

STUDIES ON HONEY BEE (*APIS MELLIFERA* L.) BEHAVIOR:  
PREFERENCES FOR ETHANOL  
SOLUTIONS *AND* SUB-LETHAL EFFECTS  
OF TWO INSECT GROWTH  
REGULATORS

By

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## LIST OF EQUATIONS

$$[3.1] \quad Y = B3*\exp(-B2*x) + B4*(1-\exp[-B2*x])$$

$$[3.2] \quad \% = \frac{e^{lsmean}}{1 + e^{lsmean}}$$

## PREFACE

This thesis is comprised of two studies which investigate learning in the honey bee (*Apis mellifera* L.). The first study is a continuation of a previous analysis evaluating the honey bee as a potential biological model for human alcoholism research (Abramson et al. 2000). The second study is designed to test for sub-lethal effects that “harmless” agrochemicals may have on the learning capacity of bees. The methods used in these experiments are based on conventional *modus operandi* for the conditioning of both harnessed bees and free-flying foragers (Bitterman et al., 1983; Getz and Smith, 1987; Marfaing et al., 1989; Ladurner et al., 2003). All methods exercise the principle of “appetitive conditioning” to train bees either to extend their proboscis to an olfactory stimulus, or to visit an artificial flower on foraging flights.

# CHAPTER I

## LITERATURE REVIEW

### **Introduction**

In the eyes of the behaviorist, the honey bee is unique among insects for its well-characterized habits and remarkable degree of trainability. Thanks to a long history of observation of this ubiquitous pollinator, scientists have a pretty good understanding of the various motivations shaping bee behavior, and can manipulate these motivations to learn more about the acquired behaviors of honey bees. Like every other living organism, much of the honey bee's activity is dictated by the environment and circumstances it experiences while performing tasks. The degree of external influence would therefore vary with "hive" versus "foraging" duties. One particular interest is the "choice" behavior of foraging bees. What makes one flower more appealing than another flower of equal nectar value? How do olfactory cues compare with visual cues in attracting bees to a nectar source? Does learning occur as a function of the number of rewards (feedings), or is it a temporal process that occurs in consecutive steps? The following sections attempt to uncover some of the aspects of honey bee learning, the methodology used to test for learning, and the applications for honey bee behavioral research.

## **Bioassay: The Proboscis Extension Reflex**

The purpose of a bioassay is to quantify an organism's responses to a stimulus in terms of some biological process or instinct of that organism. This allows consistent results to be achieved for the testing of multiple subjects of the same species. Feeding behavior is one of the strongest instincts among animals, and is often favored by the experimenter for producing a reliable mechanism for behavioral research (Menzel, 2001). For the honey bee, the feeding mechanism is the Proboscis Extension Reflex (PER), an involuntary unfurling of the proboscis when an appetitive chemical cue is presented to the antennae. The instinct is so highly developed in bees that in a classical conditioning experiment, a single training trial (neutral odor followed immediately by sucrose feeding) is sufficient to reproduce the PER in subsequent trials using the odor only (Brandes et al., 1988; Bitterman et al., 1983).

It is necessary that the PER is very acute for an insect which relies on trophallaxis (food sharing by way of regurgitation) as a means of supplying nutrients and foraging information to the other members of the colony. Studies both past and present have implied that the PER undergoes natural olfactory conditioning when foragers share their scented nectar crop with experientially naïve hive bees (Nixon and Ribbands, 1952; Ribbands, 1954; von Frisch, 1967; Gil and De Marco, 2005). PER conditioning has also been described in terms of memory formation in free-flying foraging bees. Repeated encounters with a nectar source are stored as long-term memories which can be retrieved in the laboratory using olfactory stimulation of the PER (Gerber et al., 1996). Other research has shown that there is brain-regulated behavioral plasticity in worker bees

which is mediated by the deficit of certain duties, and which affects the sensitivity of the PER throughout the life of the bee (Robinson, 1992; Schulz and Robinson, 1999).

All worker bees demonstrate the ability to learn an olfactory association using the PER mechanism, regardless of age or caste (Bhagavan et al., 1994), but the velocity of learning is determined by each individual's sucrose response threshold. In general, sucrose responsiveness increases with age, with foragers having a greater predisposition to associative learning than guard and nurse bees (Scheiner et al., 2003). Bees altogether lacking the PER behavior in laboratory conditioning trials appear to be the result of genetic predisposition (Bhagavan et al., 1994).

### **The Role of Olfactory Cues in PER Conditioning**

Karl von Frisch pioneered olfactory conditioning of the honey bee feeding response in the first half of the twentieth century using free-flying subjects and an array of odorants (von Frisch, 1967). He observed that hive bees would learn the location of a food source by associating a floral odor on a forager's body with the nectar shared by the forager through trophallaxis. Von Frisch's experiments demonstrated the importance of olfactory learning to the nourishment of the hive, but they did not give insight into the capacity individual bees possessed for learning new olfactory associations .

Pavlovian, or "classical" conditioning of the PER was developed by the Japanese scientist Kuwabara (1957) some years later to facilitate laboratory investigations of associative learning in harnessed honey bees. Classical conditioning is defined as the

training of a natural response mechanism, such as the PER, to the presentation of a conditioned stimulus (CS), i.e. a neutral odor, through the pairing of the CS with an unconditioned stimulus (US), i.e. sucrose. While Kuwabara's investigations employed visual stimuli, colored light and water vapor, as conditioned stimuli, his strategy was applied to olfactory conditioning by his successor, Takeda. Takeda trained the PER of harnessed foragers to presentations of either citral or cinnamic aldehyde through the pairing of these experimentally neutral odors with a sucrose feeding (Takeda, 1961). Not only did his experiments prove that olfaction was more closely related to the PER than visual perception, but he also demonstrated that honey bees have the ability to make a discrimination between two odors when only one of the odors was reinforced with a sucrose feeding.

Further investigation of the olfactory-induced PER revealed that wild-caught experienced foragers will produce the response in the laboratory when given food-source odors, whereas non-foraging bees which have been exposed to the same odors in the hive do not produce the PER response (Gerber et al., 1996). These results indicate that the PER is developed through first-hand experience with a food-source, not through the impregnation of hive comb with floral odor. Subsequent studies have focused on the factors influencing the rate of learning in olfactory conditioning experiments. These factors include CS intensity, the degree of chemical relatedness between two CS's in a discrimination study, the length and number of training trials given, the amount and concentration of the sucrose reward (US), and the frequency of US presentations (Erber,

1975; Getz and Smith, 1987; Brandes et al., 1988; Buchanan and Bitterman, 1989; Couvillon and Bitterman, 1993; Bhagavan and Smith, 1997).

### **PER as an Indicator of Insecticide Toxicity**

Broad-spectrum chemical insecticides have long been known to have detrimental effects on honey bees, but recently scientists have uncovered potential hazards in target-specific insecticides, namely insect growth regulators (IGR's), fungicides, and biorational insecticides (Cantwell et al., 1966; Mussen et al., 2004; Thompson et al., 2005; Decourtye et al. 2005). While most of these insecticides do not cause direct mortality, and that only when administered in high oral doses (Cantwell et al., 1966), behavioral side-effects have been observed that may result in an overall reduction in colony integrity (Smirle et al., 1984; Vandame et al., 1994). Toxicity tests have been carried out for both cuticular and oral contamination of bees, though the focus of the study presented in Chapter Three is on oral sub-lethal toxicity of insect growth regulators.

The majority of sub-lethal toxicity studies are performed as field trials using established hives and insecticide-laced sucrose feeders (Schricker and Stephen, 1970; Thompson et al., 2005; Faucon et al., 2005; Ladurner et al., 2003). This method allows for the observation of foraging behavior, communication dances, and brood production as affected by an insecticide, but it does not give good information on the impact the chemical may have on an individual honey bee brain. Whole-brain or whole-bee laboratory assays are good techniques for measuring the amount of insecticide present in the target tissue post-exposure, respective to the mode of action of the insecticide. For

example, whole-brain acetylcholinesterase analysis is used to measure the effect of organophosphate insecticides, which act as acetylcholinesterase inhibitors in the central nervous system of most insects, on enzyme activity levels (Weick and Thorn, 2002). Fat body analyses can also expose trace amounts of insecticide which are transported to these structures to be metabolized. However, the accuracy of these tests is often brought into question in cases of low-dose or sub-lethal exposure to insecticides, as trace amounts of the chemicals may not be detected.

Proboscis conditioning is considered to be a more sensitive indicator of neurophysiologic changes due to insecticide exposure (Vandame et al., 1994; Weick and Thorn, 2002; Decourtye et al., 2005). Bees that show no outward signs of sub-lethal poisoning may be experiencing retardation or disruption of nervous transmission, which may in turn be expressed as inhibition in learning. Chapter Three discusses the issue of sub-lethal insecticide poisoning in honey bees, as well as implications from preliminary proboscis conditioning studies that support the methodology.

### **Honey Bees and Alcoholism Research**

Chapter Two introduces a non-agricultural application of PER conditioning in honey bees: developing an invertebrate model for human alcoholism research. Currently, small mammalian species such as rats and mice are the predominant choice for alcoholism research, but these subjects have presented some problems to the endeavor. Among these are a natural aversion to high-content ethanol solutions, the high cost associated with genetically manipulating rodents to create an affinity for alcohol, and the

dissimilarities in ethanol metabolism between rodents and humans (Swanson et al., 2004; Höög et al., 2001). From an economic standpoint, and perhaps a biologic one, it would be beneficial to find a more suitable animal model. Various invertebrates have been sized up to the task, including *Drosophila* flies and roundworms, which both have the added bonus of completely described genomes, but lack human genetic characteristics. Murine animals have the attractive characteristic of producing mutations which resemble human genotypes, yet they are expensive to work with (Swanson et al., 2004). In general, as animal complexity increases, so does the cost of experimentation and the ethical complications of animal testing. Invertebrates would offer both a cheap and a virtually un-debated moral solution if an appropriate species existed.

Enter the honey bee. Although genomic information is lacking, the well-described behavior of honey bees coupled with their relatively large size (compared with other invertebrate models), high reproductive rate, availability, low cost, and natural ability to consume and metabolize alcohol makes this insect attractive for such research (Abramson et al., 2003). Even beyond these qualifications, the honey bee possesses social characteristics that bear an uncanny likeness to human social behaviors. Naturalists have noted the similarities in social Hymenopteran (namely bees and ants) and human behaviors for over a century, but an interesting angle observed by few is the way in which both creatures react to alcohol consumption.

The first account of insect behavior as influenced by alcohol consumption was recorded by naturalist John Lubbock (1888) under the title *On the Senses, Instincts, and*

*Intelligence of Animals*. The subjects of his experiment were feral ants. Having fed an ethanol concoction to a heterogeneous population of worker ants, he noted the treatment of the mixed group of inebriated workers by sober guard ants from one colony: “The sober ants were rather puzzled; but after examining the intoxicated individuals, they picked up the strangers and threw them into the ditch, while they carried their own friends into the nest, where they no doubt slept off the effects of the spirits (pp. 233-234)”

Lubbock (1888) remarked that his intoxicated ants exhibited behavior that would be just as well described of humans in a similar state of drunkenness. Clearly, there exists a parallel between human social interactions and those of social Hymenoptera. Honey bees are excellent examples of social Hymenoptera whose behavior can be likened to that of humans, particularly in the manner they consume and metabolize alcohol. Both bees and humans produce a similar form of the enzyme alcohol dehydrogenase along with aldehyde dehydrogenase, which are responsible for the degradation of ethanol into carbon dioxide and water.

Although honey bees do not consume pure ethanol as a part of their natural diet, it has been shown that worker bees will imbibe 190 proof ethanol under laboratory conditions. This remarkable conclusion resulted from a three-part study conducted by Abramson et al. (2000) that was designed to assess the suitability of the honey bee to human alcoholism research. The objective of the initial investigation was to determine whether ethanol was aversive to bees in either a free-choice or no-choice situation. Laboratory experiments were conducted on harnessed foragers (the preparation of harnessed foragers

is described and illustrated in Bitterman et al., 1983), and free-choice experiments in the context of a natural setting using an established hive.

## References

**Abramson, C. I., G. Fellows, B. Browne, A. Lawson and R. Ortiz. 2003.**

Development of an ethanol model using social insects: II. Effect of antabuse on consumatory responses and learned behavior of the honey bee (*Apis mellifera* L). *Psych. Rep.* **92**: 365-378.

**Abramson, C. I., S. Stone, R. Ortez, A. Luccardi, K. Vann, K. Hanig and J. Rice.**

**2000.** The development of an ethanol model using social insects I: Behavioral studies of the honey bee (*Apis mellifera* L.). *Alcohol Clin Exp Res.* **24**(8): 1153-1166.

**Bhagavan, S. and B. Smith. 1997.** Olfactory conditioning in the honey bee, *Apis mellifera*: Effects of odor intensity. *Physiol. & Behav.* **61**(1): 107-117.

**Bhagavan, S., S. Benetar, S. Cobey and B. Smith. 1994.** Effect of genotype but not of age or caste on olfactory learning performance in the honey bee, *Apis mellifera*. *Anim. Behav.* **48**: 1357-1369.

**Bitterman, M. E., R. Menzel, A. Fietz and S. Schäfer. 1983.** Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psych.* **97**(2): 107-119.

**Brandes, C., B. Frisch, and R. Menzel. 1988.** Time-course of memory formation differs in honey bee lines selected for good and poor learning. *Anim. Behav.* **36**: 981-985.

**Buchanan, G. and M. E. Bitterman. 1989.** Learning in honeybees as a function of amount of reward: Tests of the equal-asymptote assumption. *Anim. Learn. Behav.* **17**(4): 475-480.

- Cantwell, G., D. Knox, T. Lehnert and A. S. Maichael. 1966.** Mortality of the honey bee, *Apis mellifera*, in colonies treated with certain biological insecticides. *J. Invert. Path.* **8**: 228-233.
- Couvillon, P. and M. E. Bitterman. 1993.** Learning in honeybees as a function of amount of reward: Further experiments with color. *Anim. Learn. Behav.* **21**(1): 23-28.
- Decourtye, A., J. Devillers, E. Genecque, K. Le Menach, H. Budzinski, S. Cluzeau, and M.H. Pham-Delègue. 2005.** Comparative sub-lethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Arch. Environ. Contam. Toxicol.* **48**: 242-250.
- Erber, J. 1975.** The dynamics of learning in the honey bee (*Apis mellifera carnica*). I. The time dependence of the choice reaction. *J. Comp. Physiol.* **99**: 231-242.
- Faucon, J.-P., C. Aurières, P. Drajnudel, L. Mathieu, M. Ribière, A.-C. Martel, S. Zeggane, M.-P. Chauzat and M. Aubert. 2005.** Experimental study on the toxicity of imidacloprid given in syrup to honey bee (*Apis mellifera*) colonies. *Pest Manag. Sci.* **61**: 111-125.
- Gerber, B., N. Geberzahn, F. Hellstern, J. Klein, O. Kowalsky, D. Wüstenberg and R. Menzel. 1996.** Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Anim. Behav.* **52**: 1079-1085.
- Getz, W. and K. Smith. 1987.** Olfactory sensitivity and discrimination of mixtures in the honeybee *Apis mellifera*. *J. Comp. Physiol. A* **160**: 239-245.

