International Journal of Comparative Psychology, Vol. 12, No. 4, 1999

FAILURE TO FIND PROBOSCIS CONDITIONING IN ONE-DAY OLD AFRICANIZED HONEY BEES (*APIS MELLIFERA L.*) AND IN ADULT URUÇU HONEY BEES (*MELIPONA SCUTELLARIS*)

Charles I. Abramson
Oklahoma State University, USA

Italo S. Aquino
Universidade Federal da Paraiba, BRAZIL

Sherril M. Stone
Oklahoma State University, USA

ABSTRACT: The proboscis extension reflex was used to investigate behavior modification in one day old Africanized honey bees and in adult Uruçu honey bees. Experiments were designed to investigate classical conditioning, pseudoconditioning, and central excitatory state. Additional experiments examined the suitability of the proboscis extension reflex to serve as a feeding assay were carried out on Uruçu. The results indicated no classical conditioning and no pseudoconditioning in young Africanized bees or in the adult Uruçu. A large central excitatory effect was observed in young Africanized bees, but only a small effect was observed in Uruçu. The proboscis extension reflex could be used as an assay to test the suitability of artificial diets in Uruçu.

The study of the proboscis extension reflex (PER) has led to many areas of fruitful research in the honey bee (Kartzev, 1996; Menzel & Bitterman, 1983), including studies of Pavlovian conditioning (Bitterman, Menzel, Fietz, & Schäfer, 1983; Buckbee & Abramson, 1997; Menzel, 1987), discriminative punishment (Smith, Abramson, & Tobin, 1991); influence of pesticides on behavior (Mamood & Waller, 1990; Stone, Abramson, & Price, 1997; Taylor, Waller, & Crowder, 1987) olfactory discrimination (Getz & Smith, 1987; Smith & Menzel,

Address correspondence to Charles Abramson, Laboratory of Comparative Psychology and Behavioral Biology, Departments of Psychology and Zoology, Oklahoma State University, 215 North Murray, Stillwater, OK 74078. E-mail: charles@okstate.edu

1989), and as a rapid bioassay to measure detection of adulterated beeswax (Aquino, Abramson, & Payton, 1999). Additionally, the PER has served as a model system for studying the biochemistry of learning and other forms of behavior modification in honey bees and other insects (Abramson, 1994; Menzel, Hammer, Braun, Mauelshagen, & Sugawa, 1991; Mercer, 1987).

Most recently, work on the PER has been directed toward the study of Africanized honey bees. Work with the so called "Killer bee" revealed the existence of a number of classical conditioning phenomena in adults, although the asymptotic level of performance is less than that observed with the European honey bee (Abramson, Aquino, Silva, & Price, 1997). The PER has also been used to study the influence of pesticides on learning (Abramson Aquino, Ramalho, & Price, 1999) and to measure attraction to consumer products such as soft drinks and perfumes in the Africanized honey bee (Abramson, Aquino, Azeredo, Filho, & Price, 1997).

The experiments reported here are designed to study behavior modification in the day-old Africanized honey bee. Invertebrates such as the fruit fly (*Drosophilia melanogaster*) and the mollusc (*Aplysia californica*) have been used as model systems to study gerontology and developmental issues in behavior modification but there is little comparative research with honey bees (Carew, Marcus, Nolen, Rankin, & Stopfer, 1990; Bhagavan, Benatar, Cobey, & Smith, 1994; Le Bourg, 1996). The rationale behind the experiments reported here are two-fold. First, the comparative analysis of learning in the Africanized honey bee would be incomplete if confined to the study of adults. Second, we had interest in providing some data on the ability of young bees to learn a PER Pavlovian conditioning task.

During the course of the experiments with Africanized bees we had the unique opportunity to work with the stingless honey bee, Uruçu. The question naturally arose whether the PER technique, so successful with both the Africanized and European honey bees, could be adopted to the study of Uruçu. The PER is most readily studied in Africanized and European honey bee by confining them in small metal harnesses. Once harnessed, bees readily extend their mouthparts (proboscises) to feed on a sucrose solution after the solution has been briefly applied to the antennae, on which sucrose sensitive contact sensillae are found. One potential difficulty is whether Uruçu will remain viable when restrained. A second potential problem is that, once restrained, will normal feeding behavior be possible. The ability to feed regularly when restrained is a necessary condition in PER research on learning.

A successful application of PER methodology to Uruçu is important not only for what it may reveal about the learning process in this interesting honey bee but takes on an added significance because of the dramatic decline in the numbers of Uruçu in the northeast of Brazil. A major factor in this decline is the destruction of their natural habitats, food sources, and colonies by humans raiding hives for honey (Lorenzon, 1996). The honey is produced in such small quantities (about 1 liter per hive each year) it is extremely expensive at almost $100.00 a liter and therefore profitable to sell.

The Uruçu bee is slightly larger than the Africanized bee. It has an orange hairy thorax and orange tint to the antennae. In addition, it produces a very thin honey the color of which range from light green to dark yellow. The hives of Uruçu are smaller than *Apis* and consist of only a few hundred bees. They store their honey in honey pots and not in the familiar hexagonal cells so characteristic of *Apis*. Because Uruçu is a member of the family of stingless bees their defensive mechanisms are restricted to strong mandibles, small hive entrance, sticky entrance tunnels and honey, wax, and propolis that smell like unwashed socks.

GENERAL METHODS

Three series of experiments are common to both the Africanized honey bee and the Uruçu honey bee. In the first series, Pavlovian conditioning of proboscis extension is examined. An investigation of pseudoconditioning is the purpose of the second series of experiments. In the third series, the influence of central excitatory state is researched. In addition to Pavlovian conditioning, pseudoconditioning, and central excitatory state, the usefulness of the PER technique as a feeding assay was examined in Uruçu. All Africanized and stingless bees were obtained from, and studied in, the Laboratório Apícola of the Universidade Federal da Paraiba (UFPB), Bananeiras, Brazil.

