ECOLOGY, BEHAVIOR AND BIONOMICS

The Effect of Essential Oils of Sweet Fennel and Pignut on Mortality and Learning in Africanized Honeybees (*Apis mellifera* L.) (Hymenoptera: Apidae)

Charles I. Abramson[1, 2], Paulo A. Wanderley[3], Maria J.A. Wanderley[3], José C.R. Silva[3] and Lynnette M. Michaluk[1]

[1]*Lab. Comparative Psychology and Behavioral Biology, Depts. of Psychology and Zoology, Oklahoma State University 115 North Murray, Stillwater, OK 74078, USA*
[2]*Correspondent author, charles.abramson@okstate.edu*
[3]*Depto Agricultura, Univ. Federal da Paraiba, 58220-000, Bananeiras, PB, Brazil*

Neotropical Entomology 36(6):828-835 (2007)

Efeito de Óleos Essenciais de Erva-Doce e Alfazema sobre a Mortalidade e Aprendizagem de Abelhas Africanizadas (*Apis mellifera* L.) (Hymenoptera: Apidae)

RESUMO - Recentemente foi descoberto que pequenas concentrações de óleos essenciais de erva-doce (*Foeniculum vulgare* Mill) ou alfazema [*Hyptis suaveolens* (L.) Poit] podem ser usadas para controlar pulgões. O que não se sabe é se esses óleos também podem influenciar o comportamento de abelhas melíferas. Experimentos utilizando abelhas encapsuladas ou livres, em diferentes concentrações usadas para controlar pulgões, mostraram que as abelhas rapidamente associaram o odor a um estímulo aprendendo a discriminá-lo, e não foram repelidas pelo mesmo. No entanto, as abelhas melíferas não consumiriam os óleos quando misturados à sacarose para criar um estímulo incondicional. Em um experimento, em que abelhas encapsuladas foram submetidas a várias concentrações dos óleos essenciais, concentrações maiores que 50% foram prejudiciais às abelhas. Os experimentos relatados aqui reforçam a validade do uso de técnicas de condicionamento para avaliar a ação de óleos essenciais no comportamento das abelhas melíferas.

PALAVRAS-CHAVE: Repelente, encapsulada, aprendizado, comportamento

ABSTRACT - It was recently discovered that exposure to small concentrations of the essential oils of sweet fennel (*Foeniculum vulgare* Mill) or pignut [*Hyptis suaveolens* (L.) Poit] can be used to control aphids. What is not known is whether these oils also influence honeybee behavior. Experiments using both harnessed and free-flying foragers at concentrations used to control aphids showed that bees readily associated the odors with a reward, discriminated between them, and were not repelled. Honeybees, however, would not consume the oils when mixed with sucrose to create an unconditioned stimulus. An experiment in which harnessed bees consumed various concentrations showed that concentrations greater than 50% were detrimental. The experiments reported here provide further evidence supporting the use of conditioning techniques to evaluate the use of essential oils on honey bee behavior.

KEY WORDS: Repellent, free-flying, behavior

Honeybees naturally visit plants to gather nectar. Among these plants are the sweet fennel, known in Brazil as "erva-doce" (*Foeniculum vulgare* Mill) and the pignut, known in Brazil as "alfazema" (*Hyptis suaveolens* (L.) Poit).

Sweet fennel is recognized internationally for its medicinal value in treating some respiratory diseases (Torres 2004). It is also used in Brazil to season typical foods in the northeast and is used to protect fruits against post-harvest pathogenic fungi. Sweet fennel is also used by the cosmetic industry because of its soft and pleasant smell. It can be found in a variety of products ranging from shampoos, to soaps, deodorants, and lotions. It has also become popular to use sweet fennel in perfumed candles. As a result of heavy commercial use, sweet fennel is becoming an important cash crop in the northeast of Brazil.

The main constituents of sweet fennel essential oils are (*E*)-anethole (72.27% - 74.18%), fenchone (11.32% - 16.35%) and methyl chavicol (3.78% - 5.29%) (Mimica-Dokič *et al.* 2003). The method of distillation used by these authors significantly affected the essential oil yield and quantitative composition, although the antifungal activity of the oils was only slightly altered.

Pignut is a sylvan plant and is considered a weed (Severino & Christoffoleti 2001). However, it is aromatic and has been used for honey production (Lorenzon *et al*. 2003). In addition, it has been shown that the essential oil of pignut has antibacterial activities that inhibit development of certain fungi and bacteria (Okonogi *et al.* 2005). Its main constituents are sabinene, 1,8-cineole, spathulenol, (*E*)-caryophyllene and biclyclogermacrene (Campos *et al.* 2002). Because pignut is considered a weed, it is easy to obtain in large quantities and therefore the cost of extracting its essential oil is low (Lorenzon *et al.* 2003).

Recently, research from the Laboratório de Entomologia at the Universidade Federal da Paraiba, Bananeiras, Brazil, showed that the essential oils of sweet fennel and pignut can be used to control pests and diseases of crops (Malele *et al.* 2003, Mimica-Dokič *et al.* 2003, Abramson *et al.* 2006a). In addition, they produce nectar and pollen that can be collected by honeybees. The purpose of the present experiments was to determine whether exposure to various concentrations of sweet fennel and pignut was acutely toxic to honeybees; whether the amount of the oils used as botanical insecticides was repellent to bees; and finally, whether the oils affected their learning ability.