- Gil, M. and R. De Marco. 2005.** Olfactory learning by means of trophallaxis in *Apis mellifera*. *J. Exper. Biolo.* **208**: 671-680.
- Höög, J-O, J. Hedberg, P. Strömberg, and S. Svensson. 2001.** Mammalian alcohol dehydrogenase—functional and structural implications. *J. Biomed. Sci.* **8**(1): 71-76.
- Kuwabara, M. 1957.** Bildung des bedingten Reflexes von Pavlovs Typus bei der Honingbiene, *Apis mellifica*. *J. Fac. Sci. Hokkaido Univ. (Zool.)* **13**: 458-464.
- Ladurner, E., J. Bosch, S. Maini and W. Kemp. 2003.** A method to feed individual bees (Hymenoptera: Apiformes) known amounts of pesticides. *Apidologie* **34**: 597-602.
- Lubbock, J. 1888.** On the senses, instincts and intelligence of animals. D. Appleton and Company, New York. pp. 233-234.
- Marfaing, B., J. Rouault, and P. Laffort. 1989.** Effect of the concentration and nature of olfactory stimuli on the proboscis extension of conditioned honey bees *Apis mellifica Ligustica*. *J. Insect Physiol.* **35**(12): 949-955.
- Menzel, R. 2001.** Searching for the memory trace in a mini-brain, the honey bee. *Learn. & Mem.* **8**: 53-62.
- Mussen, E., J. Lopez, and C. Peng. 2004.** Effects of selected fungicides on growth and development of larval honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *Physiol. Ecol.* **33**(5): 1151-1154.
- Nixon, H. L., and C. R. Ribbands. 1952.** Food transmission within the honey bee community. *Proc. R. Soc. Lond. B.* **140**: 34-50.
- Ribbands, C. R. 1954.** Communication between honey bees. I. the response of crop-attached bees to the scent of their crop. *Proc. R. Soc. Lond. A* **29**: 141-144.

- Robinson, G.E. 1992.** Regulation of division of labor in insect societies. *Ann. Rev. Entomol.* **37**: 637-665.
- Schricker, B. and W. P. Stephen. 1970.** The effect of sub-lethal doses of parathion on honeybee behaviour. I. Oral administration and the communication dance. *J. Apic. Res.* **9**(3): 141-153.
- Scheiner, R., R. Page, and J. Erber. 2003.** Sucrose responsiveness and behavioral plasticity in honey bees (*Apis mellifera*). *Apidologie* **35**: 133-142.
- Schulz, D.J., and G. E. Robinson. 1999.** Biogenic amines and division of labor in honey bee colonies: behaviorally related changes in the antennal lobes and age-related changes in the mushroom bodies. *J. Comp. Physiol. A.* **184**: 481-488.
- Smirle, M. J., M. Winston, and K. Woodward. 1984.** Development of a sensitive bioassay for evaluating sub-lethal pesticide effects on the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **77**: 63-67.
- Swanson, K., M. Mazur, K. Vashisht, L. Rund, J. Beever, C. Counter, and L. Schook. 2004.** Genomics and clinical medicine: rationale for creating and effectively evaluating animal models. *Exp. Biol. Med.* **229**: 866-875.
- Takeda, K. 1961.** Classical conditioned response in the honey bee. *J. Insect Physiol.* **6**: 168-179.
- Thompson, H., S. Wilkins, A. Battersby, R. Waite, and D. Wilkinson. 2005.** The effects of four insect growth regulating (IGR) insecticides on honeybee (*Apis mellifera* L.) colony development, queen rearing and drone sperm production. *Ecotoxicology* **14**(7): 757-769.

**Vandame, R., M. Meled, M-E Colin, and L. Belzunces. 1994.** Alteration of the homing-flight in the honey bee *Apis mellifera* L. exposed to sub-lethal dose of deltamethrin. *Environ. Toxicol. Chem.* **14**(5): 855-860.

**von Frisch, Karl. 1967.** The dance language and orientation of bees. Harvard University Press, Cambridge, MA.

**Weick, J., and R. Thorn. 2002.** Effects of acute sub-lethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **95**(2): 227-236.

CHAPTER II  
THE DEVELOPMENT OF AN ETHANOL MODEL USING SOCIAL INSECTS III:  
PREFERENCES TO ETHANOL SOLUTIONS

**Abstract**

The following experiment was designed to determine whether free-flying honey bees have an aversion to an ethanol solution when given a choice between targets containing an ethanol solution in sucrose or sucrose only. Honey bees given a choice between a 1% ethanol solution and pure sucrose solution showed no preference to the sucrose solution in either the acquisition or extinction phase of the experiment. Bees given a choice between a 5% ethanol solution and sucrose solution showed no differences in the initial choice of targets, but some bees did switch over to the sucrose-only target as the experiment progressed. During the extinction phase, bees landed on the previously reinforced sucrose target more often than the target previously containing the 5% ethanol solution. An experiment in which bees were given a single 5% ethanol target revealed that of 20 bees, 11 returned for the entire twelve trials of the experiment. All bees returned at least six times to the 5% ethanol target. Analysis of data showed that honey bees do not have a preference for sucrose solution over 1% and 5% ethanol solutions.

## Introduction

This experiment stemmed from a multi-part effort to explore the possibilities of using honey bees as an invertebrate model for human alcoholism research. The research focused initially on the behavior of honey bees to produce a catalog of similarities and differences in the ethanol-induced behaviors of bees and humans. This comparative approach differed substantially from the simple systems approach used in fruit fly, *Drosophila melanogaster* Meigen, and nematode, *Caenorhabditis elegans* Maupas, models of alcoholism (Rothenfluh and Heberlein, 2002; Wolf and Heberlein, 2003). In these models more attention was paid to molecular and biochemical effects of ethanol than its influence on behavior. In response to a lack of empirical behavioral data, the recent research on honey bees has addressed basic behavioral issues associated with ethanol consumption.

The experiment presented in this chapter was the third in an ongoing series of behavioral research. The first experiment was a preliminary investigation of the effects of several concentrations of ethanol on locomotion, stinging response, proboscis extension, and foraging behaviors (Abramson et al., 2000). In addition, self-administration of sucrose containing ethanol was observed to determine at what concentration ethanol was aversive to bees. This series of investigations proved that ethanol works as a depressant on bees' systems much in the same way as it does on vertebrates, and that bees will self-administer ethanol solutions (in sucrose) as great as 20%. The second experiment in the series tested the effects of Antabuse®, an emetic drug used to treat alcoholics, on consumption of ethanol by harnessed foragers (Abramson et al., 2003). Although the results from this

experiment were mixed, there was some indication that bees pretreated with Antabuse® lost their appetite for ethanol over time. This was even more pronounced when ethanol-fed bees were compared with sucrose-fed bees, who were unaffected by the Antabuse® treatment.

The following experiment was designed to further examine the results of a particular component of the first study (Abramson et al., 2000)—self-administration of ethanol in free-flying marked foragers. The preliminary results showed that marked bees would return to a feeding station containing 1% or 5% ethanol solution. The present experiment determined whether free-flying honey bees had an aversion to ethanol solution when given a choice between 1% or 5% ethanol, and a sucrose solution. In this experiment, free-flying foragers were simultaneously given a target containing ethanol and a target containing sucrose. Each target was assigned a unique color and a fixed location at the experimental station. This design was based on the behavioral tendency towards “flower constancy” in foraging bees, which allows them to find food after a previous association of flower color and location, to nectar content (Hill et al., 1997). Participating bees were given a two-phase training session consisting of the acquisition and extinction of the “flower constancy” behavior. Observations were made of initial target choice, target switching based on solution preference, or target constancy based on the flower constancy behavior. The position of targets was also changed intermittently to observe for a location preference. Care was taken to control all factors except the solution (ethanol or sucrose) choice, in order to ascertain whether an aversion to the ethanol solutions was determining foraging behavior.

## Materials and Methods

In fall 2003, 60 European honey bees were selected at random from a hive located in a greenhouse (Fig. 1). Approximately 10 meters from the hive, a table was set with two grey targets constructed from disposable Petri dishes (5.5 cm in diameter). A 2.5 cm diameter patch of either yellow or orange was affixed to the targets (Fig. 2). These colored stimuli were found in previous experiments to be equally discernable to bees (Abramson et al., 1996).



**Figs.1 and 2.** The greenhouse hive (above) was placed at the far end of the greenhouse, and the experimental station was set up on a table located in the middle of the greenhouse, two tables behind the one pictured above. Pictured at left are colored targets being prepared with ethanol and sucrose solution.

Subjects were divided into 3 groups of 20. Groups 1 and 2 made a choice between a 1% ethanol solution and 1.8 M sucrose or between a 5% ethanol solution and 1.8 M sucrose, respectively. The ethanol solutions were prepared by diluting pure ethanol with 1.8 M sucrose to create final volumes of either 1% or 5% ethanol solutions. The addition of sucrose to the ethanol solution was comparable to a “mixed drink.” Previous experiments have shown that honey bees will also consume ethanol without the addition of sucrose (Abramson et al., 2000). The purpose for imbedding the ethanol in an appetitive

substance was to observe the degree of aversiveness, if any, of ethanol. If the ethanol was aversive, bees would land more frequently on the target associated with sucrose than the target associated with ethanol over the course of the experiment. Group 3 was monitored over 12 visits to the 5% ethanol only target. The purpose of Group 3 was to provide an additional check to determine if the 5% ethanol solution was aversive. If so, bees would be expected to stop feeding on the target after only a few visits. The work was done with marked foragers, which were experimentally naïve and trained in a single session. Solutions of 1% and 5% ethanol were used because previous experiments (Abramson et al., 2000, 2003) indicated that ethanol concentrations greater than 5% severely disrupt the learned behavior of honey bees. To control for calendar variables, bees from each group were run daily. The intertrial intervals and feeding durations were controlled by each individual bee. Following each visit, targets were washed thoroughly with tap water and dried to remove any pheromones or other contaminants left by the foragers. The distance between the targets (center to center) was approximately 20 centimeters.

Subjects were selected at random from a group of foragers at a feeding station providing a 0.367 M sucrose solution (Fig. 3). A single bee was obtained in a small matchbox, carried to a gray target, and while feeding on a 1.8 M sucrose solution droplet, marked with a spot of colored lacquer (Figs. 4 and 5). The experiment began when the bee returned to the gray target twice. Several placements were often required before the subject would return to the target. Occasionally, “intruder bees” would return with the subject, having been recruited during a return visit to the hive (Fig. 6). These bees were

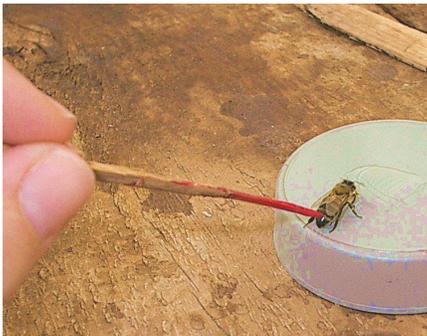
caught in a matchbox and contained until the experiment ended. Detailed instructions on how to establish a feeder and to attract a bee from the feeder to a target are available from Abramson (1990).



**Fig.3.** The sucrose feeding station.



**Fig.4.** A bee feeds from the “neutral” grey target at the experiment station during pre-training phase.



**Fig.5.** Dotting colored lacquer on the abdomen of a bee at the grey target.



**Fig.6.** A marked bee (left) has returned with an “intruder bee” (right).

The experiment consisted of a pre-training phase, an acquisition phase, and an extinction phase. The first phase of the experiment consisted of 4 pre-training visits, during which bees received exposure to either the orange or yellow target in a pseudorandom order (the

sequence “ABBA” with “A” being orange for half the animals and yellow for the remaining animals). Each target contained a large drop of 1.8 M sucrose. The rationale behind pre-training was to insure that each bee had experience with both targets prior to the acquisition phase.

Visits five through 16 comprised the acquisition phase. During the 12 acquisition trials, bees were presented with the yellow and orange targets simultaneously. One of these targets was always associated with an ethanol solution (either 1% or 5%) and the other target always associated with a 1.8 M sucrose solution. For half the bees, ethanol was associated with the yellow target; for the remaining subjects, ethanol was associated with the orange target. In addition to counterbalancing the stimuli, the left-right position of the targets were changed up in a pseudorandom order using the sequence **ABBABAABABBA**. This pseudorandom sequence insured that the ethanol and sucrose targets appeared on both sides equally. The ethanol and sucrose solutions were applied to the targets with individual eye droppers. A large drop was used, and care was taken to ensure that they were the same size. The rationale behind the use of a large drop instead of, for instance, a 5  $\mu$ l droplet was to eliminate the possibility of the ethanol evaporating. When  $\mu$ l droplets are used, the ethanol will evaporate within 30 seconds (Abramson et al., 2000). The dependent variable consisted of the initial choice made by the bee.

Following the 16<sup>th</sup> visit, the extinction phase of the experiment began and lasted for 10 minutes. During extinction, both targets contained a large drop of tap water. The dependent variable consisted of the number of landings on each target during 20

consecutive, 30 second intervals. The position of the targets during extinction was also counterbalanced as described above. Subjects in Group 3 did not participate in this phase. Following extinction, each subject was captured and eliminated.

## **Results**

### *Acquisition*

The effect of trial on the within subject factor was analyzed with a repeated measures ANOVA (SPSS, Inc., 1999). The mean proportion of landing on the 5% ethanol target and the 1% ethanol target across the 12 trials is depicted in Figure 7. There was no significant difference in mean acquisition of the 5% target across the 12 trials ( $F_{11,9} = 1.234, p = .381$ ). Similarly, there was no significant difference in mean acquisition of the 1% ethanol target across the 12 trials ( $F_{11,9} = .609, p = .784$ ).

### *Extinction*

The effect of trials on the within subject factor was analyzed with a paired samples *t*-test (SPSS, Inc., 1999). The mean cumulative frequency of landing on the target previously associated with a 5% ethanol solution across the 20 time intervals is depicted in Figure 8. There was a significant difference in mean extinction between the target previously associated with 5% ethanol ( $\underline{M} = 14.8, \underline{SD} = 11.2$ ) and the target previously not associated with ethanol ( $\underline{M} = 18.3, \underline{SD} = 12.9, t_{19} = -2.852, p = .01$ ). The mean frequency of landing on the target previously associated with 5% ethanol was significantly lower. Figure 9 illustrates the mean cumulative frequency of landing on the target previously associated with 1% ethanol across the 20 time intervals. There was no

significant difference in mean extinction between the target previously associated with 1% ethanol ( $\underline{M} = 11.9$ ,  $\underline{SD} = 10.0$ ) and the target previously not associated with ethanol ( $\underline{M} = 15.1$ ,  $\underline{SD} = 13.0$ ,  $t_{19} = -1.648$ ,  $p = .116$ ).

#### *Ethanol acquisition and extinction contrasts*

The effect of group on the between subjects factor was analyzed with an independent  $t$  test design. The mean frequency of landing on the targets associated with the 5% and 1% ethanol across the 12 trials revealed a significant difference in acquisition between the 5% ( $\underline{M} = 0.40$ ,  $\underline{SD} = .0.1$ ) and 1% ( $\underline{M} = 0.5$ ,  $\underline{SD} = 0.1$ ) ethanol groups, ( $t_{22} = -2.897$ ,  $p = .008$ ). Mean frequency of landing on the 1% ethanol target was significantly higher.

During extinction, there was no significant difference in landing between the targets previously associated with 5% ethanol ( $\underline{M} = 14.8$ ,  $\underline{SD} = 11.2$ ) and 1% ethanol ( $\underline{M} = 11.5$ ,  $\underline{SD} = 10.0$ ), ( $t_{38} = .845$ ,  $p = .403$ ). Similarly, there was no significant difference in landing between the non-ethanol targets in the 5% ethanol experiment ( $\underline{M} = 18.3$ ,  $\underline{SD} = 12.9$ ) and in the 1% ethanol experiment ( $\underline{M} = 15.2$ ,  $\underline{SD} = 13.0$ ), ( $t_{38} = .769$ ,  $p = .447$ ).