Subjects

One day old Africanized honey bees (Apis mellifera L.). Frames containing brood were brought to the laboratory at 9:00 AM on the day prior to the experiment and placed in an observation hive. The use of the observation hive assured us of the age of the bees and, as an added advantage, made them easier to capture. Within 15 seconds of emergence Africanized worker honey bees (*Apis mellifera L.*) were captured and harnessed in small metal tubes. The young bees were placed in the harness without rendering them unconscious as was done with adult Africanized bees. We did not want to render newly emerged bees unconscious because we did not know what effect it would have on the learning process. To secure the bee in the harness, a strip of duct tape was placed between the head and the thorax and fastened to the sides of the metal tube. Following harnessing, subjects were fed a 2.9 M sucrose solution to satiation and held overnight until testing at 12:00 PM the following day. The purpose of feeding was to equate motivational levels for the experiment that was to be run the following afternoon. Different sets of subjects were used for all the experiments described.

Adult Africanized honey bees (Apis mellifera L.). Adult workers (estimated to be 21 days old) were selected at random as they departed from the laboratory hive around 9:00 AM on the day prior to use and tested around 12:00 PM the following afternoon. Subjects collected in this way are a mixture of bees of different behavioral specializations that require either departure from the hive (e.g., foragers or nest cleaning bees) or remaining near the entrance (e.g., guards). Each bee was carried in a glass vial to the laboratory. Individual subjects were rendered unconscious by placing the glass vials in an ice water bath. When the bee became inactive it was immediately removed from the vial and put into a metal restraining harness. After regaining consciousness, subjects were fed a 2.9 M sucrose solution until its proboscis would no longer remain in contact with the solution, after which they were left until trained the next morning (for details see Abramson, Aquino, Silva, *et al.*, 1997). The rationale behind the prefeeding was to ensure that all subjects had the same motivation to feed.

Stingless honey bees (Melipona scutellaris). Adult worker Uruçu bees (estimated to be 15-20 days old) were selected at random from the laboratory hive around 9:00 AM on the day prior to use and tested around 12:00 PM the following afternoon. Subjects were placed in the same harness used for the Africanized bees. As with the young Africanized honey bees, the Uruçu were placed in the harness without rendering them unconscious. After being placed in the harness subjects were fed Uruçu honey. Subjects were fed honey, until satiated, and not the sucrose solution used for the Africanized bees because the sucrose solution would not elicit proboscis extension. Different sets of subjects were used for all of the experiments described.

Apparatus

Materials consisted of the metal harnessing tubes (0.6 cm inside diameter and 2 cm long), a ventilation chamber to prevent the accumulation of the conditioned stimulus (CS) and unconditioned stimulus (US) scents in the testing area, plastic 20 cc syringes to present the CS and filter paper strips (handled with tweezers) to administer the US or a distilled water CS. Three classes of CSs were used: 1) oils (obtained from Sigma Chemical Company, St. Louis, MO), 2) wax (Africanized and *Melipona*), and 3) water stimulation to an antennae.

Geraniol (Sigma Chemical product number G-5135), Citral (Sigma Chemical product number C-1645), and Hexanal (Sigma Chemical product number H-9008) constituted the oils. The oils and water were applied neat onto a piece of 1-cm^2 filter paper strips (Whatman #1).

The filter paper containing the oils was secured to the plunger of the syringe (one syringe for each oil used) with a metal thumbtack. The filter paper containing water was simply held with tweezers. The wax (AHB wax - 3.3 grams, *Melipona* - 1.9 grams) were obtained from the laboratory hives and placed into a syringe were it remained for 3 days prior to use. The conditioned stimulus was the odour of bees wax or oils and was selected based on their effectiveness shown in our previous research (Abramson, Aquino, Silva, *et al.*, 1997). To administer an olfactory CS, the syringe is depressed near the head of the subject. Following a trial the plunger of the syringe is pulled back in preparation for the next CS presentation. To administer antennae stimulation of the CS, the filter paper was dipped in distilled water and held between the jaws of a tweezer. It was then briefly applied to an antennae (the use of the left or right antennae was counterbalanced between subjects). The rationale behind the use of several CSs was not to restrict our findings to a particular CS. For young and adult Africanized honey bees the US consisted of a 2.9 M sucrose solution. Because the Uruçu bees did not respond to the sucrose solution used for the Africanized bees, Uruçu honey served as the US for the stingless bees. The honey was obtained directly from the Uruçu colony.

SERIES 1: PAVLOVIAN CONDITIONING STUDIES

In the first series of experiments we investigated Pavlovian conditioning in day old Africanized honey bees and adult stingless bees. To our knowledge no such studies have been attempted with these two species of honey bees.

Procedure

Africanized bees. One hundred and twenty young bees (one day old) were randomly assigned to one of 6 groups (20 subjects per group). The groups differed primarily in terms of the CS used (Hexanal, Africanized honey bee wax, and water stimulation of the antennae) and whether they received paired or unpaired training. In addition to the one day old bees, forty adult Africanized bees were also used and randomly assigned to one of two groups (20 subjects per group). For the adult groups, Hexanal and wax served as the CS, respectively. The adult groups were included to ensure that learning could occur during the time of year these experiments were conducted (July/August 1998). Unpaired controls were not run for the adult groups because the results of several experiments (using both paired and unpaired comparisons and discrimination procedures) rendered them unnecessary (Abramson, Aquino, Silva, *et al.*, 1997; Abramson *et al.*, 1999).

For all paired groups there were 12 acquisition trials followed by 12 extinction trials. If no learning was evident, extinction trials were not conducted. During acquisition a non-overlap procedure was used in which the CS terminated before the US was presented. Upon termination of the CS, the US was presented by first touching sucrose to the antenna and then to the now extended proboscis. The CS duration was 3 seconds and the US was a 2 second feeding of a 2.9 M sucrose solution. The intertrial interval was 10 minutes for paired animals and 5 minutes for unpaired. The unpaired groups were included to ensure that any increase in the probability of proboscis extension to the CS was the result of CS-US pairings and not a nonassociative effect such as sensitization.

Unpaired CS/US stimuli were presented in pseudorandom order. For half the unpaired animals, stimulus presentations consisted of three successive sequences of CS US US CS US CS CS US. For the remaining animals the sequence consisted of US CS CS US CS US US CS. The interval between stimulus presentations was five minutes - half the time for paired animals. The rationale behind using a five minute intertrial interval for unpaired animals was to keep the time between CSs presentations approximately 10 minutes. If a 10 minute intertrial interval was used then the time between CS presentations would be approximately 20 minutes and any difference between paired and unpaired animals might be accounted for in terms of such nonassociative effects as time spent in the apparatus.