Material and Methods

The methods utilized were identical to our previous work in Brazil (Abramson & Aquino 2002). Foraging honeybees (*Apis mellifera* L.) were captured from laboratory hives in glass vials, placed in an ice water bath, and while unconscious they were harnessed in metal tubes constructed from .32 caliber shells. Upon regaining consciousness, the bees were fed 1.8 M sucrose until satiated and set aside for use approximately 24h later. For a detailed description on harnessing, see Abramson (1990).

Citronella (*Cymbopogon winterianus* Jowitt) and sweet fennel (*Foeniculum vulgare* Mill.) were collected and turned into essential oils in the laboratory using a steam distillation process. In addition to these essential oils, cinnamon extract was used and provided by a commercial supplier (Gilbertie's, Easton, CT, U.S.A.). Each day, approximately 3 µl dropled of oil was applied to a 1-cm^2 piece of filter paper (Whatman no. 4) and attached to a 20 ml plastic syringe to create an odor cartridge. To apply the odor to a honeybee, 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 were detected (Abramson & Boyd 2001). Experiments were conducted during the months of June, July, and August of 2005. These months constitute the winter or "rainy season" in the northeast of Brazil. To control for calendar variables and fluctuating hive conditions, insects from all experiments were run simultaneously. All learning experiments employed unpaired control groups or the use of discriminative stimuli to control for non-associative effects.

The odor of cinnamon (commercially supplied by Gilbertie's, Easton, CT) and sweet fennel (*Foeniculum vulgare* Mill.) were used in addition to citronella. Cinnamon was used to provide a training odor that was shown effective in previous honeybee experiments (Abramson *et al.* 2004). Without including such a training stimulus, it would be difficult to interpret the results of our experiments if exposure to the odor of sweet fennel or pignut retarded learning. The odors of cinnamon, citronella, and sweet fennel 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. Cinnamon odor was included to provide a novel olfactory stimulus for the conditioned suppression and field studies described in Experiments 4 and 5.

A conditioning trial began by picking up a bee and placing it in front of a ventilation fan. Several seconds after being placed in front of the fan, the appropriate stimuli were introduced. After application of the stimuli, the insect was returned to a holding area and a second insect 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. The CS duration was 2 s and the US duration approximately 1 s (the time needed to consume a 1 µl droplet). If the insect 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.

The free-flying technique was used by first establishing a feeder containing 8% sucrose. Foraging bees were attracted to the feeder and an individual bee was captured in a matchbox, placed on a gray target constructed from a 5.5 cm diameter disposable petri dish and marked with nail polish while feeding on a 1.8 M sucrose droplet (0.6 ml). If the bee did not return to the target on its own, it was recaptured and returned to the gray target. When the bee returned to the gray target twice on its own accord, the gray target was replaced with the two odor targets used in training. The odor targets were also gray but had 0.5-cm holes equally spaced around the circumference of the dish. A cotton ball with 20 µl of an odor served as discriminative stimulus. In these experiments, the odors of pignut (6.25%) and sweet fennel (3.125%, diluted in 1% neutral detergent, Friboi Ltda., Luziania, GO), served as the discriminative stimuli and 1.8 M sucrose served as reward. For details on the free-flying procedure and how it is used with Africanized honeybees, see Abramson (1990) and Abramson *et al.* (1997).

Experiment 1: Mortality. In this experiment, we investigated whether consuming various concentrations of sweet fennel and pignut led to death within 240 min (4h). This time-frame was selected because we were interested in looking for acute toxic effects rather than the effects of long term exposure. Four hundred eighty bees were randomly divided into two major groups of 240 bees each. Group one was fed on sweet fennel and group two on pignut. The 240 bees within each major group were further divided into eight groups of 30 insects each. Each of these groups differed in concentration (0% - water only, 1% detergent, 3.125%, 6.25%, 12.5%, 25%, 50%, and 100% of either sweet fennel or pignut). The sweet fennel and pignut were diluted in unscented detergent (Minuano, Friboi Ltda., Luziania, GO). To get the bees to consume the essential oils, their antennae was stimulated with sucrose, and with the proboscis now extended, allowed to feed on the solutions.

We also wished to determine whether sweet fennel and pignut applied to the abdomens of honeybees would give results that differed from the feeding tests. An additional 60 bees were captured and harnessed. The abdomens of 30 bees were treated with a 1 µl droplet of 100% sweet fennel and the remaining 30 bees treated with a 1 µl droplet of 100% pignut. As a control for the effect of consuming the detergent *per se* on mortality another group of 60 bees (30 in the sweet fennel group and 30 in the pignut group) consumed a 1 µl droplet of diluted detergent. To get bees in this group to feed on the detergent, the same process was followed for bees that consumed essential oils. Once the essential oils or control treatments were consumed or applied to the abdomen, all insects were observed for mortality over the course of 11 time intervals totaling 4 h (5, 10, 15, 20, 25, 30, 45, 60, 120, 180, and 240 min).