#### *Ethanol target transition and number of trials completed*

During the course of the experiments, several instances occurred in which bees landed initially on an ethanol target, consumed a few microliters of the solution, and flew immediately thereafter to the non-ethanol target. Of the 20 bees in the 5% ethanol group, there were 6 occasions (out of a possible 240 opportunities) in which a bee left the

ethanol target and flew to the non-ethanol target. Only 1 bee in the 1% ethanol group exhibited such target transition behavior. The statistical analysis revealed a significant difference in target transition between the 5% ( $\underline{M} = 0.45$ ,  $\underline{SD} = 0.76$ ) and 1% ( $\underline{M} = 0.50$ ,  $\underline{SD} = 0.6$ ) ethanol targets ( $t_{38} = 2.26$ ,  $p = .03$ ). Finally, in the experiment in which bees were not given an alternative to a 5% ethanol target over the course of 12 visits, all animals returned for a minimum of 6 visits. Eleven of 20 animals completed all 12 visits, one completed 10 visits, six completed eight visits, one completed seven visits, and one completed six visits.

## **Discussion**

The results of the experiments show that honey bees do not have an aversion to low concentrations of ethanol, and will self administer ethanol when given an alternative choice of pure sucrose. In experiments with foragers visiting the experimental arena on their own accord (i.e., self-administration) there were no significant differences in choice behavior between a 1% ethanol target and a target containing only sucrose. The bees developed a position preference which ensured that during 6 of the 12 visits, ethanol would be consumed. In the extinction phase of the experiment, no differences were detected in the number of landings between the two targets.

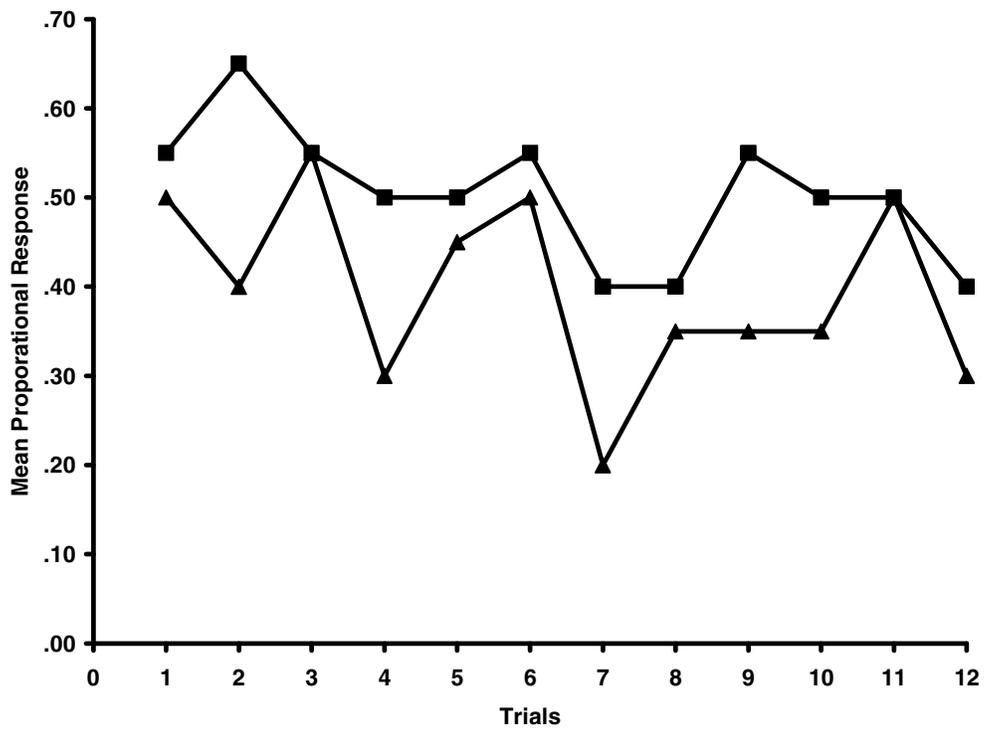
Bees given a choice between a 5% ethanol target and a sucrose target also showed no significant partiality to the sucrose-only solution when simultaneously offered both. However, there were some bees that flew off the 5% target to the sucrose-only target, and there was a difference in the extinction curves between the 5% and sucrose-only targets

when both targets were replaced with water. When considering whether a 5% ethanol solution is aversive, it is important to note that the spread between the extinction curves is not great. Moreover, 11 of 20 bees in the group given only 5% ethanol returned for the entire 12 trials, and all 20 bees returned for at least 6 trials. If the 5% ethanol solution was aversive to bees given no choice, we would expect that few bees would return to the experiment arena, for they would have gone back to the feeder where they were initially captured. It should also be noted that most of the bees feeding on the 5% solution did so until they were satiated—the equivalent of a human consuming 11 liters (Abramson et al. 2003). By any measure, such a level of consumption is remarkable—especially so when the bees are repeatedly required to fly 10 meters between the hive and the experimental arena, interact with nest mates, and unload their alcoholic beverage.

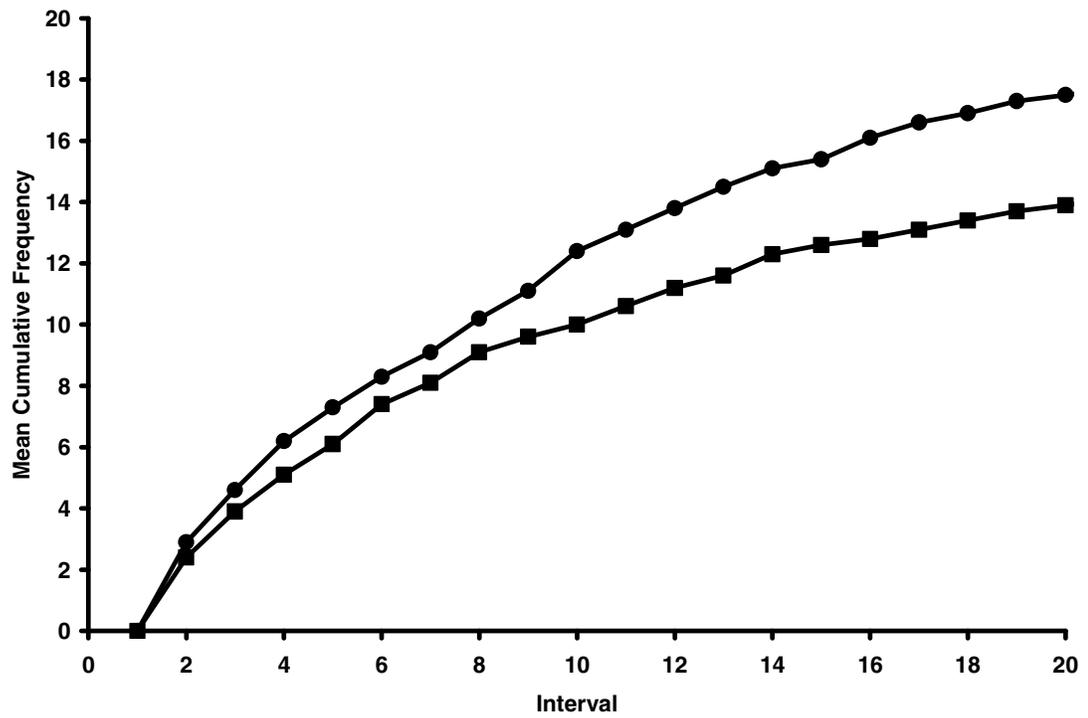
The results of the experiments reported in this chapter justify continued development of a social insect model. At first glance the use of honey bees to study alcohol consumption may seem unnecessary and premature, but bees have several advantages over conventional models. Unlike fruit flies and nematodes, honey bees have a “language” and a social structure. Much information is also known about their natural history, learning, genetics, and biochemistry (Menzel, 2003). In addition, honey bee colony structure suggests many novel experiments, including looking at the social transmission of ethanol between colony members and the possibility of developing a line of “alcoholic honey bees” for genetic experiments.

The present and previous work on the honey bee model has revealed several similarities between the behavior of bees and humans. One thing lacking is definitive data that honey bees will “compulsively” consume ethanol. Developing a line of honey bees with this compulsive behavior is possible, yet technically difficult, as has been proven successful with other animal species (Handler and O’Brochta, 1991; Swanson et al., 2004). The work would require continuation of the genomic mapping of the honey bee, which is an expensive and time-consuming process. Even without the support of data on compulsive drinking of ethanol in honey bees, the current model can certainly be used to study aspects of social drinking including the effect of ethanol on motor behavior, learning, social skills, and aggression.

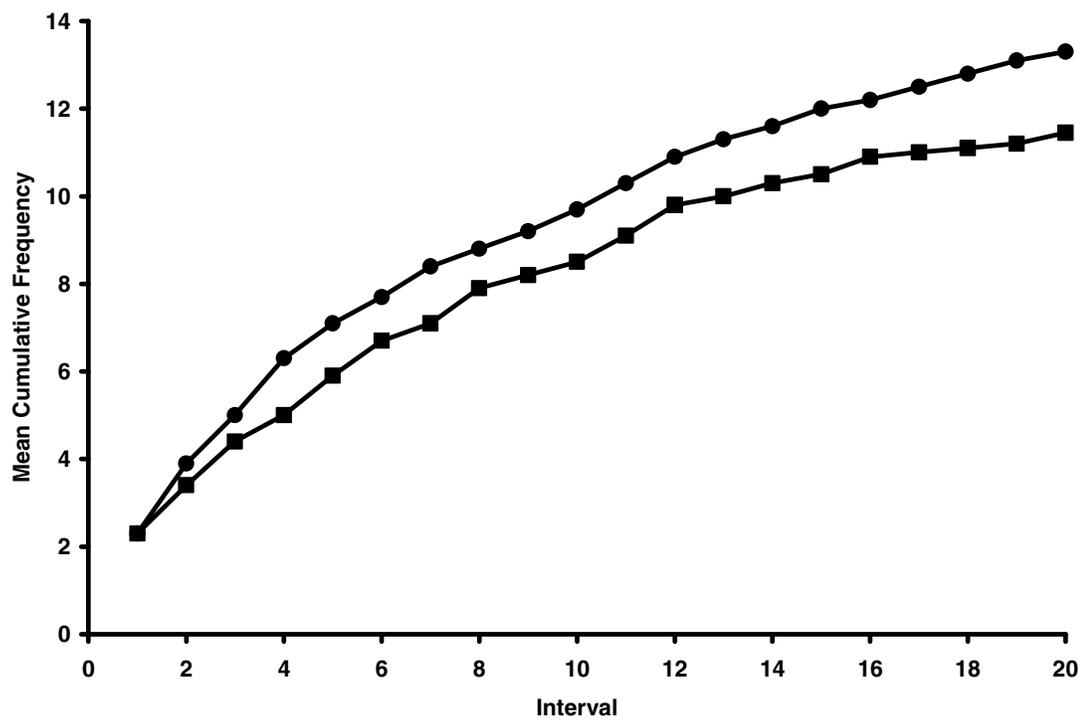
Presently, studies are being conducted on social phenomena such as food exchange, influence of ethanol on honey bee language, and the effect of ethanol on aggressive behavior. In addition, progress has been made on assessing the effect of ethanol on the mushroom bodies of the honey bee brain. Finally, preliminary experiments have begun to test the feasibility of creating an “alcoholic” line of honey bees.



**Fig. 7.** Mean proportion of landing responses to a 1% (triangles) and 5% (squares) target containing ethanol.



**Fig. 8.** Mean cumulative frequency of landing on a target previously associated with 5% ethanol (squares) and on a target previously associated with sucrose (circles).



**Fig. 9.** Mean cumulative frequency of landing on a target previously associated with 1% ethanol (squares) and on a target previously associated with sucrose (circles).

## References

- Abramson, C. I. 1990.** *Invertebrate learning: a laboratory manual and source book.* Washington, DC: American Psychological Association.
- Abramson, C. I., D. A. Buckbee, S. Edwards, and K. Bowe. 1996.** A demonstration of virtual reality in free-flying honey bees: *Apis mellifera*. *Physiology & Behavior*, 59, 39-43.
- Abramson, C. I, S. M. Stone, R. A. Ortez, A. Luccardi, K. L. Vann, K. D. Hanig, and J. Rice. 2000.** The development of an ethanol model using social insects I: Behavior studies of the honey bee (*Apis mellifera* L.). *Alcoholism: Clinical and Experimental Research*, 24, 1153-1166.
- Abramson, C. I., G. W. Fellows, B. L. Browne, A. Lawson, and R. A. Ortiz. 2003.** The development of an ethanol model using social insects: II. The effect of antabuse on consumatory responses and learned behavior of the honey bee (*Apis mellifera* L.). *Psychological Reports*, 92, 365- 378.
- Handler, A. and D. O'Brochta. 1991.** Prospects for gene transformation in insects. *Annu. Rev. Entomol.* 36: 159-183.
- Hill, P., P. Wells, and H. Wells. 1997.** Spontaneous flower constancy and learning in honey bees as a function of colour. *Anim. Behav.* 54: 615-627.
- Menzel, R. 2003.** Associative learning and memory processing in bees. In J. H. Byrne, H. Eichenbaum, H. Roediger III, & R. F. Thompson (Eds.), *Encyclopedia of learning and memory* (2<sup>nd</sup> ed.). New York: Macmillian. Pp. 273-277.
- Rothenfluh, A. and U. Heberlein. 2002.** Drugs, flies, and videotape: the effects of

ethanol and cocaine on *Drosophila* locomotion. *Current Opinion in Neurobiology*, 12, 639-645.

**SPSS Inc. 1999.** SPSS Base 10.0 for Windows. SPSS Inc., Chicago IL.

**Swanson, K., M. Mazur, K. Vashisht, L. Rund, J. Beever, C. Counter, and L.**

**Schook. 2004.** Genomics and clinical medicine: rationale for creating and effectively evaluating animal models. *Exp. Biol. Med.* **229**: 866-875.

**Wolf, F. W. and U. Heberlein. 2003.** Invertebrate models of drug abuse. *Journal of Neurobiology*, 54, 161-178.

## CHAPTER III

### THE EFFECT OF INSECTICIDES CONSIDERED HARMLESS TO HONEY BEES (*APIS MELLIFERA* L.): PROBOSCIS CONDITIONING STUDIES USING THE INSECT GROWTH REGULATORS TEBUFENOZIDE AND DIFLUBENZURON

#### **Abstract**

The following experiments were designed to examine the effects of the insect growth regulators tebufenozide and diflubenzuron on Pavlovian conditioning of harnessed foragers. In one set of experiments, bees learned a simple task in which they associated a conditioned stimulus with a sucrose feeding. A second set of experiments required bees to learn a complex odor discrimination task. Within each experiment, separate groups of bees were pretreated with the insecticide, while others received the insecticide imbedded in a sucrose solution used as the unconditioned stimulus. Results indicated that exposure to both tebufenozide and diflubenzuron influence the performance of honey bees at least mildly. Bees pretreated with 10  $\mu$ l of tebufenozide, 10 minutes prior to learning a simple Pavlovian task, produced lower levels of acquisition when the concentrations were administered in dosages equivalent to 0.22 L/ha, 0.29 L/ha, and 1.0 L/ha. When animals were given a series of 1  $\mu$ l droplets of diflubenzuron over the course of 12 training trials the effect on acquisition and extinction were even more pronounced. No such effects were seen when tebufenozide was used. Discrimination learning was also influenced in

bees pretreated with the insecticides or having the insecticides imbedded in the unconditioned stimulus.

## **Introduction**

The use of Pavlovian conditioning as a sensitive and reliable bioassay to test for the effect of lethal and sub-lethal levels of agrochemicals on behavior is well established (Decourtye and Pham-Delègue, 2002; Mamood and Waller, 1990; Taylor et al., 1987; Weick and Thorn, 2002). Studying the influence of sub-lethal amounts of insecticides on honey bee (*Apis mellifera* L.) behavior is important for the survival of honey bees, public policy issues, honey bee population regulation, environmental degradation, and the use of biological controls. What the literature currently lacks are experiments specifically designed to survey the effects of insecticides specifically acclaimed not to “harm” honey bees. These compounds may include some of the new generation pyrethroids, insect growth regulators, and fermentation by-products, all of which are currently used in formulation of new products.