Conditioned responses to the CS were categorized visually into one of two states during each trial. If a subject extended its proboscis after the onset of the CS, but before its antennae were touched with the sucrose, a response was registered. Otherwise, a non-response was recorded.

Stingless bees. Two hundred and forty adult Uruçu honey bees were randomly assigned to one of 12 groups (20 subjects per group). As in the Africanized bee experiments the groups differed primarily in terms of the CS used and whether they received paired or unpaired training. The major difference between the Africanized bee experiment and the Uruçu experiment is that a greater range of CSs were used (Hexanal, Geraniol, Citral, Uruçu wax, Africanized honey bee wax, and water stimulation to the antennae). Geraniol, Citral, and Uruçu wax were included because we wanted to test a range of CSs as was done in our original work with Africanized bees (Abramson, Aquino, Silva, *et al.*, 1997). In addition to using a greater variety of CSs, the second difference between the Africanized and Uruçu experiments was the use of Uruçu honey as a US. The change was necessary because the Uruçu did not drink the sucrose solution so effective with Africanized bees. With the exceptions already noted, all training variables and the use of unpaired control groups were identical to those used in the Africanized experiments.

RESULTS AND DISCUSSION

Figure 1 shows the mean proportion of animals responding to paired presentations of a Hexanal, AHB wax, or antennae stimulation CS with a sucrose US in one-day old Africanized honey bees. To insure that conditioning can occur during the time these experiments were run, the results of two adult Africanized honey bees groups are also presented. The adult animals showed a learning curve typical of our previous studies of Pavlovian conditioning in Africanized honey bees. In contrast, the one-day old honey bees showed no evidence of learning despite responding to the sucrose US on each training trial. As would be expected from these data, performance of unpaired animals did not differ from paired animals. Figure 2 presents the results of the 3 unpaired groups. As in the paired groups there is little responding to the CS and all one-day old honey bees responded to each presentation of the US.

As would be expected from the figures, the results of the repeated measures ANOVA for the adult AHB indicates a significant Group effect $F(1, 38) = 4.753$, $p = .036$, a Trial effect $F(11, 418) = 10.603$, $p = .000$, and a non-significant Group x Trial interaction $F(11, 418) = .240$, $p = .995$. A similar analysis conducted on the data for the three paired one-day old AHB indicates no significant Group effect $F(2, 114) = 2.544$, $p = .087$, no Trial effect $F(11, 627) = 1.772$, $p = .055$, and no Group x Trial interaction $F(22, 627) = .641$, $p = .896$. Overall comparisons between one-day old paired and unpaired groups reveal no significant Group differences $F(1, 114) = 2.232$, $p = .138$.

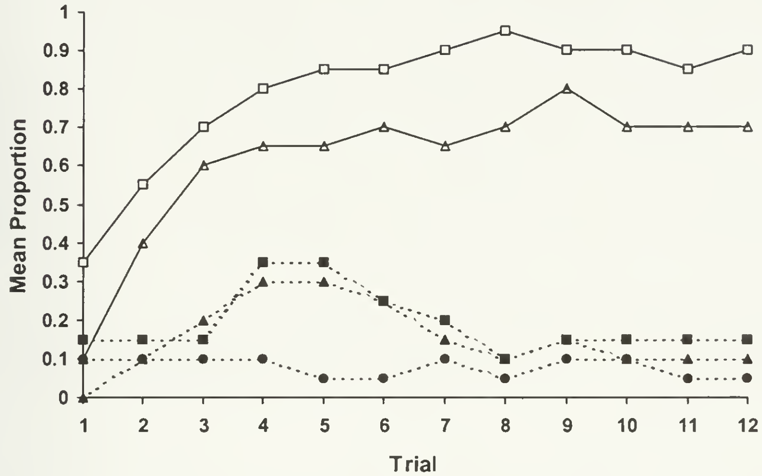

Figure 1. Mean proportion of adult and one day old Africanized honey bees responding to a CS over the course of 12 acquisition trials. Performance is shown in AHBs for both adults (unfilled symbols) and day olds (filled symbols). Different CSs were used as follows: Beeswax (squares); hexanal (triangles); and antennae stimulation (circles). No conditioning is evident in day old bees.

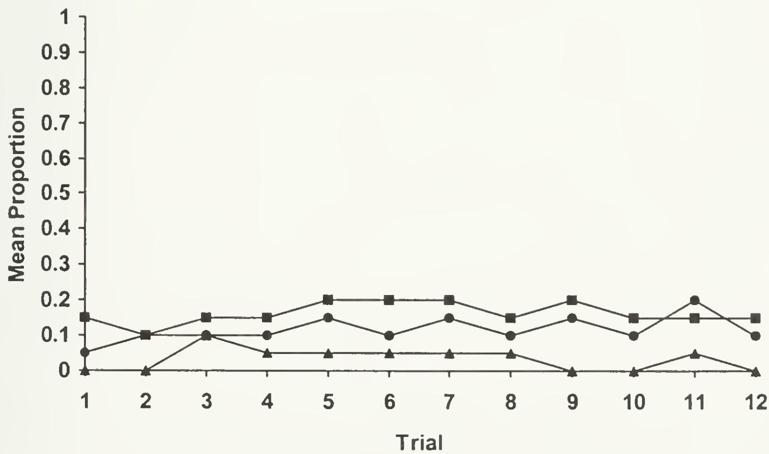

Figure 2. Mean proportion of one day old Africanized honey bees responding to a CS which was explicitly unpaired with a US. Performance using different CSs is represented as follows: Beeswax (squares); hexanal (circles); and antennae stimulation (triangles).

In Figure 3 the data are again plotted to show the comparison between adults and one-day old AHBs when beeswax and hexanal served as the CSs. Both wax and hexanal were readily associated with the US in adults but not in day old bees. We find this result interesting because both adults and young bees responded to each presentation of the US although none acquired an association with the CS. The differences between adults and young bees are supported by the results of a repeated measures ANOVA which indicated a significant Group effect $F(1, 76) = 112.191$, $p = .000$, a Trial effect $F(11, 836) = 7.950$, $p = .000$, and a Group x Trial interaction $F(11, 836) = 5.029$, $p = .000$.