Experiment 2: Simple classical conditioning. In the second experiment we investigated whether bees could associate the odor of citronella with a feeding of either sweet fennel or pignut in a simple Pavlovian experiment. Twenty-four bees were selected from a group of approximately 100 harnessed the previous day. All insects 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 insect was used.

The 24 insects were randomly divided into two groups consisting of 12 bees each. Group 1 received 12 paired presentations of a citronella CS with a 1 µl droplet of 3.125% sweet fennel diluted in 1.8 M. sucrose. The remaining 12 insects comprised Group 2 and received 12 paired presentations of citronella with a 1 µl droplet of 3.125% pignut diluted in 1.8 M. sucrose. The CS duration was 2 s and the US duration approximately 1 s. The time between the end of the US and the next CS (technically known in the conditioning literature as the intertrial interval or ITI) was 10 min.

It is customary in our research on learning in Africanized bees to include unpaired control groups and a minimum of 18 subjects per group. We did not do so here because it was obvious that insects were not feeding consistently on either essential oil following the first training trial and therefore the experiment was terminated (see results).

Experiment 3: Discriminative stimuli. In this experiment we investigated whether the odor of these essential oils could serve as a cue for harnessed honeybees in a complex learning task as represented by the ability of honeybees to discriminate between two conditioned stimuli – one of which was paired with a US and the other which was not. Forty bees were randomly divided into two groups of 20 each. One group received a CS+ of 3.125% sweet fennel odor (diluted with detergent) paired with a feeding of 1.8 M sucrose and a CS- of 3.125% pignut odor (diluted with detergent) that was not followed by a feeding. For the remaining 20 insects, the CS+ was the odor of pignut and the CS- the odor of sweet fennel. Learning would be evidenced by a statistical difference between CS+ and CS- responding. The order of CS+ and CS- was pseudorandom, CS duration 2 s, US duration approximately 1 s, and the ITI was 5 min.

Experiment 4: Proboscis suppression. In this experiment, we investigated whether the odor of sweet fennel or pignut could suppress an extended proboscis when the proboscis was extended by learning or by reflex. To determine this, we used a variation of the conditioned suppression technique. Originally developed by Estes & Skinner (1941) to estimate the impact of emotional responses produced by classical conditioning on behavior controlled by its consequences, we wished to determine whether exposure to these odors would suppress an already extended proboscis. We have successfully used this strategy in an earlier paper assessing citronella odor as a bee repellent (Abramson *et al.* 2006b).

Four groups of 20 insects each were used. Those in Groups 1 and 2 were harnessed and maintained as in the previous experiments. Groups 1 and 2 assessed whether exposure to the odor of 3.125% sweet fennel or 3.125% pignut respectively, 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 insect was allowed to drink a 1 µl droplet. This continued for five trials. Following the 5th trial, insects received eight test trials, four with the odor of citronella and four with the odor of sweet fennel. The duration of citronella and sweet fennel was 2 s. Insects in Group 2 received identical training with the exception that the stimuli were citronella and pignut.

Citronella 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 assess 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 insect 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 sweet fennel (or pignut) was pseudorandom following the order ABBABAAB with A being sweet fennel (or pignut) and B citronella. The ITI both during the five sucrose only trials and the eight test trials was 10 min. The insects were not allowed to feed during any of the eight test trials.

The selection process for insects in Groups 3 and 4 was more complicated. We needed a population of 40 insects that always responded to a cinnamon odor CS. All of our previous research on Africanized honeybees in Brazil has consistently showed lower levels of learning than is typically reported with European honeybees (Abramson & Aquino 2002). To acquire our sample of 40 bees, we trained approximately 200.

The basic experimental design for insects in Groups 3 and 4 was conceptually similar to those in Groups 1 and 2. Prior to receiving eight test trials, all insects 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 five training trials. Insects were allowed to feed on the US droplet and this was the reason why insects in Group 1 were permitted to feed on the five sucrose-only trials prior to receiving their eight 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 sweet fennel or pignut based on the same pseudorandom schedule used for insects in Groups 1 and 2. The ITI was 10 min. The durations of the citronella, sweet fennel, and pignut test stimuli were 2 s – the same duration used in Groups 1 and 2.

Experiment 5: Repellent test. In order to provide a complete evaluation of sweet fennel and pignut as potential repellents, a field test was conducted. Our test differs from the more traditional approach of placing a potential repellent in some type of container and assessing its effect on a group of bees because we applied the suspected repellent directly to each individual bee. Moreover, controls were employed to rule out the effect of novelty *per se*.

Fifteen bees foraging near the laboratory on several patches of sulphur cosmos (*Cosmos sulphureus,* Cav., Asteraceae) flowers were studied. When a bee landed on a flower it received a 2 s presentation of the odor of either sweet fennel or pignut or of cinnamon. The odor of cinnamon was used to provide a control for the effect of stimulation *per se*. 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 honeybee was disrupted in response to the two odors. Disruption was defined as flying off the flower, although any behavior of interest was recorded.