Many of these new products are considered by the Environmental Protection Agency and other regulatory bodies as user-friendly, target-specific and environmentally safe. However, little is known about their effects, if any, on honey bee behavior, particularly learned behavior. In order to use these chemicals effectively and without injuring the ubiquitous and valuable pollinators, it is important to know what effects these agrochemicals have on honey bee behavior.

In addition to providing data on the effect of “harmless” chemicals on honey bee behavior, this work takes on added significance when considering that it is increasingly popular to seek “fast track” approval for exemption labeling. The two compounds studied in the following experiments fall under this category. Fast track labeling is a potentially dangerous precedent because the effects of these materials on the complete spectrum of honey bee behavior are unknown at this time.

Previous experiments on the study of chemicals considered “not harmful” to honey bees investigated the compound, dicofol. This product is a chlorinated hydrocarbon insecticide and a chemical analog of DDT. It is considered nontoxic to most insects and is used primarily as an acaricide. The proboscis conditioning paradigm was employed to detect any deleterious effects of the insecticide on learning. Training consisted of pairing a neutral olfactory cue (conditioned stimulus, or CS) with sucrose (unconditioned stimulus, or US) to produce a learned response to the conditioned stimulus alone. Each bee tested received twelve acquisition trials, followed by twelve extinction trials for which the unconditioned stimulus was omitted. To control for pseudoconditioning, the appearance of learning when bees are actually responding to a time sequence rather than the presented stimulus, “unpaired” experiments were run, in which bees received an equal number of separate conditioned stimulus and unconditioned stimulus presentations in a pseudorandom sequence. The toxic effects from insecticides were observed in the inability of bees to learn to extend their proboscis to an olfactory cue. Results showed that honey bees pretreated with dicofol exhibited significantly lower levels of learning than honey bees not pretreated (Stone et al. 1997).

The purpose of the present set of experiments is to examine the effects of the insect growth regulators tebufenozide and diflubenzuron on Pavlovian (a.k.a. “classical”) conditioning of harnessed foragers. These insecticides were chosen on the basis of their extensive usage on honey bee foraged crops, such as cotton and improved pastures. Tebufenozide is a molting disruptor whose mode of action is to mimic the natural insect molting hormone 20-hydroxyecdysone. When ingested by targeted insect larvae, the insecticide acts as an agonist in the chemical processes concerned with cuticle formation. A new but malformed cuticle is created, leading to an excess of chitin under the exoskeleton and eventual death by dehydration and starvation (Walgenbach 1999). The active ingredient is tebufenozide, which is described as “being safe for pollinators such as the honey bee” (Dhadialla et al. 1998). This statement referred to a field study which recorded the number of foraging bees, dead adults, and dead pupae following exposure to ten times the recommended rate of application. The results indicated that exposure to tebufenozide did not kill or disrupt foraging behavior, and had no effect on either eclosion or larval development of honey bees (Heller et al. 1992).

Diflubenzuron is a chitin inhibitor which disrupts the synthesis of the chitin exoskeleton that is required before a larval molt. The new cuticle is weakened, leading to an unsuccessful molt and eventually, death. There is also a secondary effect in which eclosion is prevented, or if larvae emerge, they die shortly thereafter (Tripathi 1996). The active ingredient, diflubenzuron, is described as having no effect on honey bees following exposure (Tripathi 1996). Yet, this report fails to state *how* the toxicity of the insecticide was measured. A more detailed investigation reviewed studies that used rates

ranging from 30  $\mu\text{g}/\text{bee}$  for oral and contact  $\text{LD}_{50\text{'s}}$  (IPSC Health and Safety Guide No. 99, 1995) to 115  $\mu\text{g}/\text{bee}$   $\text{LD}_{50\text{'s}}$  (Information Ventures, Inc.1995). The IPSC study noted that colonies were not affected after aerial application of 350g of diflubenzuron/ha.

For the following experiments, the amount of insecticide delivered to bees was determined by labeled rates for a ground application. Six rates were chosen representing the highest, lowest, and three intermediate concentrations that may be applied in an intensive pest control program. These labeled rates were then diluted with equal parts of a 1.8 M sucrose solution to improve palatability and to compensate for chemical repellency (Thompson, 2003). Therefore, the quantities of insecticide fed to bees are representative of a 50% dilution of the labeled rates.

## **Materials and Methods**

### *Capturing and harnessing honey bees*

Worker honey bees were selected randomly from the exterior surface and landing board of a single colony (Stillwater, OK) between May and November (2001). Bees collected in this way were a mixture of different behavioral specializations that required either departure from the hive (i.e., foragers or nest-cleaning bees) or remaining near the entrance (i.e., guards). They were captured individually in perforated-lid glass vials, beginning around 7:00 a.m. on the day prior to use. Approximately 100 bees were captured each time and taken to the laboratory, where the vials were placed in an ice water bath (Figs. 10 and 11). No attempt was made to determine the age of the subjects.



**Figs. 10 & 11.** Bees are collected from hive entrance in the morning and transported back to the lab, where they are anesthetized in an ice bath.

When the bees became inactive enough to permit handling, they were removed from the ice bath and secured in individual restraining harnesses constructed from .38 caliber shells using a small strip of duct tape placed between the head and thorax and fastened to the sides of the shell (Fig. 12). Once harnessed, the bees were fed a 1.8M sucrose solution until satiated and left overnight. This ensured that all subjects would have approximately the same motivation to feed during training the following morning.



**Fig. 12.** A close look at a harnessed forager.

### *Overview of experimental design*

Two types of conditioning experiments were performed—acquisition and discrimination—and within each experiment the insecticide was administered either

before or during training trials. When an insecticide was administered during training, it was imbedded in the sucrose US. The rationale behind the application of the insecticide before or during testing was to explore the effect of a single dose (administered prior to training) or of several smaller doses (administered during training in US presentations) on learning in honey bees. Bees were fed the insecticides via a Hamilton microliter syringe. To serve as a control against experimenter bias, the six rates of each insecticide were placed in containers randomly assigned a number 1 through 6.

A conditioning trial began by picking up a bee and placing it in front of a ventilation fan (Figs. 13-15). The purpose of the fan was to remove CS and US odors from the training area. Several seconds after being placed in front of the fan, a bee was administered the appropriate stimulus. Following application of a stimulus, the bee was returned to a holding area and a second bee was run. This continued until all the subjects scheduled to be run on that day received the required number of training trials. An attempt was made to control for calendar variables and fluctuating hive conditions by running bees from several different treatment groups daily.

Experiment 1 investigated the effects of tebufenozide and diflubenzuron on simple classical conditioning, wherein honey bees were trained to associate a CS odor with a US presentation. Following a twelve trial acquisition (training) phase, an extinction phase was initiated during which the CS was presented twelve times without the US. The rationale for including an extinction phase was to determine if the insecticide influenced persistence of a conditioned response when the response was no longer followed by a

feeding. The performance of paired bees was evaluated against an “unpaired” group of bees receiving an identical number of CS and US presentations that were explicitly separate.

The unpaired group was included to ensure that any learning observed in the insecticide groups was actually the result of the pairing between the CS and US, and not due to a central excitatory state in which the honey bee extends its proboscis to the presentation of an olfactory stimulus because it has been sensitized by a sucrose feeding (Abramson 1994). In addition to serving as a control for the paired groups, unpaired experiments were also analyzed for any effects the insecticides may have on unpaired responses. These effects would manifest as a high level of CS responding in unpaired bees treated with the same insecticide concentrations as their match paired group.

Experiment 2 investigated the effects of tebufenozide and diflubenzuron on complex classical conditioning where honey bees were trained to discriminate between two different CS odors, one of which was always paired with a US. The rationale behind including a discrimination experiment was to determine if exposure to the insecticide influenced different types of learning in the honey bee. Discrimination learning is considered to be more complex than the simple CS acquisition experiment because the honey bee must learn to respond to a unique stimulus while disregarding another, similar type of stimulus. The discrimination experiment also served as a check for the paired and unpaired experiments because in the discrimination experiment, each honey bee served as its own control. It should be noted that the honey bee was also required to discriminate in

the simple learning situation. The discrimination in the simple conditioning experiment was between the explicit CS odor used in training and all background stimulation.

#### *Preparation of CS and US delivery devices*

In the simple learning situation, the CS was the odor of cinnamon oil (Gilbertie's, Southampton, NY). In the complex learning situation, one CS consisted of cinnamon odor and the second CS consisted of the odor of citral (Sigma Chemical, St. Louis, MO, product number C-1645). The odors of cinnamon and citral were selected for the discrimination experiment because previous work indicated that bees consider these stimuli equally salient (Abramson et al. 1996). The CS scent was first transferred to a 1 cm<sup>2</sup> piece of filter paper (Whatman #4) using a wooden dowel. The paper was dabbed with scent to the point of saturation, and then secured to the plunger of a 20 cc plastic syringe with a metal thumbtack.

The US varied per method of insecticide treatment (Treat in US or Pretreat), and was either a 1.8 M sucrose feeding or one of six levels of insecticide, respectively. One of these levels was a sucrose control. All levels of the insecticide were diluted with 1.8 M sucrose prior to administration to produce a 50% concentration of the labeled rate, and fed directly to the honey bee with a Hamilton microliter syringe. The insecticide imbedded in the US attempted to mimic a situation in which a honey bee consumed insecticide-laced nectar. The microliter syringe was used whenever bees were given insecticide in a sucrose US. In experiments where the insecticide solution was administered prior to training (Pretreat), there was no need for the syringe. Instead, the

US was administered as filter paper strips dipped in 1.8 M sucrose. The filter paper strips were handled with tweezers.

*Experiment 1: Simple Classical Conditioning (Acquisition and Extinction)*

One thousand two hundred honey bees were divided into 2 equal groups: 600 were used in the tebufenozide experiment and the remaining 600 were used in the diflubenzuron experiment. The two 600 bee subgroups were further divided into 2 groups of 300 bees each. One of these groups received paired CS-US presentations, and the other group received unpaired CS/US presentations and served as a control for pseudoconditioning. Both groups of 300 bees were further divided into 2 groups of 150 bees each. For one group of 150, bees received 10 µl of insecticide solution 10 minutes before training. The remaining 150 bees received 10 µl of sucrose solution 10 minutes prior to training, and received 1 µl of the insecticide solution imbedded in the US. Each group of 150 bees was further divided into 6 groups of 25 bees each. These 6 groups corresponded to the 6 levels of insecticide which are based on a 186.94 L/ha spray volume, as administered by a ground applicator. Those in the tebufenozide groups received rates of 0 L/ha, 0.15 L/ha, 0.22 L/ha, 0.29 L/ha, 0.55 L/ha, or 1.0 L/ha, corresponding to active ingredient levels of 0 µg/bee, 16 µg/bee, 24 µg/bee, 32 µg/bee, 69 µg/bee, and 131 µg/bee. Bees in the diflubenzuron groups received 0 L/ha, 0.027 L/ha, 0.07 L/ha, 0.15 L/ha, 0.29 L/ha, or 0.55 L/ha, corresponding to active ingredient levels of 0 µg/bee, 3 µg/bee, 9 µg/bee, 16 µg/bee, 32 µg/bee and 69 µg/bee. Bees in the paired groups received 12 acquisition trials followed by 12 extinction trials. A non-overlap procedure was used in which the CS was terminated before the US was

presented. The CS interval was a 3 second presentation of cinnamon odor; the US was either a 2 second feeding when filter paper strips were used, or when the microliter syringe was used, the amount of time needed for the consumption of the entire 1  $\mu$ l droplet (bees typically consumed the droplet in about 2 seconds). The interstimulus interval (time between the onset of the CS and onset of the US) was 3 seconds. When a non-overlap procedure is used, the interstimulus interval is identical to the CS duration. The intertrial interval (the time interval between the end of the US and the beginning of the next CS) was 10 minutes.



**Fig. 13.** The CS odor and US feeding are presented simultaneously.

Bees in the unpaired groups received 12 CS presentations and 12 US presentations in a pseudorandom order. For half of the unpaired bees, stimulus presentations consisted of three successive sequences of CS US US CS US CS CS US. For the remaining bees the sequence consisted of US CS CS US CS US US CS. The interval between stimulus presentations was 5 minutes—half the time of that used for the paired group. The rationale behind using a 5 minute intertrial interval (ITI) for unpaired bees was to keep the time between CS presentations at approximately 10 minutes, as in the paired

experiments. If a 10 minute ITI was used, 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 the time spent harnessed. Following the 12 CS and US presentations, the unpaired experiment was terminated (no extinction trials).

Responses to the CS and US presentations were visually categorized for each trial using a binary system. This system was used for both paired and unpaired experiments. For example, in paired groups, if a subject extended its proboscis after the onset of the CS, but before the US was presented, a “1” was recorded for the CS response. Otherwise, a “0” was recorded. A separate recording was made for the subject’s response to the US presentation.

### *Experiment 2: Complex Classical Conditioning (Discrimination)*

The design differed from the simple learning experiment in that two CS’s were used—one of which was paired with a feeding—and extinction was not tested. Also, there was no unpaired group. The unpaired group was unnecessary in discrimination experiments because each subject serves as its own control.

One CS was the odor of cinnamon used in the simple learning experiments, and the second was the odor of citral. The two CS’s were identified as CS+ and CS-: the CS followed by a feeding was the CS+, while the CS- was not followed by a feeding. For

half of the bees, the CS+ was cinnamon and the CS- citral; for the remaining bees, the CS+ was citral and the CS- cinnamon.

Six hundred and twenty-four honey bees were divided into two main groups. Three hundred twelve bees were used in the tebufenozide experiment and the remainder was used in the diflubenzuron experiment. Both groups of 312 bees were further divided into 2 groups of 156 bees. In one group of 156, 10 µl of insecticide solution was administered 10 minutes before training. The remaining 156 bees received 10 µl of sucrose 10 minutes prior to training, and received 1 µl of the insecticide solution imbedded in the US. Each group of 156 bees was again divided into 6 groups of 26 bees. These 6 groups corresponded to the 6 levels of insecticide used in Experiment 1.

Acquisition consisted of 12 presentations of both the CS+ and CS- so that a total of 24 trials were conducted. For 13 of the 26 bees, the presentation of the CS's consisted of three successive sequences of CS+ CS- CS- CS+ CS- CS+ CS+ CS-. For the remaining 13 bees, CS+ and CS- presentation was reversed. Learning was considered to have taken place if two conditions were met: 1) a bee extended its proboscis after the onset of the CS+ but before the presentation of the US, and 2) the bee stopped responding to the CS-, which was not paired with a feeding.

As in the previous experiment, a nonoverlap procedure was used. The CS duration was 3 seconds and the US duration 2 seconds. The ITI was reduced from 10 minutes to 5 minutes. The rationale behind using a 5 minute ITI in the discrimination experiments

was to keep the time between CS+ presentations approximately 10 minutes. If a 10 minute ITI was used, the time between CS+ presentations would have been approximately 20 minutes and any difference between our simple and complex learning experiments could be accounted for in terms of such nonassociative effects as time spent harnessed.

As in the simple learning experiments, responses to the CS and US presentations were visually categorized for each trial using a binary system. This system was used for both paired and unpaired experiments. For example, in paired groups, if a subject extended its proboscis after the onset of the CS+, but before the US was presented, a “1” was recorded for the CS+ response, otherwise, a “0” was recorded. A separate recording was made for the subject’s response to the US presentation and the CS- presentation.



**Fig. 14.** The experimental station for the “Discrimination: Pretreat Insecticide” study includes a data recording sheet, ventilation chamber, stopwatch, a US delivery device, two CS delivery devices, prefeeding station, and harnessed bees.