Figure 4 shows the mean proportion of Uruçu honey bees responding to paired presentations of Hexanal, Geraniol, Citral, Uruçu wax, AHB wax, or antenna stimulation. No conditioning is evident in any of the groups despite each animal responding to the US. The results of the repeated measures ANOVA for the adult Uruçu indicated no significant Group effect $F(1, 38) = 2.652$, $p = .112$, no Trial effect $F(11, 418) = .918$, $p = .523$, and no Group x Trial interaction $F(11, 418) = 1.541$, $p = .114$.

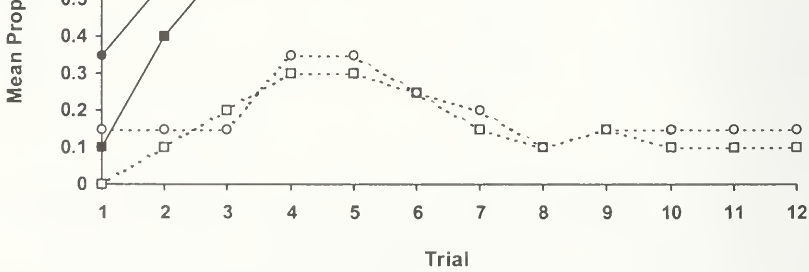

Figure 3. Mean proportion of adult (filled symbols) and one day old Africanized honey bees (open symbols) responding to a CS of either beeswax (circles) or hexanal (squares) during paired training.

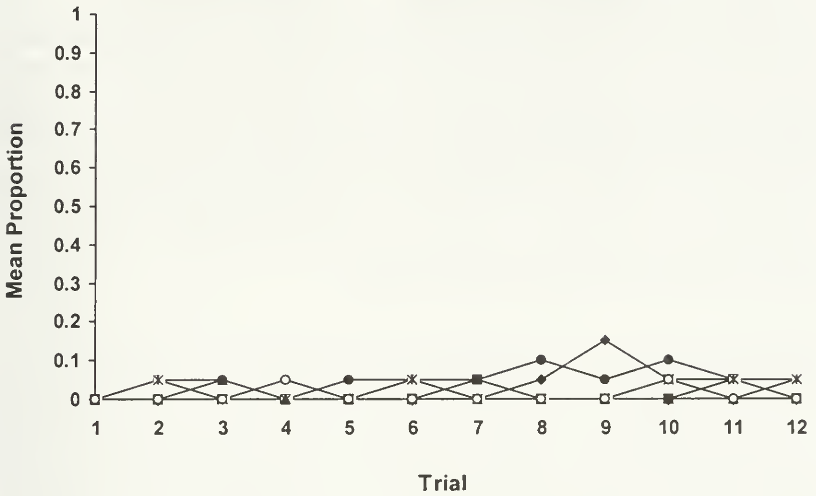

Figure 4. Mean proportion of adult Uruçu honey bees responding to different CS's over the course of 12 acquisition trials. Performance using six different CS's is represented as follows: AHB wax (filled squares); antennae stimulation (filled circles); geraniol (filled triangles); hexanal (filled diamonds); Uruçu wax (stars); and citral (unfilled circles).

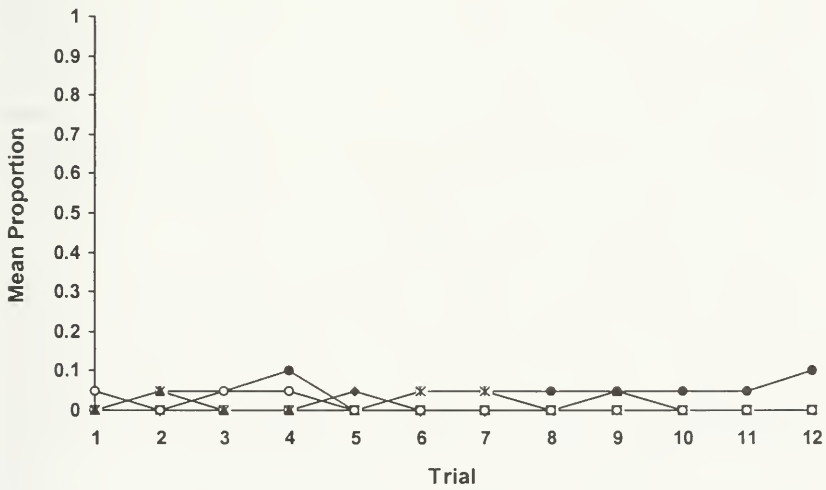

Figure 5. Mean proportion of adult Uruçu honey bees responding to a CS which was explicitly unpaired with a US. Performance is shown for six different CS's as follows: AHB wax (filled squares); antennae stimulation (filled circles); geraniol (filled triangles); hexanal (filled diamonds); Uruçu wax (stars); and citral (open circles).

Figure 5 presents the results of the six unpaired Uruçu groups. As is the case of one-day old bees and the adult Uruçu, few Uruçu respond to the CS when the CS and US are explicitly unpaired. Overall comparisons between adult paired and unpaired Uruçu revealed no significant Group differences $F(1, 228) = .691$, $p = .407$. It should be noted that the original design of the one-day old AHBs and Uruçu experiments included 12 CS-only extinction trials. Because no acquisition was obtained in the one-day old AHBs nor in the Uruçu bees, extinction trials were not necessary.

SERIES 2: PSEUDOCONDITIONING STUDIES

In the previous series we found no evidence for Pavlovian conditioning in day old Africanized honey bees nor in adult Uruçu. The present series of experiments looks for another type of behavior modification known as pseudoconditioning.

Pseudoconditioning refers to any "conditioning" that is not shown to have been dependent upon the correlation between the CS and US (Grether, 1938; Harris, 1943). It is most readily studied by exposing animals to a series of US presentations followed by a CS only test trial. If the animal responds to the CS even though the CS was not explicitly paired with the US, pseudoconditioning is indicated. Traditional controls for pseudoconditioning include explicitly unpaired CS/US presentations and discrimination training. Two explanations have been offered to account for pseudoconditioned responses. One explanation suggests that the unconditioned response generalizes to stimuli resembling the unconditioned stimuli. The alternative explanation suggests that exposure to an unconditioned stimulus produces a change within the organism that would cause the organism to respond to any external stimulus (Mackintosh, 1974).