Thirty bees were used. Fifteen were exposed to sweet fennel and cinnamon and the other 15 were exposed to pignut and cinnamon. Each insect received a minimum of two stimuli presentations (one each of sweet fennel, 3.125%, and cinnamon, 100% or of pignut, 3.125% and cinnamon, 100%) and most received at least four stimulus presentations. Bees were individually marked and tracked in order to obtain multiple observations on each individual. Eight of the bees in the sweet fennel/cinnamon group were first presented with the odor of sweet fennel and the remaining seven bees with the odor of cinnamon first. In the pignut/cinnamon group, eight of the 15 bees first received the odor of pignut followed by cinnamon and the remaining seven insects received cinnamon followed by pignut. The experiment on an individual bee was terminated when it returned to the hive and the same bee was not used on successive visits.

Experiment 6: Free flying discrimination. In this experiment, the free-flying procedure was used to determine whether under natural conditions honeybees could discriminate the odor of sweet fennel and pignut. Twelve insects were randomly divided into two groups of six. The training odors were 3.125% sweet fennel and 3.125% pignut, respectively. Each insect received 24 training trials in which it was confronted with two targets differing in odor (known in the conditioning literature as a simultaneous discrimination). One odor target always contained a drop of sucrose (S+) and the other always contained a drop of water (S-). The drops were 0.6 ml. For six of the insects the S+ was sweet fennel and the S- was pignut. In the remaining insects the S+ was pignut and the S- was sweet fennel. The targets were positioned approximately 10 cm apart and all insects received 24 training trials with one trial per visit. Following a trial, the targets were removed and thoroughly washed with care being taken not to contaminate the targets. The position of S+ and S- was pseudorandom from trial to trial. The dependent variable was initial choice. A correct choice was landing on the S+ target.

Following the 24 training trials, each insect received a 10 min extinction test where both targets now contained water (0.6 ml) and the number of landings on each target was counted over the course of twenty 30 s intervals. Persistence during the extinction test is one method to estimate the strength of any learned association formed during the previous 24 training trials. Following extinction, the insect was captured and not permitted to return to the hive.

Statistical analysis. SPSS for Windows (2002) was utilized to perform analyses for all experiments. Alpha was set at 0.05 for all experiments (except where noted), unless heterogeneity of variances or heteroschedasticity of within groups variance was present, in which case alpha was set at 0.01.

Experiment 1. Due to computer error, it was not possible to statistically analyze results from this experiment, therefore, summary statistics are presented.

Experiment 2. A mixed 2 (group) x 12 (trial) analysis of variance (ANOVA) was utilized to assess differences between the sweet fennel and pignut groups on both CS acquisition responses and US responses.

Experiment 3. Differences in CS+ and CS- responding between the sweet fennel and pignut groups were analyzed using univariate analysis of variance (ANOVA). Before comparing differences in CS+ and CS- responses, responses to the 12 CS+ trials were combined to make up a mean CS+ response, and responses to the 12 CS- trials were combined to make up a mean CS- response.

Experiment 4. Within-groups responses were analyzed using the general linear model (GLM) for repeated measures, and for between-groups responses, the GLM for univariate analyses of variance was employed.

Experiment 5. Due to experimenter error, only summary statistics were recorded for this experiment and it was necessary to analyze the results using separate binomial tests for each of the odors. First, the observed proportion of bees that remained on flowers or feeding when the control stimulus was presented was computed. Then, proportions for the control conditions were compared to the observed proportions in the experimental groups. Alpha was set to 0.01 for this experiment.

Experiment 6. Responses across trials were first transformed into a single mean for each type of response: S+ and S- for acquisition and S+ and S- for extinction intervals. Mean responses for acquisition and extinction were then analyzed using multivariate analysis of variance (MANOVA) for mixed designs.

Results

Experiment 1. Topical application or ingestion of sweet fennel or pignut oils at concentrations used to control aphids did not harm Africanized bees over the course of the intervals tested. At high concentrations, however, many insects died. As the concentration of oil decreased below 50%, few if any insects died in either the sweet fennel or pignut oil groups. Two or fewer bees died in each control group, and in both the sweet fennel and pignut oil groups, at oil concentrations of ≤ 25% (n = 30 per group). However, at 50% concentration, 11 bees in the sweet fennel group and 12 bees in the pignut group died. At concentrations of 100%, 18 bees in the sweet fennel group and seven bees in the pignut group died. When 100% concentrations were applied directly to the abdomens of bees, six in the sweet fennel group died, but only two in the pignut group died.