## **Results**

### *Acquisition Experiment: Initial problems with statistical analyses*

Flaws in experimental design can be unapparent until the collected data is analyzed using statistical methods. In this study, for example, the binary nature of the data (1=proboscis extension, 0=no proboscis extension) was not suited to the ANOVA procedure (SAS Institute 2003, v 9.1.2), an otherwise appropriate test for comparing mean responses of multiple comparisons. An attempt was made to use the general linear model repeated measures test to calculate means and variances for each concentration of insecticide, but the convergence criteria for this procedure was not met by at least half of the dataset. This was due in part to the “bee effect”, a measure of individual bee responses which factored into generating a mean for each group of bees.

### *A new approach*

The solution to the analysis problem was owed, in part, to other researchers who encountered the same obstacles in similarly designed experiments (Weick and Thorn 2002) and tackled them with a simple pair-wise *t*-test (SAS Institute 2003, v 9.1.2). This required tabulating all of the 1’s and 0’s for each concentration of insecticide at each treatment level (Pretreat and Treat in US, Tables 1&2), and then comparing individual concentrations with the likewise tabulated “control” data (no insecticide). Next, a test was run to generate chi-square values for each comparison of insecticide dose to control, the results of which were examined for a significant dose effect across the twelve trials of the experiment. This test provided the first numerical representation of statistical trends in the data, but was too generic to make inferences about the results.

The data needed to be fit to a nonlinear model in order to make comparisons in trends across experimental groups. Mean estimates were taken from a population of 25 bees for each dose of insecticide at each treatment level. To account for the variation in individual bee responses, a random effect parameter was calculated using the MIXED procedure, but the data failed to converge with other parameters in a unique descriptive model.

The convergence issue was a serious setback that necessitated a reorganization of the empirical data. This was achieved by setting up a data filter that recognized “learning”, which was based on the measured frequency of responses throughout the duration of the experiment. Learning was defined as “the occurrence of two consecutive positive PER responses per bee over the course of 12 trials”, and the filter thus described enabled SAS to remove the bees that did not learn from the overall analysis (Table 3). The convergence issue was finally resolved for individual bee response, and a nonlinear mathematical model was fit to describe the trends in learning for each concentration of insecticide and treatment.

The “Learning and Memorization Model” below was borrowed from Stepanov and Abramson (2005), who validated this model for the assessment of memorization performance of a variety of animal subjects, including humans.

$$Y = B3*\exp(-B2*x) + B4*(1-\exp[-B2*x]) \quad [3.1]$$

The model is described by three parameters: B2—the velocity of learning; B3—the predisposition to the next learning (memorization) before the beginning of testing; B4—an asymptotic volume of learned objects. When applied to the PER data sets, this equation generated a predicted learning curve corresponding to an actual response curve for each treatment level and concentration of insecticide, based on the best estimated values of B2, B3, and B4.

### *Computations*

These “best estimates” were derived from the iteration of the parametric values given for each coefficient. In this case, B2, B3, and B4 were assigned the same range of starting values, according to the restrictions set by Stepanov and Abramson (2005): 0 to 3, in increments of 0.2. The iteration process produced a grid of the 10 combinations of coefficient values with the lowest sums of squares. The combination with the lowest of these values was submitted to the Gauss-Newton iteration process, which configured the coefficient estimates at which convergence occurred.

Graphical representations of these fitted models allowed for a simple comparison of the “predicted” versus “actual” outcome for each data set (see Figs. 15-50). Statistical values for the goodness of fit of the model were calculated for the CS data of each insecticide\*dose\*treatment combination in terms of probability of the F statistic ( $p > F$ ). Consequently, three instances of a “bad fit”, or failed convergence, were found for the insecticide diflubenzuron in the “Treat in US” condition: 3  $\mu\text{g}/\text{bee}$  ( $F = 0.23$ ,  $p > F =$

0.796) (Fig. 30), 32  $\mu\text{g}/\text{bee}$  ( $F = 2.48, p > F = 0.139$ ) (Fig. 36) and 69  $\mu\text{g}/\text{bee}$  ( $F = 1.98, p > F = 0.194$ ) (Fig. 38) There was no dose effect observed in this analysis. A subsequent test was run to determine whether the models for the various doses of insecticide differed from one another within a treatment condition (Pretreated or Treat in US). The test attempted to fit a common set of parametric values to all of the dose levels, including the control. The results concluded that each dose level was significantly different and could not fit a common set of parametric values for every comparison within treatment for both tebufenozide ( $F = 3.28$ ) and diflubenzuron ( $F = 5.30$ ).

#### *Extinction and Unpaired data analyses*

The extinction data was eliminated from the analysis due to the failure of bees to extinguish the learned response from the acquisition phase. The literature supports the observation that bees do not easily “unlearn” a trained behavior, and to achieve this state, the length of the extinction phase would need to have been greatly increased (Marfaing et al., 1989; Gerber et al., 1996; Decourtye et al., 2005). Furthermore, the current study was concerned with the effects of two chemicals on the ability of honey bees to make a novel association, not to terminate an existing one.

Unpaired data for this study was consistent with the literature as well (Abramson, 1997), and therefore was not included in this manuscript. The unpaired experiments served as a check for the acquisition experiments, and the predicted outcome for the former was simply that bees would not learn to respond to the unpaired CS odor. This prediction held true for all doses and treatment conditions for both tebufenozide and diflubenzuron.

### *Discrimination Experiments*

Testing for learning in discrimination experiments presented a whole new challenge. The variable “trial” had to be discarded, because the irregular presentations of CS+ and CS- stimuli over 24 trials cancelled time as a factor influencing memorization. Discrimination experiments were evaluated on the proportion of positive responses (proboscis extension to odor) to the CS+ versus negative responses (no extension) to the CS-.

### *Analysis of discrimination data*

Analysis procedures for discrimination data began with the removal of bees that responded to 0 out of 12 presentations of the CS+ odor. These bees were removed from analysis because they were unable to make the initial association of odor to sucrose feeding, and therefore could not produce valid odor discrimination behavior. The number of bees removed for tebufenozide and diflubenzuron analyses differed enormously: 26 removed for the former versus 70 for the latter. The ratio of “In US” to “Pretreated” bees removed was also noteworthy. In the tebufenozide analysis, this ratio was approximately 1:1, but in the diflubenzuron analysis it was greater than 2:1.

The data not extracted by the above filter was run through a GLIMMIX procedure to generate LS MEANS for each of these interactions or effects: stimulus, stimulus\*dose, stimulus\*treatment and stimulus\*dose\*treatment. The means were calculated to reflect positive PER responses only. Significant interactions were found for stimulus ( $F_{1, 6840} = 1319.69, p < .0001$ ), stimulus\*dose ( $F_{5, 6840} = 16.11, p < .0001$ ) and stimulus\*treatment ( $F_{1,$

$F_{6840} = 5.34, p = 0.0209$ ) effects in the tebufenozide analysis (Figs. 51 and 52), but the diflubenzuron analysis showed a three-way interaction for stimulus\*dose\*treatment ( $F_{5, 5784} = 3.70, p = 0.0024$ ) (Figs. 53 and 54).

The GLIMMIX procedure was designed to handle binary data, and was therefore a better choice than the MIXED procedure for integrating the “random bee effect” into the means estimates. The random effect statement produces a more reliable estimation of mean PER response, because it considers individual variance in a population of known size. The response estimates generated by the GLIMMIX procedure are not represented as percentages, as in the MIXED procedure, but were converted to percent positive response using the equation:

$$\% = \frac{e^{\text{lsmean}}}{1 + e^{\text{lsmean}}} \quad [3.2]$$

The logarithmic transformations produced values that were very close to the means computed by the MIXED procedure, but even these fine-line adjustments caused changes in probability values which affected the statistical significance of the measured effect.

Discrimination experiments were designed to further investigate the effects of insecticides on memorization, not to establish a learning curve. The acquisition study gave us a general idea of how each concentration of insecticide would affect the shape of the learning curve, as compared to a control experiment. We called this a “simple learning” paradigm, because it was based upon a single association—odor to food—

which improved over time until stimulus saturation occurred. Conversely, the discrimination study was based upon *two* unique associations—odor A to food; odor B to no food—which had no relevance to time, only to each other. The absence of a “time” factor eliminated the option of bees making a “programmed response” to either stimulus. Therefore, it was possible to observe whether bees treated with insecticide, who had already demonstrated they were able to learn to some degree in the acquisition experiments, could distinguish randomly placed positive stimuli (CS+) from equally random negative stimuli (CS-).

## **Discussion**

In acquisition experiments, tebufenozide and diflubenzuron significantly diminished the learning curves of bees when compared with the control. This was evidenced by the significant differences in the learning curves for each insecticide dose (see *Computations* above). Yet, results indicated that these two insecticides did not disrupt the *mechanism* of learning simple associations; most of the given dose and treatment interactions fit the learning model [3.1]. Only diflubenzuron produced results that could not be fit to the learning model in the experiments where insecticide was imbedded in the US. There was no evidence of dose-dependent learning suppression in this case; non-fitting concentrations were 3, 16, and 69 $\mu$ g/bee, while fitted concentrations were 0, 9, and 32 $\mu$ g/bee.

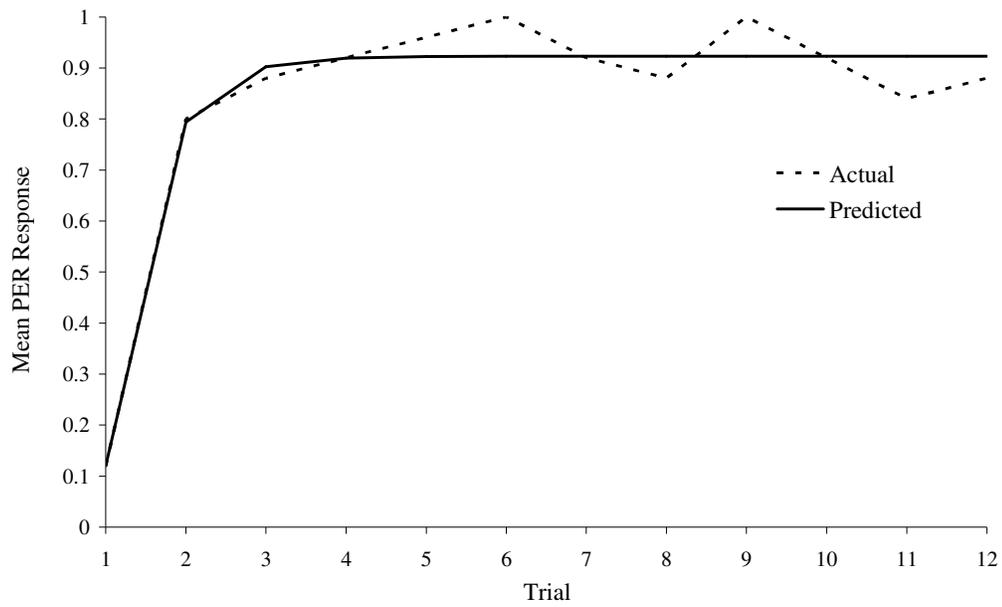
In the comparison of the two treatment levels—“Pretreated” and Treatment US”—bees generally performed better when insecticide was given before trials. This may have been

due to the reluctance of bees to feed from the end of a microliter syringe during the Treat in US trials, or stimulus saturation may have been occurring with the insecticide - impregnated sucrose. Neither tebufenozide nor diflubenzuron proved to be unpalatable to bees, which drank freely of both insecticide solutions in the “Pretreated” experiments.

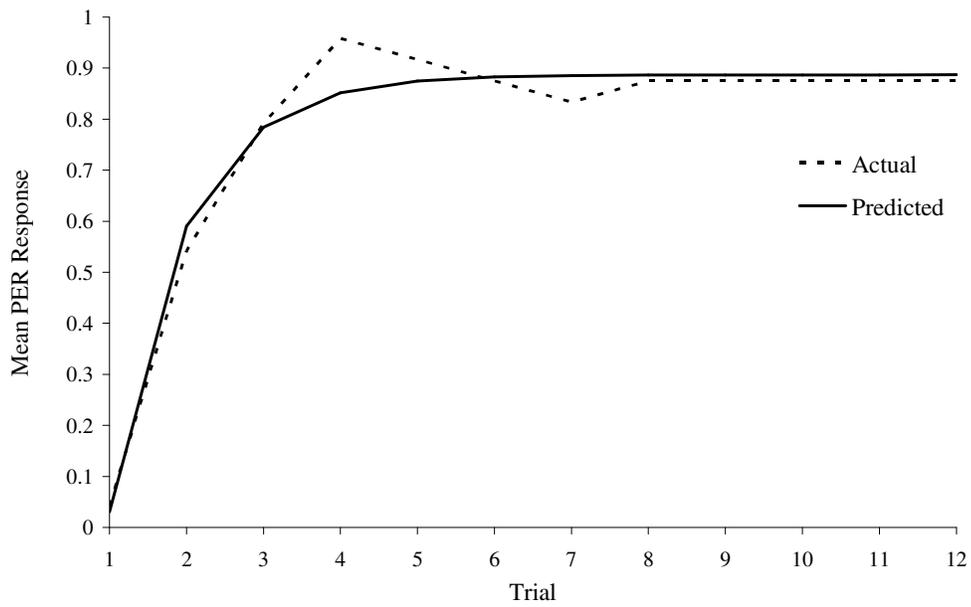
Discrimination experiments also supported an insecticide effect, as there was statistical evidence that the “complex learning” mechanism was disrupted at all insecticide concentrations of tebufenozide and diflubenzuron. The most notable results were found in the comparison of CS+ and CS- responses within a treatment. For tebufenozide, there was a significant dose effect for CS+, but the CS- responses seemed to follow an arbitrary response pattern. The erratic stimulus response pattern was even more pronounced in the diflubenzuron analysis. It should be noted, however, that nearly three times as many diflubenzuron-treated bees were eliminated from the analysis than tebufenozide-treated bees, thus creating a “small sample size” problem for the diflubenzuron chemical group. The remaining diflubenzuron subjects should be considered “exceptional” learners, whereas tebufenozide subjects were more representative of the entire population.

Clearly, both tebufenozide and diflubenzuron have sub-lethal effects on associative olfactory conditioning of the PER, but the effects are not consistent for simple and complex conditioning paradigms. This could be explained by the theory that simple and complex learning are moderated by different areas of the honey bee brain. For example, when the regular temporal presentation of CS+ and US were randomized in the discrimination experiments, this seemed to create great difficulty for bees in making a

single association of odor to feeding. The process of memory formation in honey bees has been lightly explored in terms of temporal dynamics and the organization of neuromeres in the brain (Erber, 1975; Brandes et al., 1988; Menzel, 2001), but more work is needed to understand the specific interactions of insect growth regulators and other “harmless” insecticides on the central nervous system.