Pseudoconditioning is readily observed in invertebrates including polychaete worms (Evans, 1966a, b) and octopuses (Young, 1960) and is considered a major form of behavior modification in invertebrates (Well, 1968). A study of olfactory conditioning in the earthworm revealed substantial amounts of pseudoconditioning despite significant differences between animals receiving paired and unpaired training (Abramson & Buckbee, 1995). Pseudoconditioning in proboscis conditioning studies with European bees has been assessed by unpaired and discrimination procedures but was not the subject of investigation in its own right. Pseudoconditioning has been the object of investigation in adult Africanized honey bees and although, the amount of pseudoconditioning observed in those experiments was minimal, it was argued that some component of acquisition of proboscis conditioning in Africanized honey bees is based on pseudoconditioned responses (Abramson, Aquino, Silva, *et al.*, 1997). The present experiment examined pseudoconditioning in young Africanized honey bees and in adult Uruçu. To our knowledge no studies are reported in the literature that specifically examined pseudoconditioning in young Africanized and stingless honey bees.

Procedure

Eighty, one day old Africanized honey bees were captured from cells, maintained, and harnessed as in the previous series and randomly placed into one of four groups (N = 20). Separate groups of animals received 2, 5, 8, or 11 US presentations before receiving a single CS only test trial. The test trials for each of the 4 groups appeared on trials 3, 6, 9 and 12, respectively. The CS was Africanized honey bee wax, the US a 2 second feeding of sucrose and the intertrial interval 10 minutes.

For the Uruçu experiments, eighty adult animals were captured, maintained, and harnessed as in the previous series. The same experimental design used with day old Africanized bees was adopted with adult Uruçu with the exception of the CS and US. The CS used was the odour of Uruçu wax, the US was a 2 second feeding of Uruçu honey.

The rationale behind selecting beeswax as a CS over the alternatives presented in the first series of experiments is that we wanted to find some effect. For adult Africanized bees, beeswax is potent enough to serve as both a CS and US (Abramson, Aquino, Silva, *et al.*, 1997). We also wanted to take advantage of the fact that beeswax is used in the construction of cells in which the larvae develop and therefore the animals should be familiar with its odour.

RESULTS AND DISCUSSION

Figure 6 shows the mean proportion of one day old AHBs and Uruçu honey bees responding to a single CS only test trial. The figure indicates no pseudoconditioning at any of the 4 test trials.

The low level of pseudoconditioning when beeswax is used is in direct contrast to the results obtained with adult AHBs. In our earlier pseudoconditioning study performed with adults (Abramson, Aquino, Silva, *et al.*, 1997) approximately 50 percent of the subjects (9 of 18 subjects) responded to each of the CS only test trials when beeswax was used.

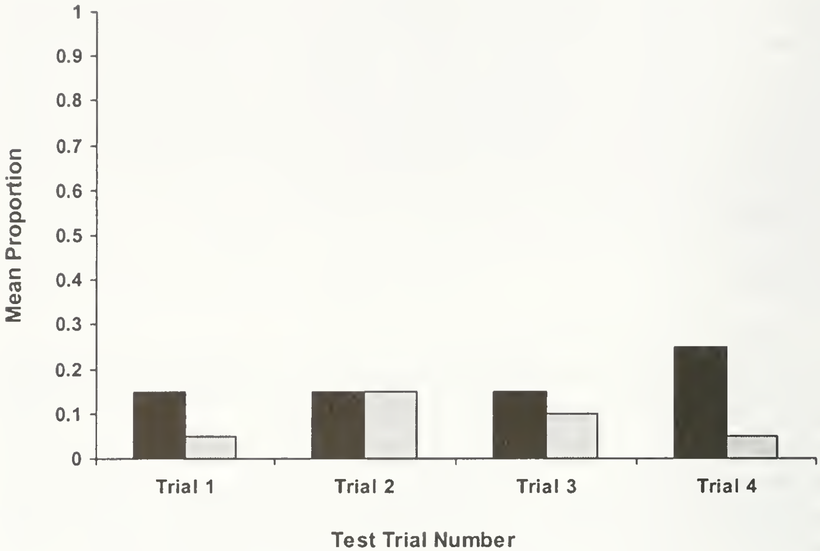

Figure 6. Mean proportion of one day old Africanized honey bees and adult Uruçu honey bees responding to a single CS only test trial. Black histograms represents performance of day old AHBs. Gray histograms represents performance of adult Uruçu bees. No pseudoconditioning is evident.

Results of the ANOVA indicated no Trial effect for the one-day old AHB $F(3, 79) = .768$, $p = .516$, no Trial effect for Uruçu bees $F(3, 79) = 1.034$, $p = .382$, and no Group effect between the AHBs and Uruçu bees $F(3, 152) = .628$, $p = .598$.

SERIES 3: CENTRAL EXCITATORY STATE STUDIES

In the previous two series neither Pavlovian conditioning nor pseudoconditioning was found to occur in day old honey bees or in adult Uruçu. In the present series of experiments the role of central excitatory state was studied. Central excitatory state (CES) refers to the temporary state of "excitement" generated in the nervous system of invertebrates following exposure to a US such as that provided by feeding on sucrose or honey. CES may serve as the basis of pseudoconditioning effects in invertebrates (Terry & Hirsch, 1997).

One hundred and twenty young Africanized honey bees (one day old bees) were selected from the laboratory hives and randomly placed into one of 6 groups consisting of 20 subjects each. They were captured and maintained as in the previous experiments. The primary difference between the groups was the amount of elapsed time between a 2 second feeding of sucrose and antennae stimulation with spring water. Under normal conditions honey bees will not extend their proboscis to water stimulation. Following a 2 second feeding of sucrose, one group was stimulated three seconds later with spring water, a second group seven seconds later, a third group 15 seconds later, a fourth group 30 seconds later, a fifth group 60 seconds later, and a sixth group 120 seconds later. Stimulation consisted of two strokes of the antennae with spring water saturated filter paper. If the proboscis extended the animal was allowed to feed on spring water for 2 seconds. The filter paper was handled with tweezers.

For the Uruçu experiments, one hundred and twenty adult animals were captured, maintained, and harnessed as in the previous series. The same experimental design used with day old Africanized bees was adopted with adult Uruçu with the exception of the US. The US was a 2 second feeding of Uruçu honey.