Experiment 2. The results of simple Pavlovian conditioning with a citronella CS and either 3.125% sweet fennel or pignut were disappointing. The experiment did not progress because very few insects fed on the US; therefore extinction trials were not run. Of the 12 insects that received sweet fennel, there were only 45 cases where a honeybee actually consumed the US. Only on the first trial did all insects respond, as training progressed, the number of insects feeding on the US rapidly declined. The situation for insects receiving pignut US was even worse. No insect responded on the first trial and only three times did an insect consume the US. For a proper assessment of classical conditioning, the vast majority of insects should consume the US on every trial. In this experiment, for example, the total number of unconditioned stimuli expected to be consumed across all insects is 144 (12 insects x 12 US presentations). A 2 (group) x 12 (trial) mixed Analysis of variance showed significant differences in consumption of the US between groups over trials, ($F = 18.00$; df = 1, 22; $P = 0.0001$). The sweet fennel group consumed the US significantly more often ($M = 0.40$, $SD = 0.44$) than did the pignut group ($M = 0.02$, $SD = 0.07$). However, a 2 (group) x 12 (trial) mixed analysis of variance showed no significant differences among groups in response to the CS during acquisition, ($F = 3.07$; df = 1, 22; $P = 0.09$). The means and standard deviations for the sweet fennel group and for the pignut group were 0.14 (0.35) and 0.007 (0.02) respectively.

Note that under natural conditions honeybees would never be exposed to such high concentrations of sweet fennel and pignut. The 3.125% concentration used in these experiments is not toxic to honeybees yet toxic to aphids (Abramson *et al.* 2006a).

Experiment 3. When the odors of sweet fennel and pignut were used to signal food rather than consumed as a US, the situation was very different. Harnessed bees readily learned to associate the odors with food. Fig. 1 shows the results of the harnessed discrimination experiment. As the number of training trials progressed, insects learned to respond to the odor signaling food and to decrease responding to the odor not signaling food. Statistical analyses revealed significant differences between CS+ and CS- responding for both the sweet fennel and pignut groups, ($F = 47.96$; df = 1, 19; $P < 0.0001$; $F = 128.18$; df = 1, 19; $P < 0.0001$) respectively. The mean and standard deviation for the sweet fennel group was: CS+ ($M = 0.75$, $SD = 0.31$), CS- ($M = 0.25$, $SD = 0.32$). For the pignut group, they were CS+ ($M = 0.82$, $SD = 0.17$), CS- ($M = 0.32$, $SD = 0.26$).

Experiment 4. Fig. 2 shows the results of the experiments testing whether sweet fennel and pignut had repellent properties and whether these properties differentially affected learned and reflexive behaviors. The results indicated no repellent effect. When sweet fennel and pignut were applied to an already extended proboscis, the proboscis stayed extended relative to the control odor and there was no effect across repeated applications ($F = 0.28$; df = 3, 76; $P = 0.84$).

Experiment 5. The laboratory results were supported by the field tests. Of 21 total applications of sweet fennel to

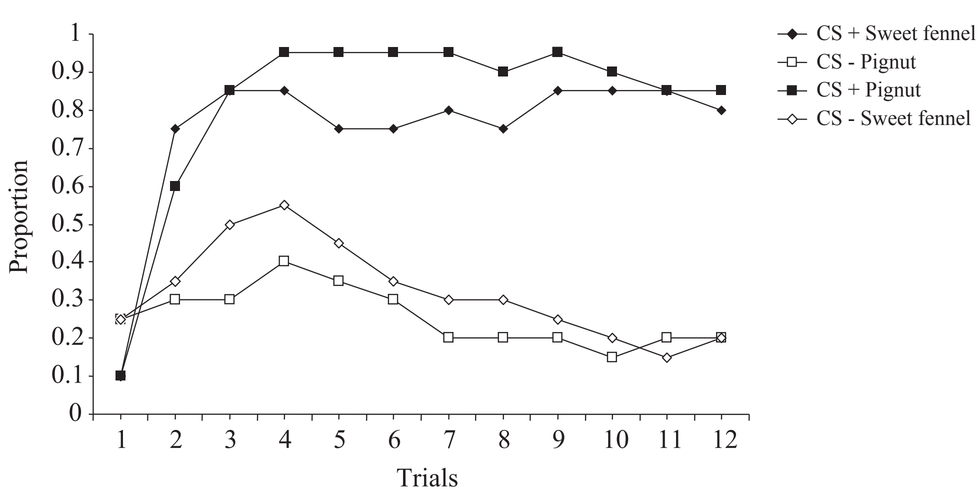

Fig. 1. Proportion of insects withdrawing their proboscis to the presentation of sweet fennel or pignut. The proboscis was extended by either a prior learned association with cinnamon odor or reflexively by stimulating the antennae with sucrose.