**Fig. 15.** CS responses for 0 µg/bee tebufenozide, pretreated.



**Fig. 16.** CS responses for 0 µg/bee tebufenozide, treated in US.

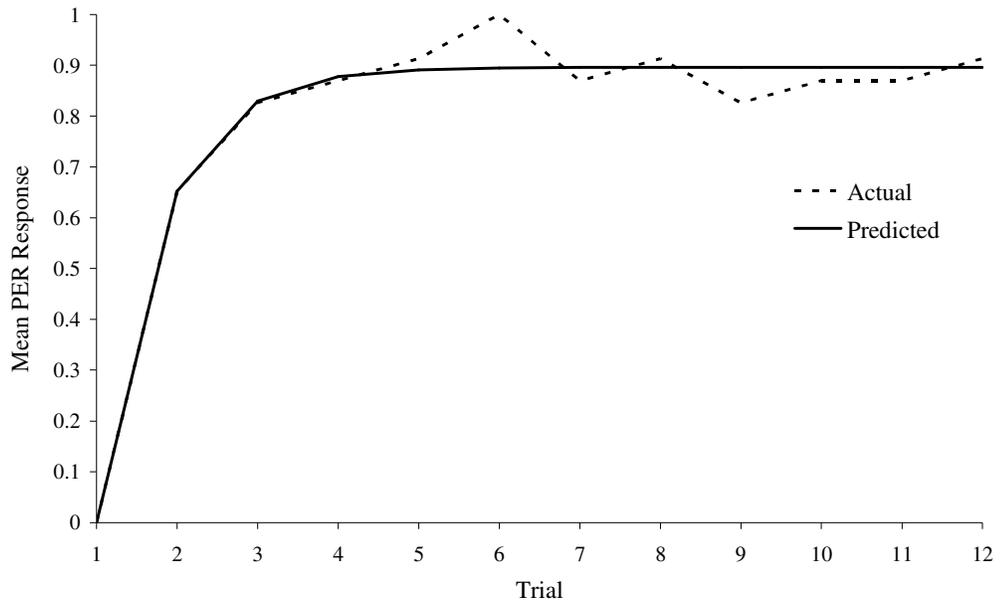


Fig. 17. CS responses for 0 µg/bee diflubenzuron, pretreated.

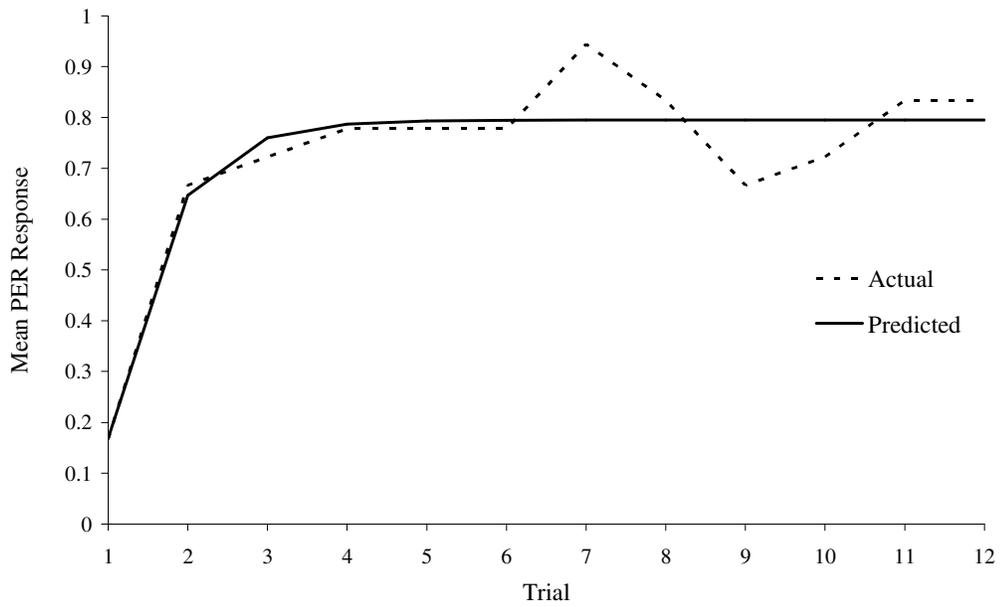
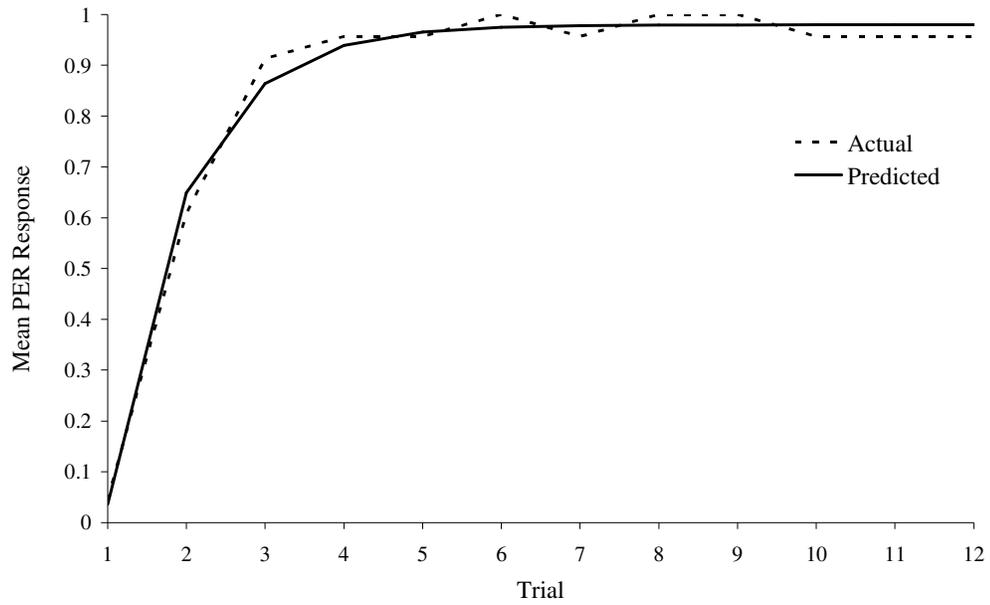
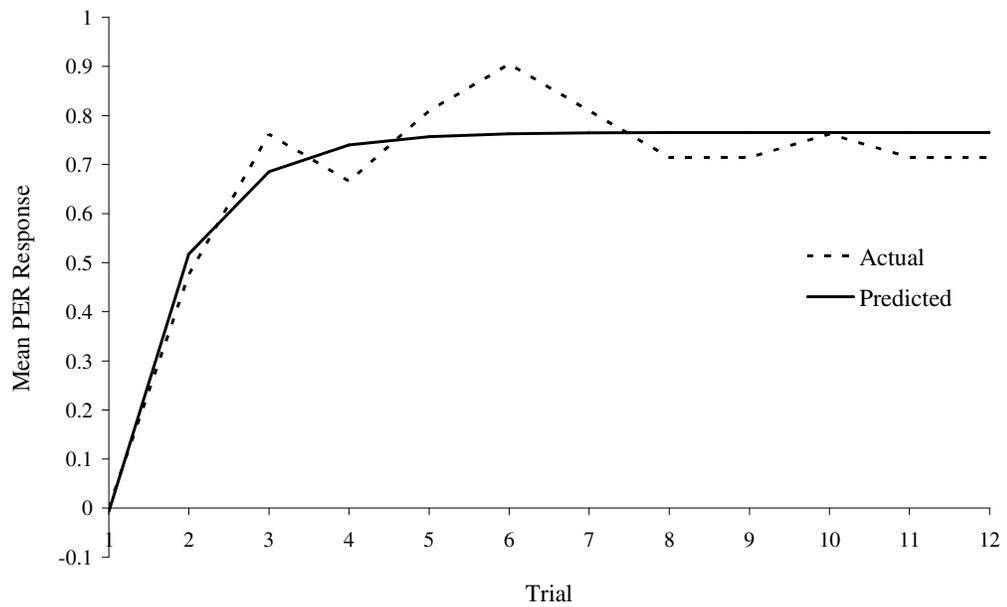


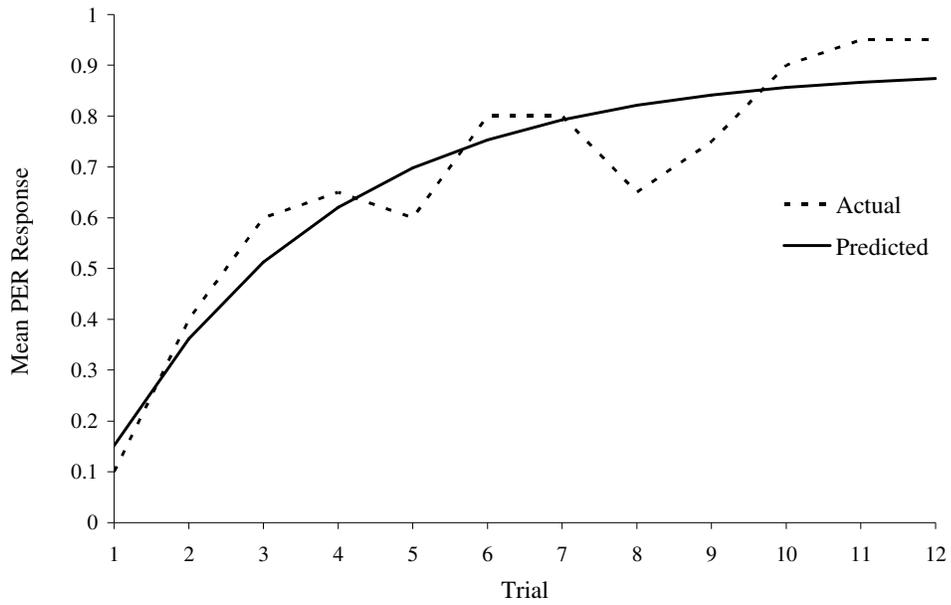
Fig. 18. CS responses for 0 µg/bee diflubenzuron, treated in US.



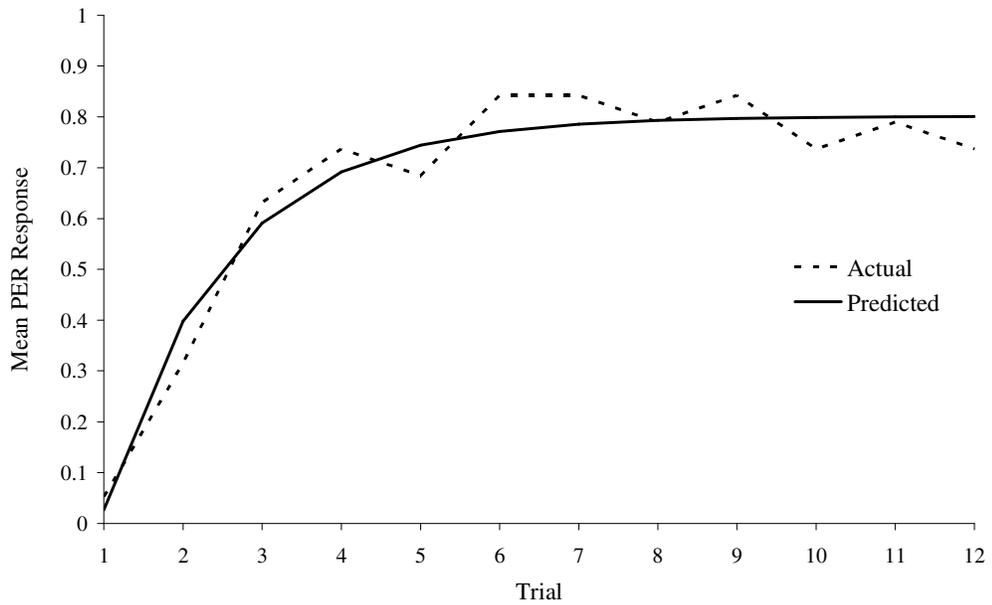
**Fig. 19.** CS responses for 16 µg/bee tebufenozide, pretreated.



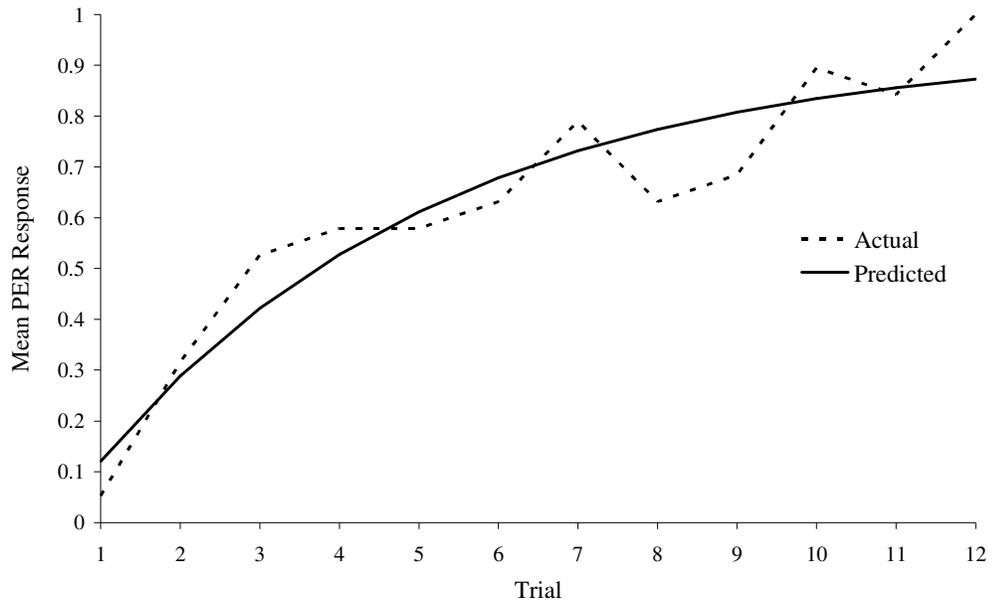
**Fig. 20.** CS responses for 16 µg/bee tebufenozide, treated in US.



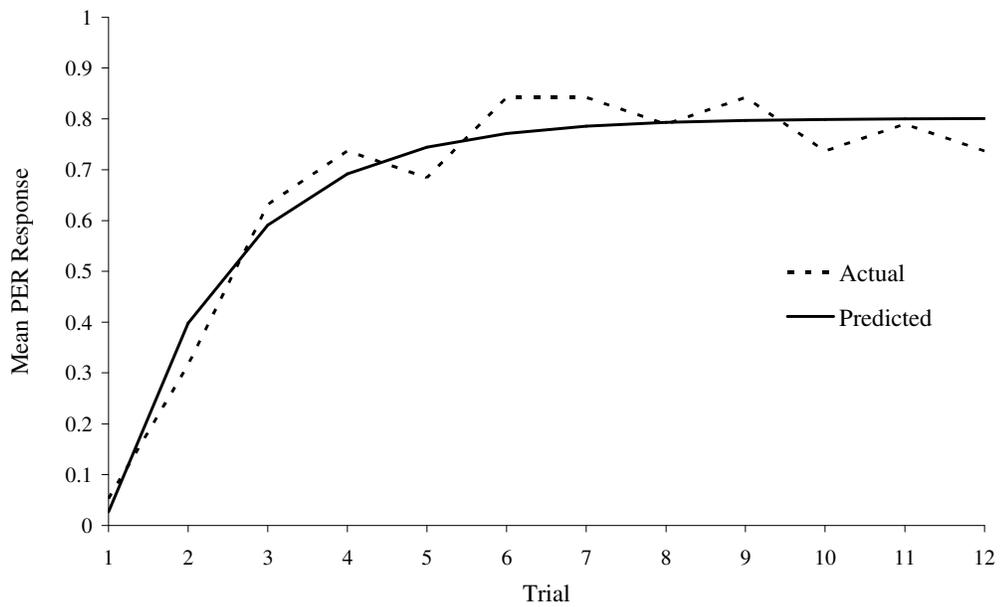
**Fig. 21.** CS Responses for 24 µg/bee tebufenozide, pretreated.