RESULTS AND DISCUSSION

In contrast to the Pavlovian and pseudoconditioning findings the results of the central excitatory state experiments are interesting. Figure 7 shows the mean proportion of one day old AHBs and Uruçu honey bees responding at each of the 6 post feeding intervals. Over 70% of the one-day old AHBs in our sample responded to water stimulation following a sucrose feeding at each of the post feeding intervals. Of the experiments reported here this is the first evidence for behavior modification in one day old Africanized honey bees.

The Africanized results are especially intriguing when compared to the Uruçu results. Figure 7 shows that about 50% of the Uruçu bees responded to water stimulation three seconds after a honey feeding. This percentage dropped to 20% seven seconds after the initial feeding. In contrast to the Africanized results no Uruçu bee responded 15, 30, 60, or 120 seconds following the honey feeding.

The results of an ANOVA indicate no Trial effect for the one-day old AHB $F(5, 114) = .348$, $p = .882$ and a Trial effect for the Uruçu bees $F(5, 114) = 13.918$, $p = .000$. A comparison between the one-day old AHBs and Uruçu bees revealed a significant Group effect $F(1,228) = 64.794$, $p = .000$, a significant Trial effect $F(5,228) = 2.296$, $p = .191$, and a significant Group x Trial interaction $F(5, 228) = 2.299$, $p = .046$.

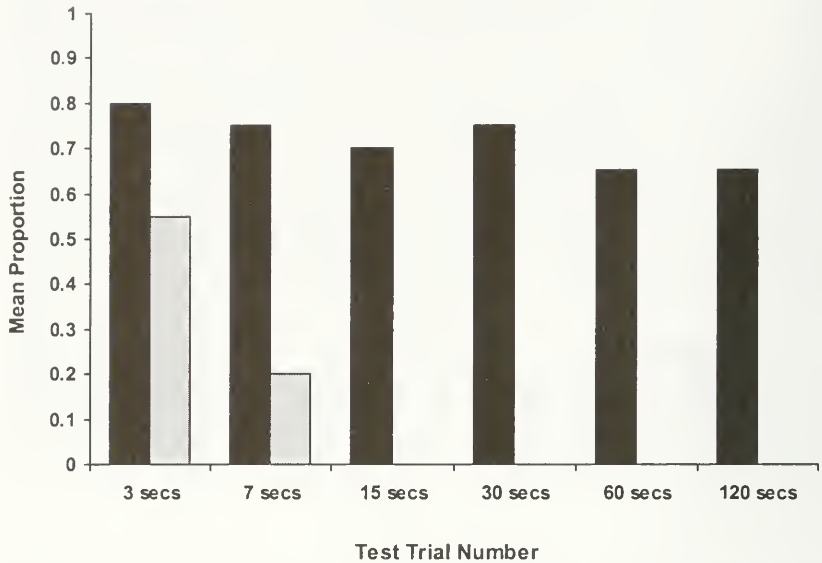

Figure 7. Mean proportion of one day old Africanized honey bees and adult Uruçu honey bees responding to water stimulation at each of the six post feeding intervals. Black histograms represents performance of day old AHBs. Gray histograms represents performance of adult Uruçu bees. Central excitatory state is evident in day old AHBs throughout all intervals tested but is restricted to the first interval in Uruçu bees.

SERIES 4: THE USE OF THE PROBOSCIS EXTENSION REFLEX AS A FEEDING ASSAY IN URUÇU

The previous series of experiments suggest that the proboscis extension reflex may not be useful for studies of Pavlovian conditioning, pseudoconditioning nor central excitatory state. The purpose of this series is two-fold. First we wanted to determine if the PER can be used as an assay to develop artificial diets for Uruçu. Second, we wanted to document the inability of sucrose to serve as a US for this honey bee.

Feeding experiments I: Discrimination between sucrose (2.9 M) and dark Melipona honey.

With the exception of one study using softdrinks (Abramson, Aquino, Azeredo *et al.*, 1997) and another using beeswax (Abramson, Aquino, Silva *et al.*, 1997) proboscis conditioning studies use some type of sucrose solution as a US or reward. In our initial attempts at proboscis conditioning we were surprised to find that sucrose failed to elicit a PER in Uruçu. The purpose of this experiment was to document this failure and to provide evidence that the proboscis extension technique can be used to study feeding preferences in Uruçu.

Twenty subjects were selected from the laboratory colony. Each animal received four exposures to sucrose and honey in a pseudorandom order (ABBA BAAB) where A and B represent the two substances (exposure to the substances were counterbalanced). The duration of feeding time was 2 seconds and there was a 10 minute intertrial interval.

Feeding experiments II: Discrimination between two solutions shown to be effective in Uruçu field experiments.

In attempts to find artificial diets for Uruçu the standard technique is to test the solution under field conditions in which foragers are observed to drink the solution and to return to the hive where the potential food source is placed in honey pots. If the pot is cap the food is considered acceptable to the colony. Such a field test can take two weeks. We believe the proboscis extension technique will provide a more rapid method for testing suitable artificial diets. To examine this idea we used the PER technique to see how readily animals will drink two solutions known to be effective from field studies (Aidar, 1996a, 1996b). One solution is composed of one part water to two parts honey. The other solution is composed of one part water, one part sugar, and one part honey (Alves, 1996). Twenty subjects were selected from the laboratory colony. Each animal received four exposures to both solutions in a pseudorandom order (ABBA BAAB) where A and B represent the two solutions (exposure to the substances were counterbalanced). The duration of feeding time was 2 seconds and there was a 10 minute intertrial interval.

RESULTS AND DISCUSSION

Figure 8a shows the mean proportion of animals feeding on either sucrose or honey (Feeding Experiment 1) and Figure 8b shows the mean proportion of animals feeding on Formula 1 or Formula 2 (Feeding Experiment 2). The two bars of Figure 8a indicate that Uruçu honey bees clearly do not feed on sucrose solutions but will feed on honey. The sucrose results were surprising given how readily *Apis* feeds on sucrose and points to the necessity of having a range of USs available for conditioning studies.