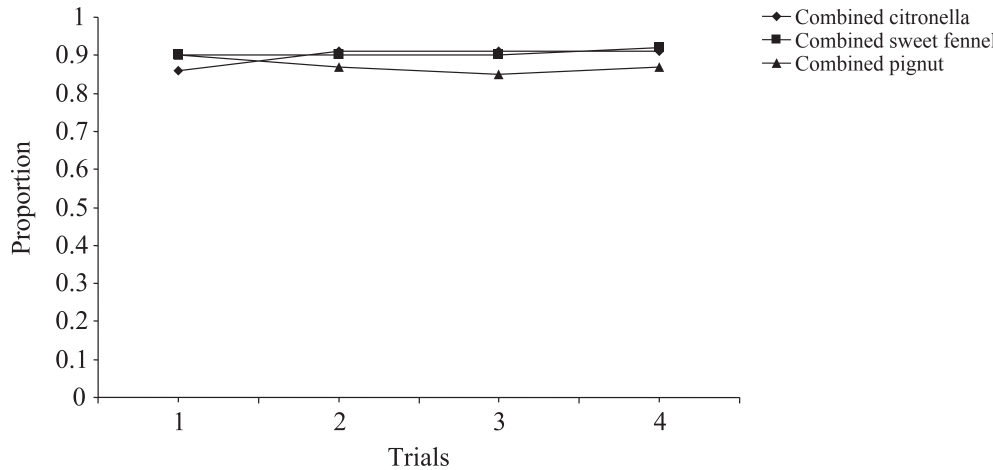

Fig. 2. Harnessed discrimination experiment. Proportion of insects responding to a CS+ and CS-.

honeybees feeding on the nectar of sulphur cosmos only two flew away. This compared favorably with the 21 total applications of cinnamon odor to the same 15 bees where only two flew away. The observed proportion of 0.90 for bees that continued to feed when sweet fennel was applied was not significantly from the test proportion of 0.81 for bees that continued feeding when cinnamon was applied (one-tailed P = 0.21). When sweet fennel was applied to bees on flower petals the results were similar to the feeding results. Of 34 total applications of sweet fennel and cinnamon, five and six respectively, produced flight responses. The observed proportion of 0.85 for bees that remained on flower petals when sweet fennel was applied did not significantly differ from the test proportion of 0.82 for bees that remained on petals when cinnamon was applied (one-tailed P = 0.41).

The results with sweet fennel were similar to those found with pignut. Of nine total applications of pignut and cinnamon to honeybees feeding on the nectar of sulphur cosmos, only1 flight response was recorded for each stimulus. The observed proportion of 0.89 for bees in the pignut group was equal to the test proportion of 0.89 for the cinnamon group (one-tailed P = 0.65). Of 23 total applications of either pignut or cinnamon to bees on petals, only five and three flight responses were recorded, respectively. Finally, the observed proportion of 0.78 for bees in the pignut group was not significantly different from the test proportion of 0.87 for the cinnamon group (one-tailed, P = 0.17).

Experiment 6. Fig. 3 shows the acquisition results of free-flying bees trained to discriminate the odors of sweet fennel and pignut. Analyses showed no significant differences during training when either sweet fennel or pignut was used as S+ or S- respectively (F = 0.41; df = 1, 10; P = 0.54), therefore the S+ groups were combined and the S- groups were combined for subsequent comparisons. Free-flying bees readily associated the odor of sweet fennel or pignut with a sucrose reward and learned to discriminate. Multivariate analysis of variance showed significant differences in mean S+ and S- responses, (F = 163.55; 1, 10; P < 0.001) Means and standard deviations for S+ and S- responses were 0.69 (0.11) and 0.15 (0.00) respectively. The acquisition results were supported by the extinction results, illustrated in Fig. 4, in which both targets now contained water. There are clear differences between the two curves. As the duration of extinction progressed over 10 min, bees landed more frequently on the target previously associated with reward. The difference between S+ and S- responding was significant (F = 105.60; df = 1, 10; P < 0.001). The means and standard deviations for the S+ and S- responses were 0.93 (0.22) and 0.50 (0.27) respectively.

Discussion

We began this research by asking the question whether the essential oils of sweet fennel and pignut are safe for Africanized honeybees. The question is important because these essential oils are used to control the aphids *Hyadaphis foeniculi* Passerini and *Aphis gossypii* Glover that attack plants of the Umbeliferae and Malvaceae families (Abramson *et al.* 2006a, Trusheva *et al.* 2006). The results of harnessed and free-flying experiments support the view that exposure to the odors of sweet fennel and pignut are not harmful to honeybees. The insects readily associated the odors with food, easily discriminated among them under both harnessed and free flying conditions, and neither sweet fennel nor pignut had a repellent effect in the laboratory or in the field. Consumption of these two essential oils at high levels, however, brought about death. We do not view the latter as a problem because honeybees would not normally encounter such high concentrations in the field.

Of particular interest, harnessed bees in a Pavlovian experiment did not feed on a compound US composed of essential oil and high molarity sucrose. A similar refusal to feed was observed in an earlier experiment investigating the effect of a pesticide composed of essential oils (wintergreen, soybean, thyme, clove, sesame) and suggests a possible limitation of the proboscis conditioning procedure (Abramson *et al.* 2006c). Successful Pavlovian conditioning critically depends upon a US that consistently elicits a response. If such a response is not forthcoming the technique cannot properly be employed to assess agrochemicals and alternative learning paradigms must be used.