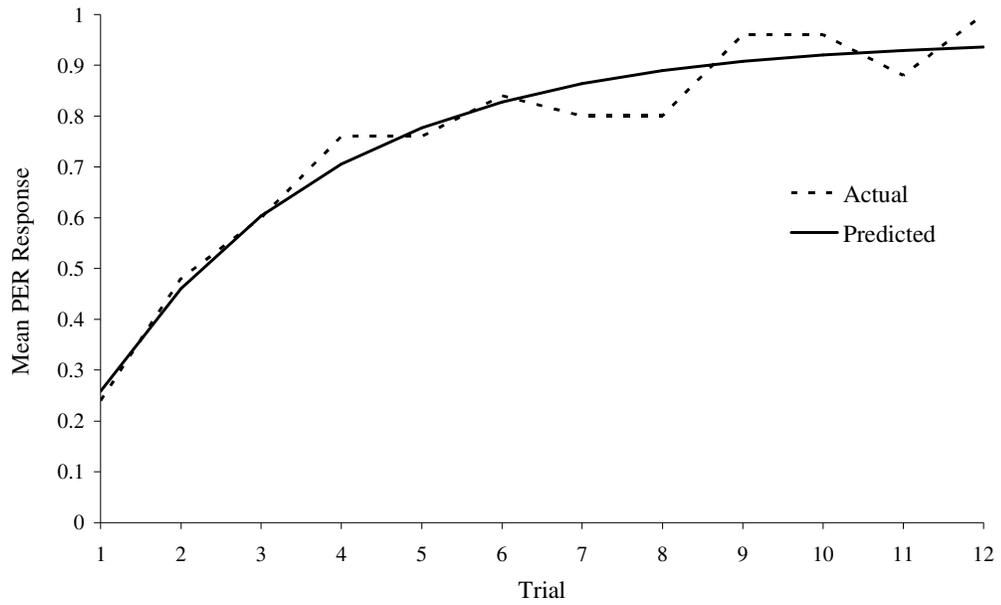
**Fig. 22.** CS Responses for 24 µg/bee tebufenozide, treated in US.



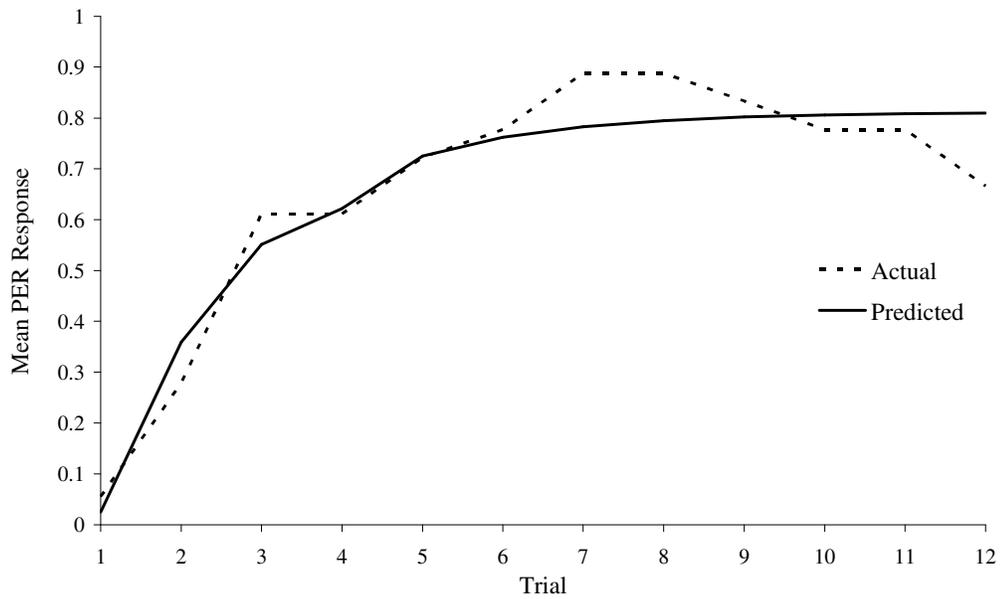
**Fig. 23.** CS responses for 32 µg/bee tebufenozide, pretreated.



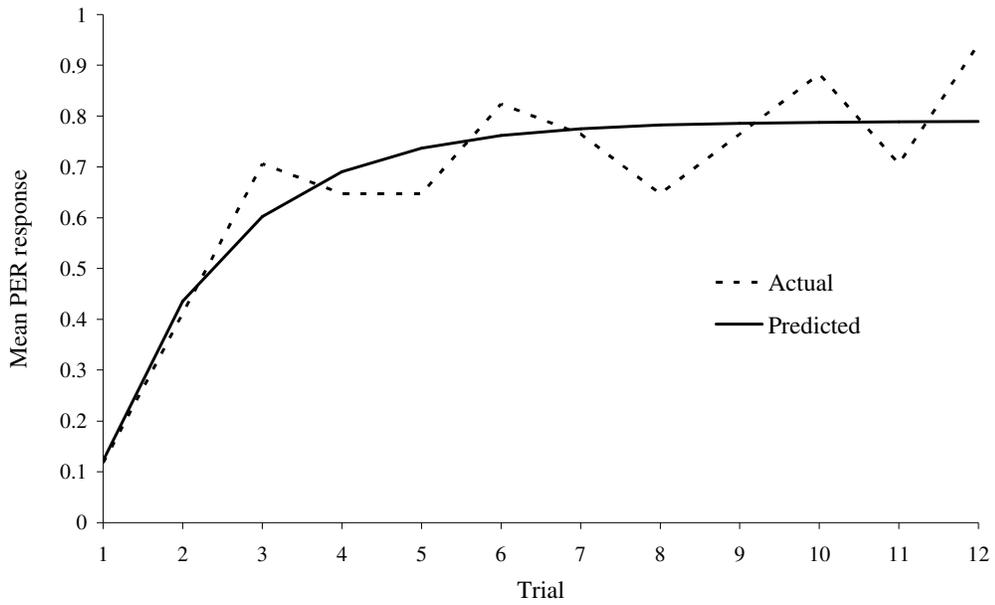
**Fig. 24.** CS responses for 32 µg/bee tebufenozide, treated in US.



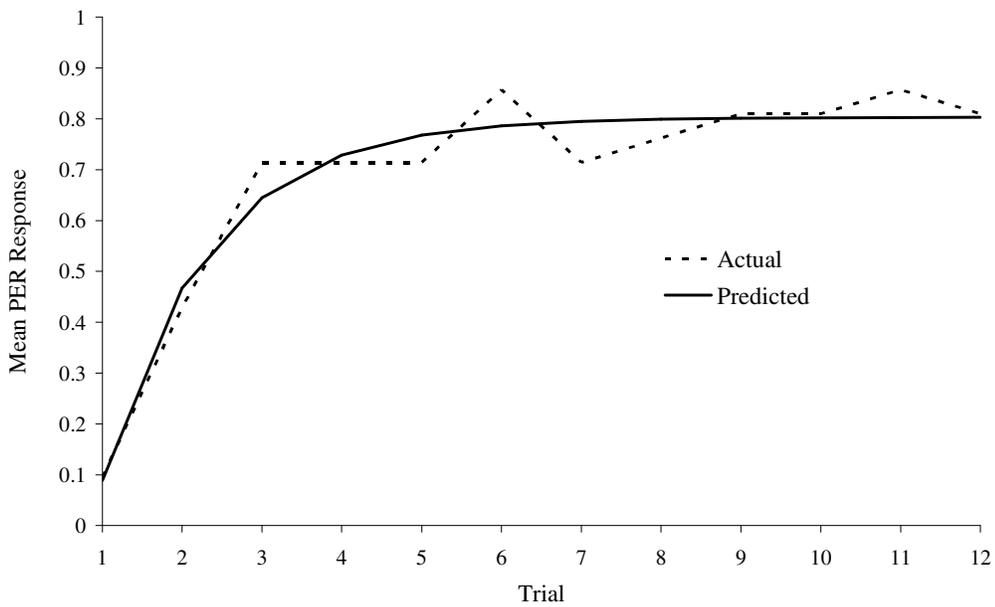
**Fig. 25.** CS responses for 69 µg/bee tebufenozide, pretreated.



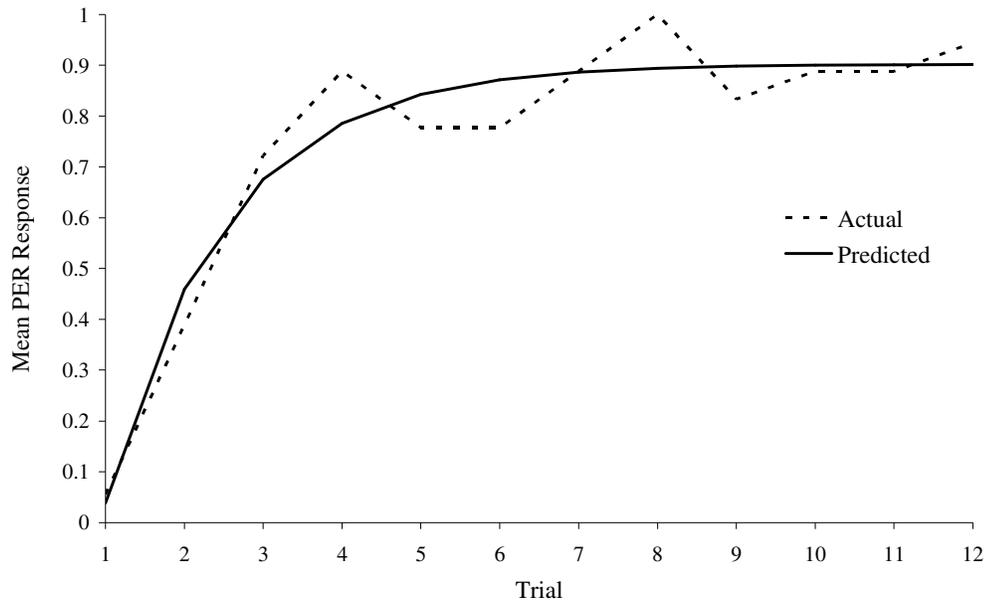
**Fig. 26.** CS responses for 69 µg/bee tebufenozide, treated in US.



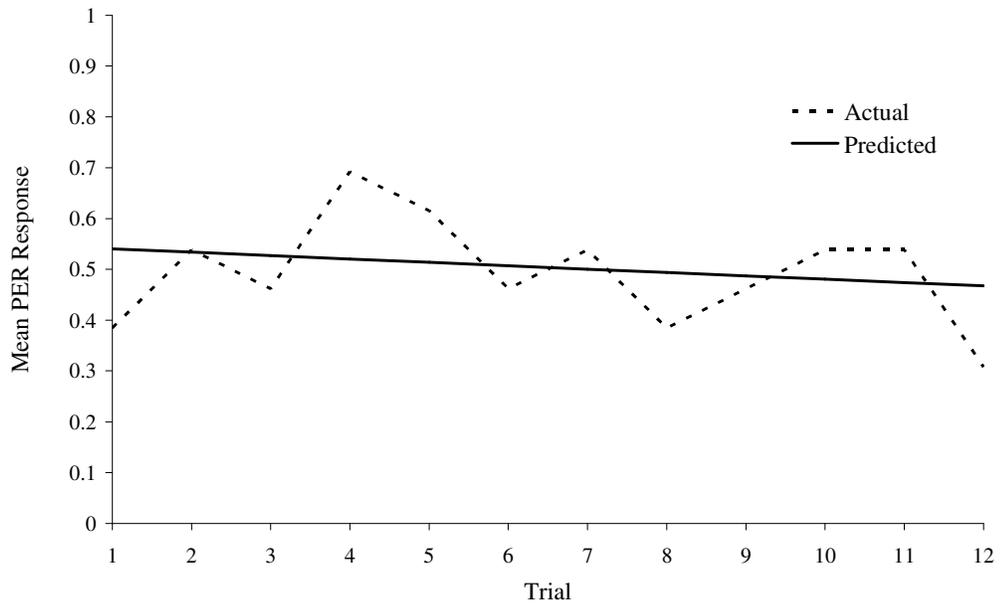
**Fig. 27.** CS responses for 131 µg/bee tebufenozide, pretreated.



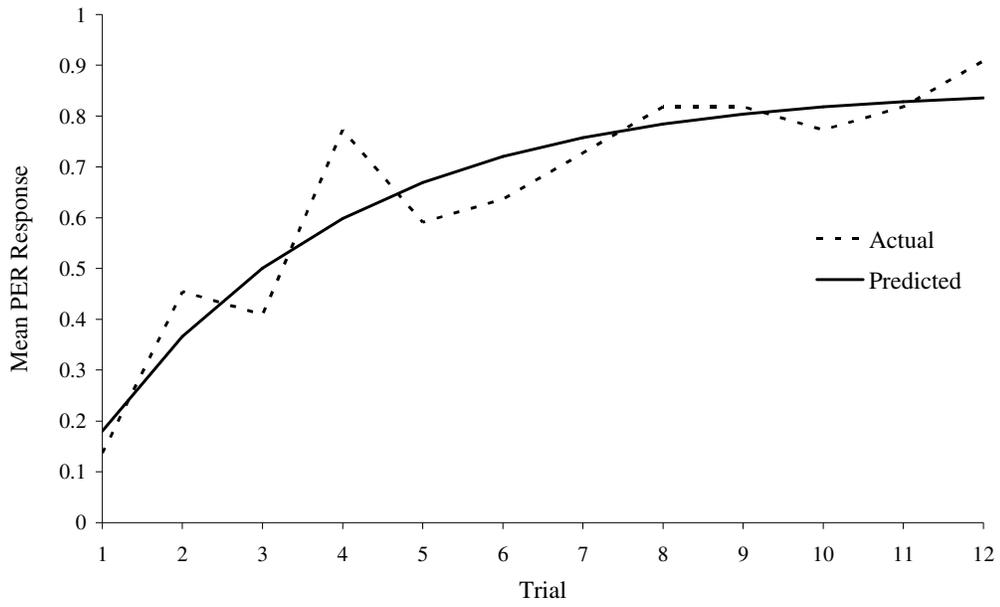
**Fig. 28.** CS responses for 131 µg/bee tebufenozide, treated in US.



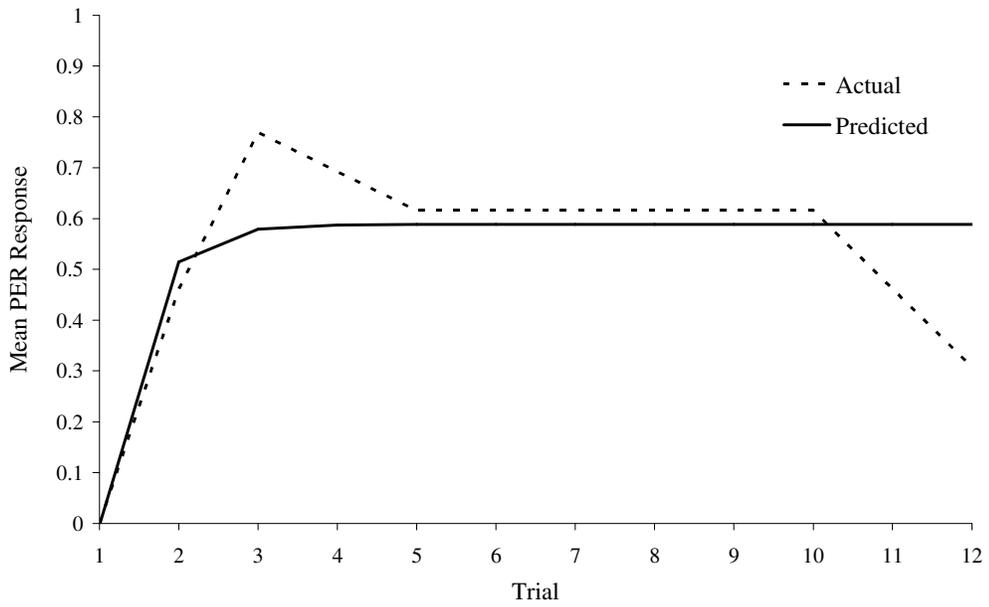
**Fig. 29.** CS responses for 3 µg/bee diflubenzuron, pretreated.