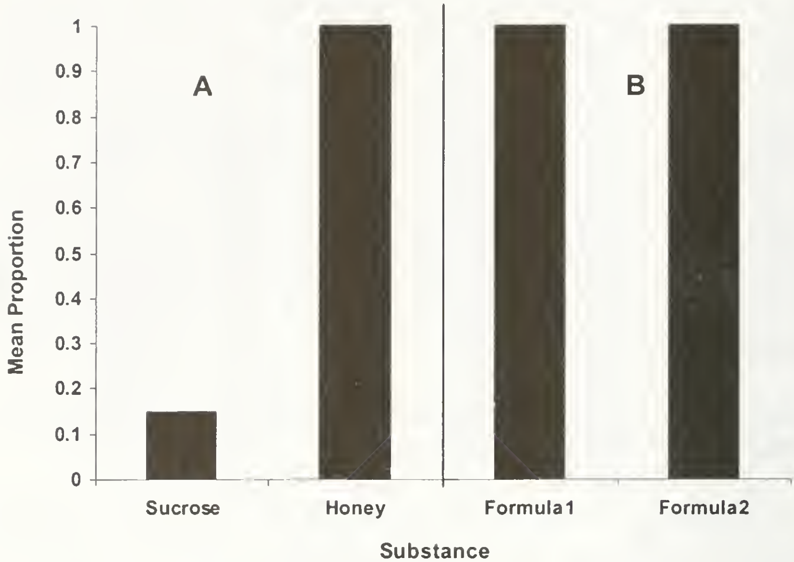

Figure 8. Panel A: Mean proportion of adult Uruçu honey bees responding to sucrose or Uruçu honey in a within subject design. Panel B: Mean proportion of adult Uruçu honey bees responding to two artificial diets in a within subject design. Sucrose is not an effective feeding stimulus for Uruçu bees.

Figure 8b shows that the two artificial diets, shown to be effective in field tests, also elicit feeding in restrained Uruçu. The results of the ANOVA revealed a significant Substance difference between Sucrose and Honey $F(1, 57) = 406.741$, $p = .000$. Because there was no variance between Formula 1 and Formula 2 and between Formula 1, Formula 2, and Honey, no meaningful statistical analysis could be run. It is evident from Figure 8, however, that these substances are readily consumed in contrast to sucrose.

We believe that the proboscis conditioning situation, though not effective in studying conditioning, can be successfully applied as a bioassay to rapidly screen for potential artificial diets suitable for field testing.

GENERAL DISCUSSION

The results of our experiments on the use of the proboscis extension technique to demonstrate learning in one day old Africanized bees and adult Uruçu were disappointing. In the Africanized experiments two different CSs were tried that were known to produce substantial levels of conditioning in adults (beeswax and Hexanal) as was a qualitatively different CS (water stimulation of the antennae). With all three CSs the performance of paired animals were low (no individual learned) and failed to differ from animals given unpaired CS/US presentations. The Uruçu experiments also employed a wide range of CSs (Hexanal, Geraniol, Citral, Uruçu wax, Africanized honey bee wax, and water stimulation to the antennae) and, as with the Africanized experiments, no conditioning was demonstrated.

The most obvious conclusion from such failures is that the proboscis conditioning technique is not effective with one day old Africanized subjects and adult Uruçu and new procedures need to be developed. There is also the possibility that day old Africanized subjects and adult Uruçu simply do not learn. Bhagavan *et al.* (1994), however, reported that five day old European honey bees could learn a Pavlovian discrimination task - one day old bees were not tested.

Negative results are never appealing and before it can be concluded that day old honey bees and adult Uruçu do indeed fail to learn under proboscis conditioning situations variations in training variables such as intertrial interval, CS and US duration, and stimulus intensity need to be examined. The results of the CES experiment (Series 3) in which day old AHBs responded consistently for two minutes suggests, for example, that lowering the intertrial interval would produce pseudoconditioning.

In addition to the manipulation of training variables we would suggest that training task be manipulated also. It is interesting to note that despite a failure to generate a consistent pattern of conditioned responses, the pattern of unconditioned responses was consistent with previous experiments - all animals responded to the US. The ability to record a consistent US response to sucrose (or honey in the case of Uruçu) suggests that the PER technique may be useful for studies of nonassociative learning such as habituation and sensitization and for associative learning studies involving instrumental conditioning situations such as punishment. The Central Excitatory State experiments clearly show that day old bees are able to modify their behavior. Such behavior is clearly adaptive and has been used to explain some examples of learning in *Drosophila* (Terry & Hirsch, 1997). In contrast to the Africanized experiments, Central Excitatory State in Uruçu was short lived.

The results of the Uruçu feeding experiments were more successful than the conditioning experiments and indicates that the PER technique will be useful in studies of feeding preferences of *Melipona.* As Aidar (1996a, 1996b) and Aidar & Campos (1998) showed, field tests of artificial diets can take two weeks before results are obtained. The PER method used here is a rapid alternative method of testing artificial diets in which results can be obtained in a single day of experimentation.

ACKNOWLEDGEMENTS

This research was funded by CNPq - Coordenação de Zootecnia e Veterinária (Grant # 451241/98-3). We would like to thank Francisco and Fábio Silva, and Paula Cristiane for their assistance and Dr. Davi S. Aidar for information on *Melipona* bees.

REFERENCES

Abramson, C. I. (1994). *A primer of invertebrate learning: The behavioral perspective.* APA: Washington, D.C.

Abramson, C. I., Aquino, I. S., Silva, M. C., & Price, J. M. (1997). Learning in the Africanized honey bee *Apis mellifera L. Physiology & Behavior, 62*, 657-674.

Abramson, C. I., Aquino, I. S., Azeredo, G. A., Filho, J. R. M., & Price, J. M. (1997). The attraction of Africanized honey bees (*Apis mellifera L.*) to soft drinks and perfumes. *Journal of General Psychology, 124*, 166-181.

Abramson, C. I., Aquino, I. S., & Ramalho, F. S. (1999). The effect of pesticides on the Africanized honey bee (*Apis mellifera L.)* on learning. *Archives of Environmental Contamination and Toxicology, 37*, 529-535.

Abramson, C.I., & Buckbee, D.A. (1995). Pseudoconditioning in earthworms (*Lumbricus terrestris*): Support for nonassociative explanations of classical conditioning phenomena through an olfactory paradigm. *Journal of Comparative Psychology, 109*, 390-397.