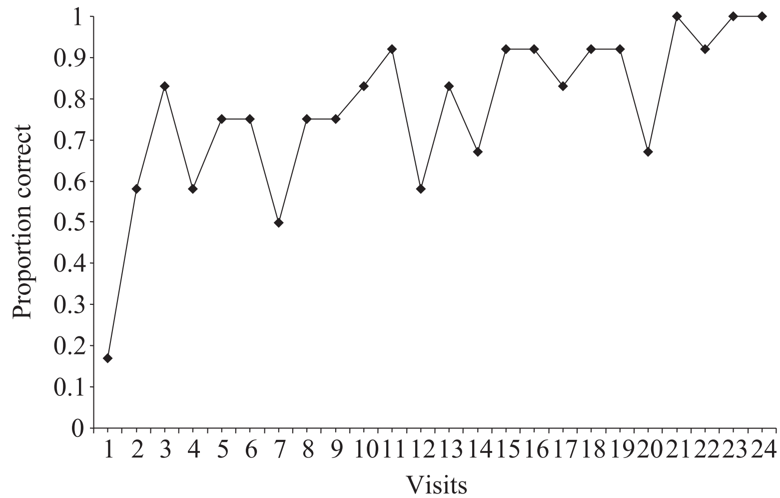

Fig. 3. Free-flying discrimination acquisition results. Proportion of correct choices where an insect landed on a target paired with sucrose.

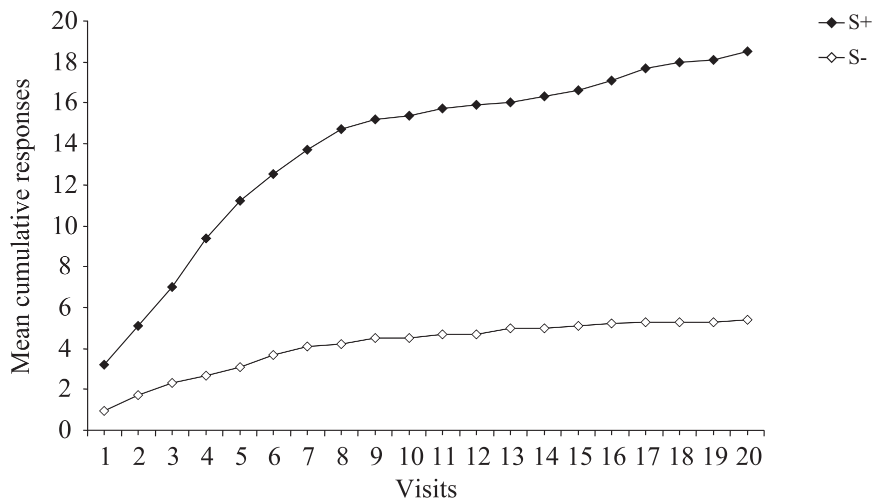

Fig. 4. Free-flying discrimination extinction results. Mean cumulative responses of insects landing on either the S+ or S- target when both targets contain water.

In previous research we used Pavlovian paradigms to assess several pesticides commonly used in Brazil to control cotton pests (Abramson *et al.* 1999). As discussed in the previous paragraph we also had the opportunity to use Pavlovian methods to evaluate a pesticide made from essential oils. This work, also conducted in Brazil, revealed that Africanized honey bees have the ability to discriminate between targets, both of which contain high concentrations of sucrose, but one of which contains a pesticide. This ability to avoid an artificial flower even though it contained a compound reward consisting of pesticide and high molarity sucrose could never have been revealed by relying on toxicity tests alone (Abramson *et al.* 2006c).

The present series of experiment are also unique in that they support the use of "conditioned suppression" in addition to Pavlovian conditioning of proboscis extension to evaluate potential repellent properties of an agrochemical. We have used the conditioned suppression and Pavlovian techniques to conclusively show that citronella odor is not repellent to Africanized honeybees (Abramson *et al.* 2006b).

We believe our strategy of using conditioning methods is superior to the present method of applying a suspected repellent to a cloth or some other medium and counting the honeybees in the area (e.g., Free *et al.* 1989). Such techniques, for example, cannot separate a decrease in honeybees brought about by the influence of a potential repellent from a decrease brought about by exposure to a novel odor. Nor can such techniques evaluate the effect of a potential repellent on learned behavior or take into account the highly evolved learning ability of the honeybee. A suspected repellent, in our view, is similar to an aversive stimulus and should therefore retard the learning of a Pavlovian conditioned response and lead to a retraction of an extended proboscis (i.e., conditioned suppression).

In conclusion, our mortality studies showed that bees survived exposure to sweet fennel and pignut at concentrations used to control aphids. As the concentration increased, however, so did mortality with the highest mortality occurring at concentrations above 50%. In addition, data collected using both conditioning of harnessed bees and field tests conclusively showed that exposure to sweet fennel and pignut at concentrations harmful to aphids did not disrupt the learned behavior of honeybees nor did they serve as a repellent.

We believe our results illustrate the advantages of using behavioral paradigms developed for the study of learning to the assessment of essential oils on honeybees. As the use of essential oils as agrochemicals increase, more research is needed on how these oils influence honeybee behavior. Pavlovian and related conditioning paradigms are easy to use and offer an elegant method for such assessment (Abramson 1994).

Acknowledgments

This work was partially supported by grants from CNPq and Petrobras. Dr. Abramson's participation was made possible by grant SES-0244142 from the National Science Foundation.

References

Abramson, C.I. 1990. Invertebrate learning: A laboratory manual and source book. Washington, D.C., American Psychological Association, 100p.

