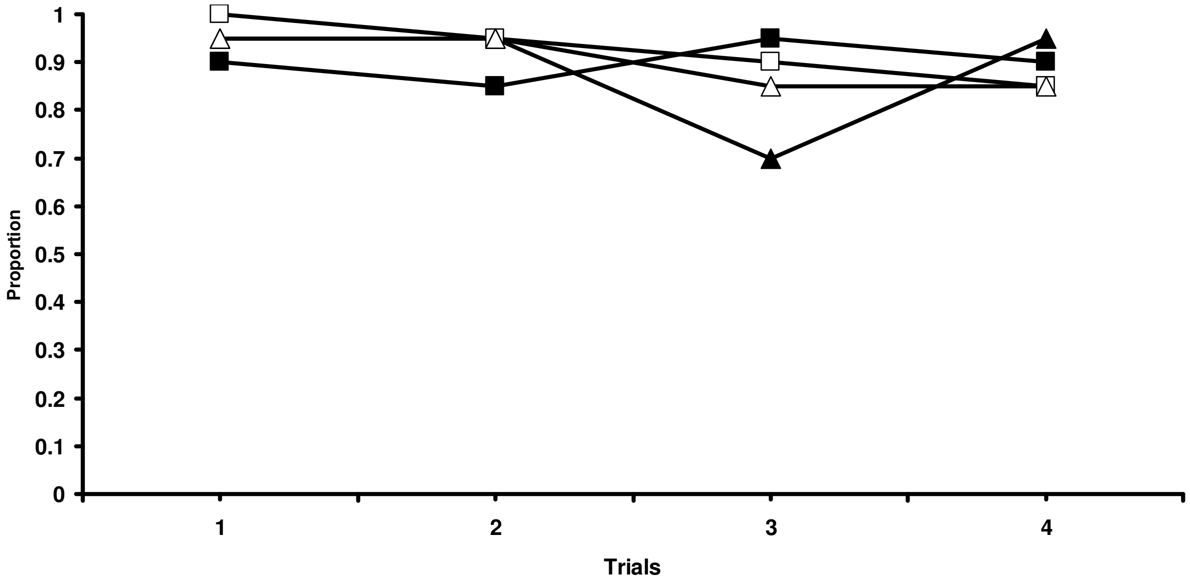

Figure 2. Proportion of honey bees (*Apis mellifera*) withdrawing their extended proboscis. The proboscis was extended by either a prior learned association with cinnamon odor or reflexively by stimulating the antennae with sucrose. Filled square – citronella L; open square – fennel CL; filled triangle – citronella R; open triangle – fennel CR.

In addition to showing that citronella was not repellent to Africanized honey bees, these results support the use of learning in harnessed honey bees to study potential repellents and that the conditioned suppression and individual bee field test employed can serve as a new model for the assessment of bee repellents. Moreover, because the animals are harnessed, biochemical and physiological manipulations using a potential repellent can now be performed and the precise manipulation of training variables can be under experimenter control. The incorporation of laboratory based learning paradigms also allows researchers to separate the effect of novelty on performance from real repellent effects.

We would also like to note that this field test method permits greater experimental control over present methods in which the suspected repellent is sprayed on flowers or flower substitutes such as a diaper. The suspected repellent can be accurately applied to any part of the bee and in controlled amounts.

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