Aidar, D. S. (1996a). Multiplição e alimentação artifical de colônias de meliponíneos (Hymenoptera, Apidae, Meliponinae). *XI Congresso Brasileiro de Apicultura*, Teresina, PI, p. 99.

Aidar, D. S. (1996b). A mandaçaia: Biologia de abelhas, manejo e multiplicação artificial de colônias de *Melipona quadrifasciata* Lep. (Hymenoptera, Apidae, Meliponinae). *Sociedade Brasileira de Genetica*, Ribeirão Preto, SP.

Aidar, D. S. & Campos, L. A. O. (1998). Manejo e multiplicação artificial de colônias de *Melipona quadrifasciata* Lep. (Hymenoptera, Apidae, Meliponinae). *Anais da Sociedade Entomológica do Brasil. 27*, 157-159.

Alves, R. M. O. (1996). Meliponicultura: Aspectos práticos. XI Congresso Brasileiro de Apicultura. Teresina, PI, p. 95.

Aquino, I. S., Abramson, C. I., & Payton, M. E. (1999). A rapid bioassay for detection of adulterated beeswax. *Journal of Entomological Science, 34*, 265-272.

Bhagavan, S., Benatar, S., Cobey, S., & Smith, B. H. (1994). Effect of genotype but not of age or caste on olfactory learning performance in the honey bee, *Apis mellifera. Animal Behaviour, 48*, 1357-1369.

Bitterman, M.E, Menzel, R, Fietz, A, & Schäfer, S (1983) Classical conditioning of proboscis extension in honeybees *(Apis mellifera). Journal of Comparative Psychology*, 97, 107-119.

Buckbee, D.A., & Abramson, C.I. (1997). Identification of a new contingency-based response in honey bees (*Apis mellifera*) through revision of the proboscis extension conditioning paradigm. *Journal of Insect Behavior, 10*, 479-491.

Carew, T. J., Marcus, E. A., Nolen, T. G., Rankin, C. H., & Stopfer, M. (1990). The development of learning and memory in Aplysia. In J. L. McGaugh, N. M. Weinberger & G. Lynch (Eds.) *Brain organization and memory: Cells, systems, and circuits* (pp. 27-51). Oxford: Oxford University Press.

Evans, S.M. (1966a). Non-associative avoidance learning in Nereid polychaetes. *Animal Behaviour, 14*, 102-106.

Evans, S.M. (1966b). Non-associative behavioural modifications in the polychaete *Nereis diversicolor. Animal Behaviour, 14*, 107-119.

Getz, W. M., & Smith, K. B. (1987). Olfactory sensitivity and discrimination of mixtures in the honey bee, *Apis mellifera. Journal of Comparative Physiology, 160*, 239-245.

Grether, W. F. (1938). Pseudo-conditioning, without paired stimulation encountered in attempted backward conditioning. *Journal of Comparative Psychology, 25,* 91-96.

Harris, J. D. (1943). Studies of nonassociative factors inherent in conditioning. *Comparative Psychology Monographs, 18* (1, Serial# 93).

Kartzev, V. M. (1996). Local orientation and learning in insects. In C. I. Abramson, Z. P. Shuranova & Y. M. Burmistrov (Eds.) *Russian contributions to invertebrate behavior* (pp. 177-212). Westport, CT: Praeger.

Le Bourg, E. (1996). Hypergravity and aging in *Drosophila melanogaster. Gerontology, 42*, 235-240.

Lorenzon, M. C. (1996). Programa de proteção das abelhas indígenas sem ferrão na Paraiba. *XI Congresso Brasileiro de Apicultura*. Teresina, PI, p. 352.

Mackintosh, N. J. (1974). *The psychology of animal learning*. Academic Press: New York.

Mamood, A. N., & Waller, G. D. (1990). Recovery of learning responses by honeybees following a sublethal exposure to permethrin. *Physiological Entomology, 15*, 55-60.

Menzel, R. (1987). Memory traces in honey bees. In R. Menzel & A. Mercer (Eds.) *Neurobiology and Behavior of Honeybees*, (pp. 310-326). New York: Springer-Verlag.

Menzel, R. & Bitterman M.E. (1983). Learning by honeybees in an unnatural situation. In F. Huber & M. E. Mark (Eds.) *Neuroethology and behavioral Physiology: Roots and Grouping Points* (pp 206-215). Berlin: Springer-Verlag.

Menzel, R, Hammer, M, Braun, G, Mauelshaugen, J, & Sugawa M (1991). Neurobiology of learning and memory in honeybees, in L. J. Goodman & R. C. Fisher (Eds.) *The Behavior of and Physiology of Bees* (pp 323-353). Slough, UK: CAB International.

Mercer, A. (1987). Biogenic amines in the bee brain, in R. Menzel & A. Mercer (Eds.) *Neurobiology and Behavior of Honeybees*, (pp 214-233). Berlin: Spinger-Verlag.

Smith, B.H., Abramson, C.I., & Tobin, T.R. (1991). Conditioned withholding of proboscis extension in honey bees (*Apis mellifera*) during discriminative punishment. *Journal of Comparative Psychology, 105*, 345-356.

Smith, B. H., & Menzel, R. (1989). The use of electromyogram recordings to quantify odourant discrimination in the honey bee, *Apis mellifera. Journal of Insect Physiology, 35*, 369-375.

Stone, J.C., Abramson, C.I., & Price, J.M. (1997). Task dependent effects of dicofol (Kelthane) on learning in the honey bee (*Apis mellifera*). *Bulletin of Environmental Contamination and Toxicology, 58*, 177-183.

Taylor, K. S., Waller, G. D., & Crowder, L. A. (1987). Impairment of a classical conditioning response of the honeybee (*Apis mellifera L*) by sublethal doses of synthetic pyrethroid insecticides. *Apidologie, 18*, 243-252.

Terry, M. G., & Hirsch, J. (1997). The problem of measuring associative learning in *Drosophila melanogaster* with proper procedures to control for nonassociative effects. In G. Greenberg & E. Tobach (Eds.) *Comparative psychology of invertebrates: The field and laboratory study of insect behavior* (pp. 223-244). Garland Publishing, Inc: New York.

Wells, M. (1968). *Lower animals*. Weidenfeld and Nicolson: London.

Young, J. Z. (1960). Unit processes in the formation of representations in the memory of Octopus. *Proceedings of the Royal Society (Series B), 153*, 1-17.