Abramson, C.I. 1994. A primer of invertebrate learning: The behavioral perspective. Washington, D.C., American Psychological Association, 273p.

Abramson, C.I. & B.J. Boyd. 2001. An automated apparatus for conditioning proboscis extension in honey bees (*Apis mellifera* L.). J. Entomol. Sci. 36: 78-92.

Abramson, C.I. & I.S. Aquino. 2002. Behavioral studies of learning in the Africanized honey bee (*Apis mellifera* L.). Brain Behav. Evol. 59: 68-86.

Abramson, C.I., I.S. Aquino, F.S. Ramalho & J.M. Price. 1999. Effect of insecticides on learning in the Africanized honey bee (*Apis mellifera* L.). Arch. Environ. Contam. Toxicol. 37: 529-535.

Abramson, C.I., I.S. Aquino, M.C. Silva & J.M. Price. 1997. Learning in the Africanized honey bee: *Apis mellifera* L. Physiol. Behav. 62: 657-674.

Abramson, C.I., J.B. Singleton, M.K. Wilson, M.K., P.A. Wanderley, F.S. Ramalho & L.M. Michaluk. 2006c. The effect of an organic pesticide on mortality and learning in Africanized honey bees (*Apis mellifera* L.) in Brasil. Am. J. Environ. Sci. 2: 33-40.

Abramson, C.I., J. Squire, A. Sheridan & P.G. Mulder Jr. 2004. The effect of insecticides considered harmless to honey bees (*Apis mellifera* L.): Proboscis conditioning studies using the insect growth regulators Confirm®2F (Tebufenozide) and Dimilin®2L (Diflubenzuron). Environ. Entomol. 33: 378-388.

Abramson, C.I., M.K. Wilson, J.B. Singleton, P.A. Wanderley, M.J.A. Wanderley, & L.M. Michaluk. 2006b. Citronella is not a repellent to Africanized honey bees *Apis mellifera* L. (Hymenoptera: Apidae). BioAssay 1:13, 7p. Available online: www.bioassay.org.br/articles/1.13.

Abramson, C.I., P.A. Wanderley, M.J.A. Wanderley, A.J.S. Miná & O. Baracho de Souza. 2006a. Effect of essential oil from citronella and alfazema on fennel aphids *Hyadaphis foeniculi* Passerini (Hemiptera: Aphididae) and its predator *Cycloneda sanguinea* L. (Coleoptera: Coccinelidae). Am. J. Environ. Sci. 3: 9-10.

Campos, I.F.P., H.D. Ferreira, T.A. Portes, J.C. Seraphin, J.R. Paula, J.G. Silva, N.R. Azevedo, S.C. Santos & P.H. Ferri. 2002. Volatile constituents of Hyptis suaveolens (I.) Poit from Brazilian cerrado. Acta Horticulturae 569: 195-201. http://www.actahort.org/books/569/569_31.htm.

Estes, W.K. & B.F. Skinner. 1941. Some quantitative properties of anxiety. J. Exp. Psychol. 29: 390-400.

Free, J.B., A.W. Ferguson & J.R. Simpkins. 1989. Honeybee responses to chemical components from the worker sting apparatus mandibular glands in field tests. J. Apic. Res. 28: 7-21.

Lorenzon, M.C.A., C.A.R. Matrangolo & J.H. Schoereder. 2003. Flora visited by the eusocial bees (Hymenoptera, Apidae) in a savanna of the south of Piaui, Brazil. Neotrop. Entomol. 32: 27-36.

Malele, R.S., C.K. Mutayabarwa, J.W. Mwangi, G.N. Toithi, A.G. Lopez, E.I. Lucini & J.A. Zygadlo. 2003. Essential oil of *Hyptis suaveolens* (L.) Poit. from Tanzania: Composition and antifungal activity. JEOR 15: 438-440.

Mimica-Dokič, N., S. Kujundžč, M. Sokovič, & M. Couladis. 2003. Essential oil composition and antifungal activity of *Foeniculum vulgare* Mill obtained by different distillation conditions. Phytotherapy Res. 17: 368-371.

Okonogi, S., S. Chansakaow, S. Vejabhikul, P. Tharavichitkul, J. Lerphokanont, A. Nakano & F. Ikegami. 2005. Antimicrobial activity and pharmaceutical development of essential oil from *Hyptis suaveolens*. Acta Hort. 678: 163-169.

Severino, F.J. & P.J. Christoffoleti. 2001. Banco de sementes de plantas daninhas em solo cultivado com adubos verdes. Bragantia 60: 201-204.

SPSS. 2002. SPSS 11.5 for Windows, Chicago, SPSS Incorporated.

Torres, S.B. 2004. Teste de envelhecimento acelerado em sementes de sweet fennel. Rev. Bras. Sementes 26: 20-24.

Trusheva, B., M. Popova, V. Bankova, S. Simova, M.C. Marcucci, P.L. Miorin, F. da Rocha Pasin & I. Tsvetkova. 2006. Bioactive constituents of Brazilian red propolis. eCAM. 3: 249-254.

Received 15/VIII/06. Accepted 13/VII/07.