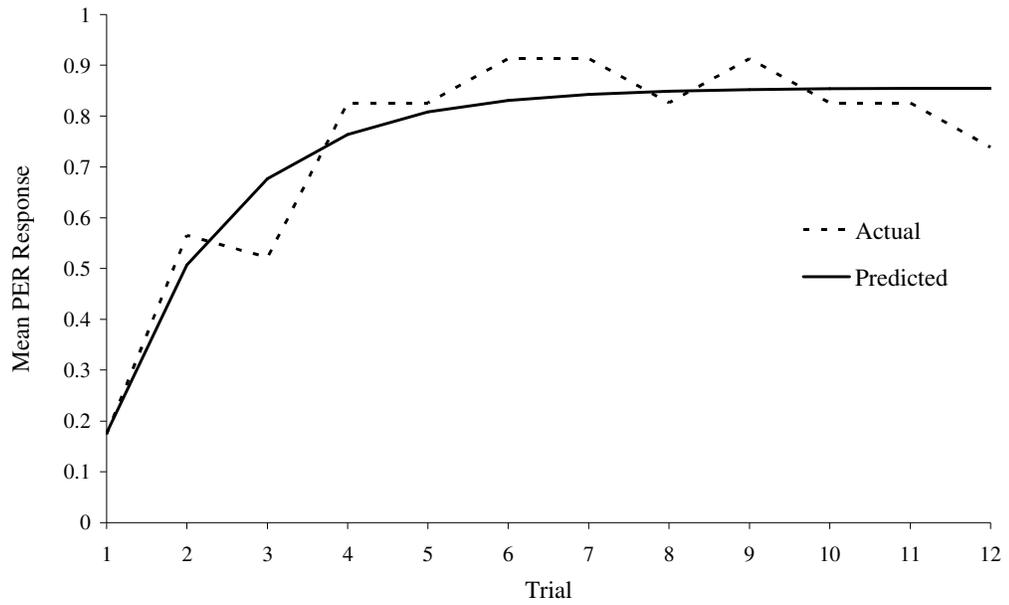
**Fig. 30.** CS responses for 3 µg/bee diflubenzuron, treated in US.



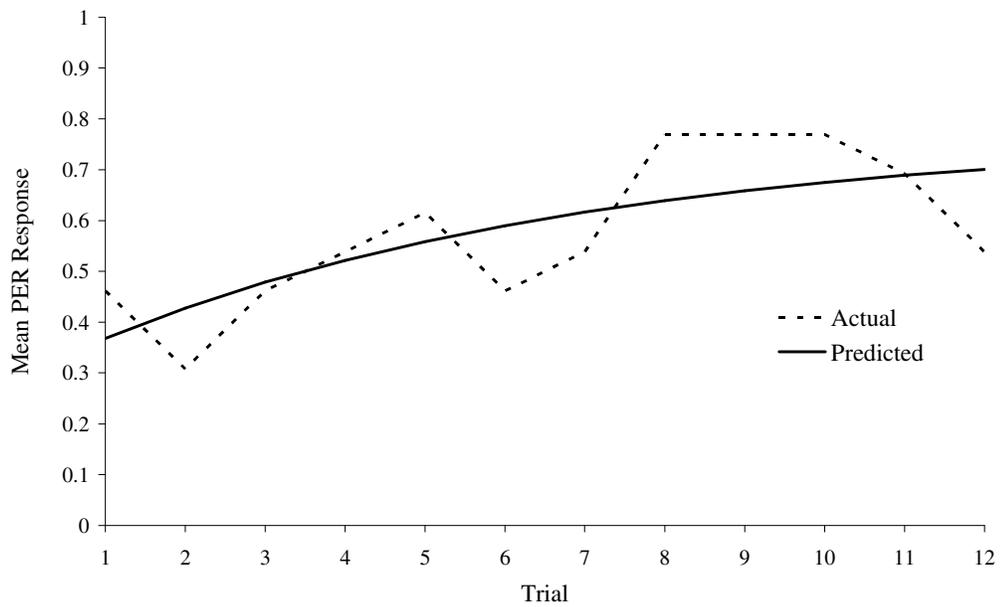
**Fig. 31.** CS responses for 9 µg/bee diflubenzuron, pretreated.



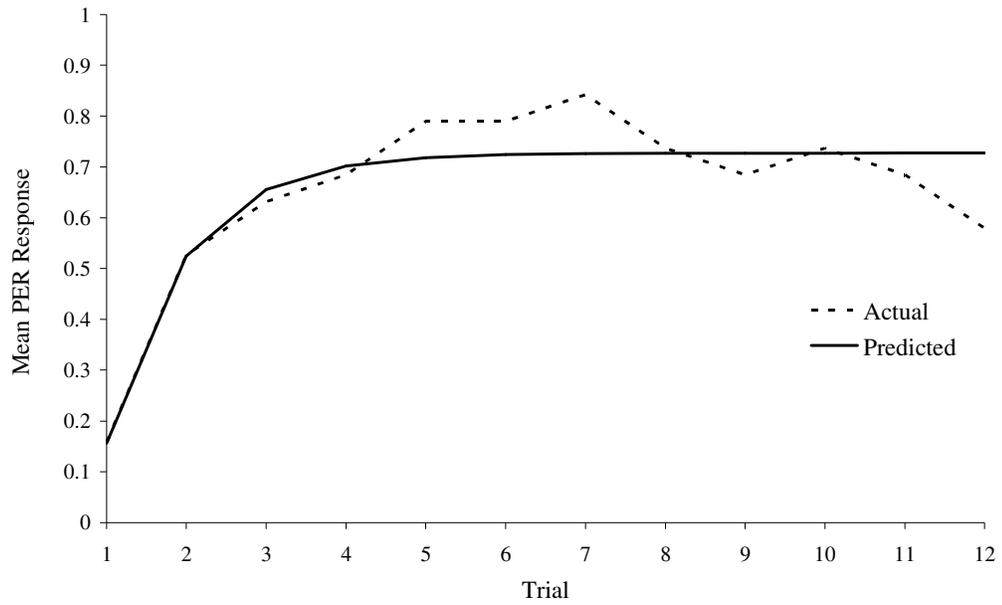
**Fig. 32.** CS responses for 9 µg/bee diflubenzuron, treated in US.



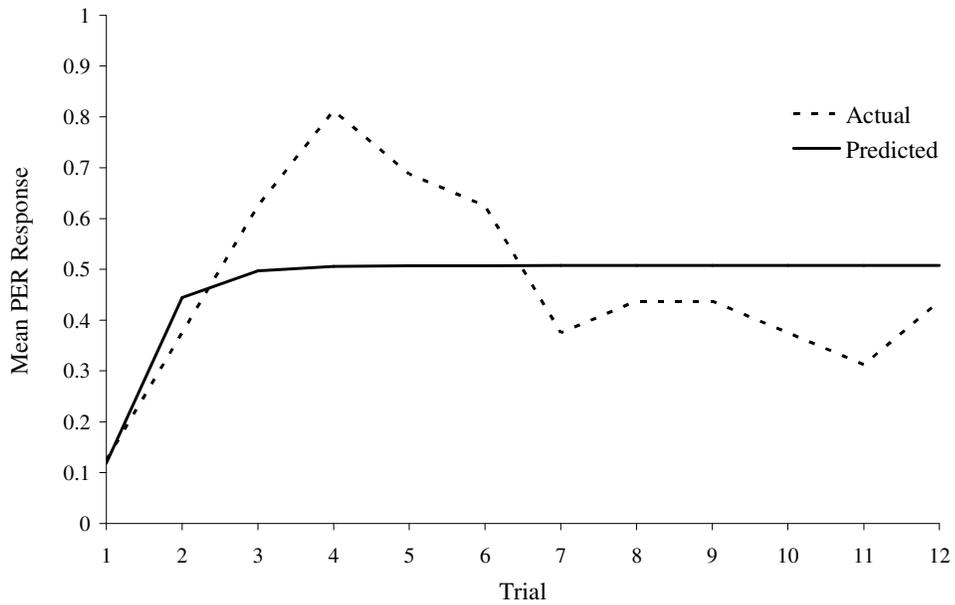
**Fig. 33.** CS responses for 16 µg/bee diflubenzuron, pretreated.



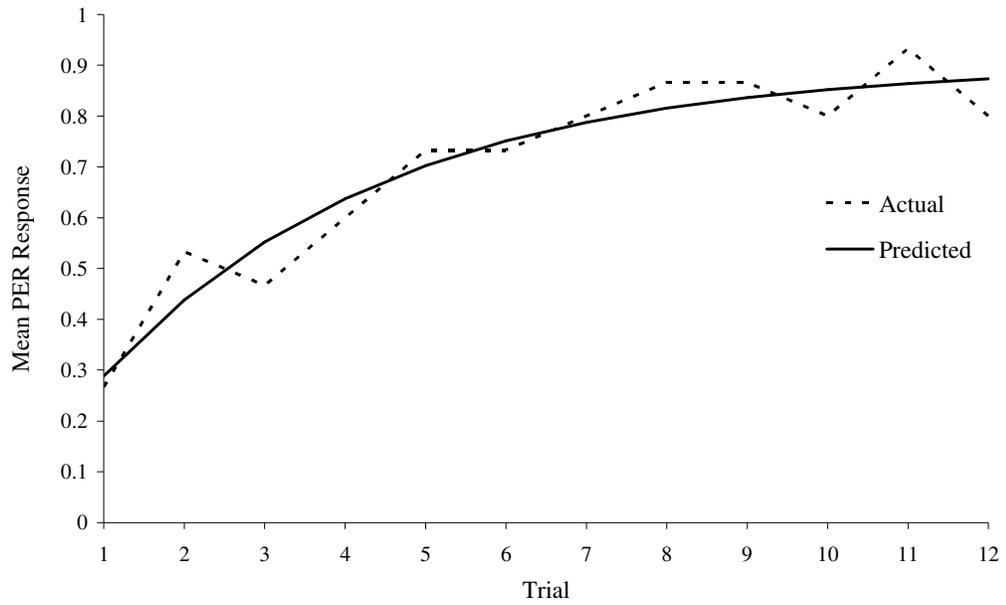
**Fig. 34.** CS responses for 16 µg/bee diflubenzuron, treated in US.



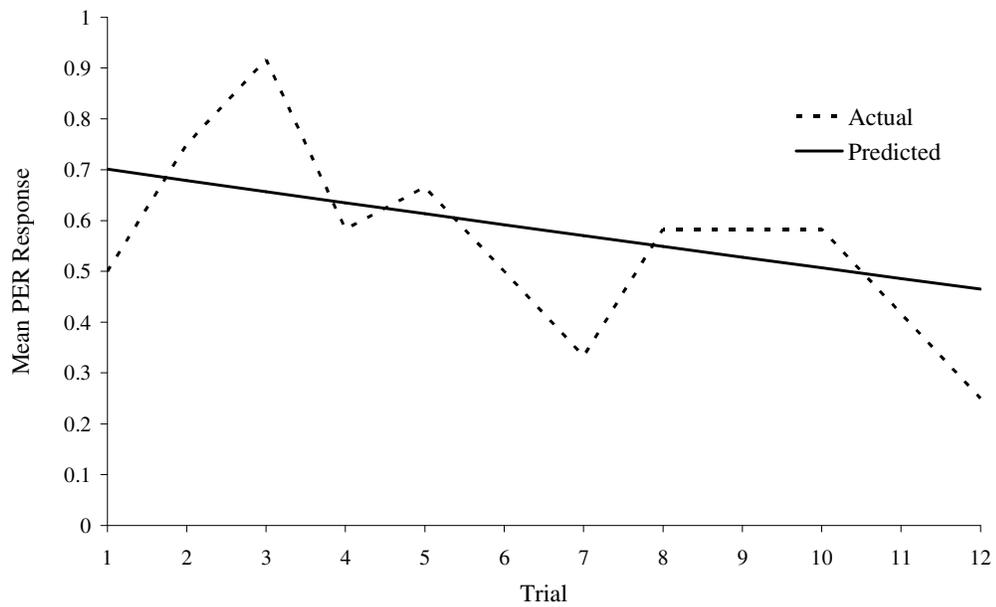
**Fig. 35.** CS responses for 32 µg/bee diflubenzuron, pretreated.



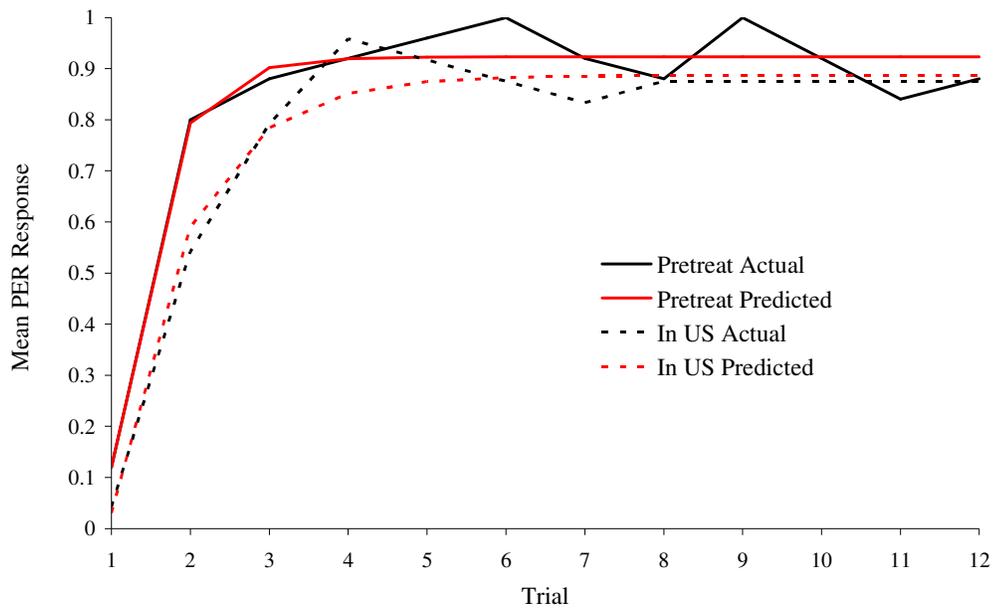
**Fig. 36.** CS responses for 32 µg/bee diflubenzuron, treated in US.



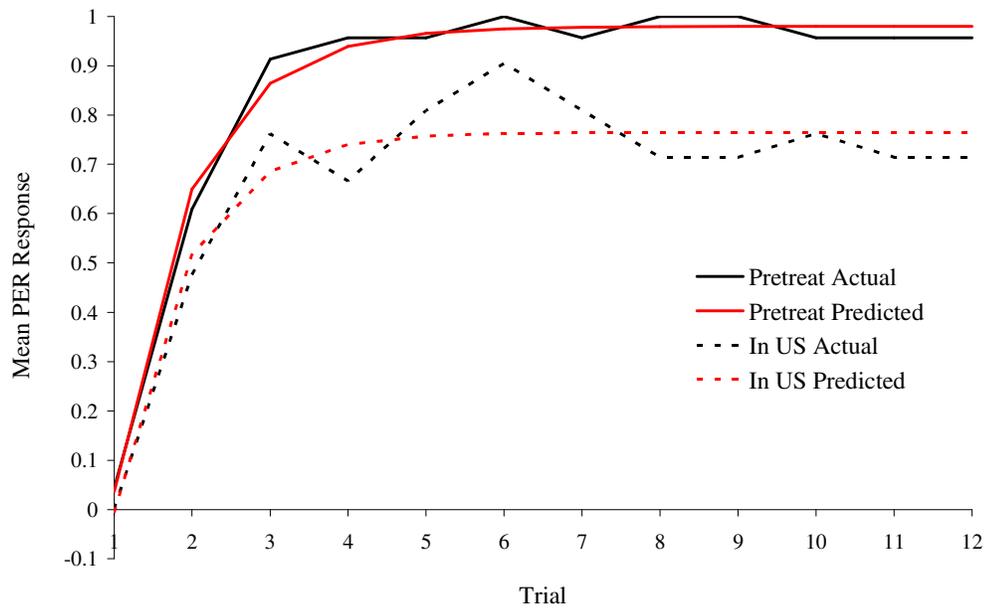
**Fig. 37.** CS responses for 69 µg/bee diflubenzuron, pretreated.



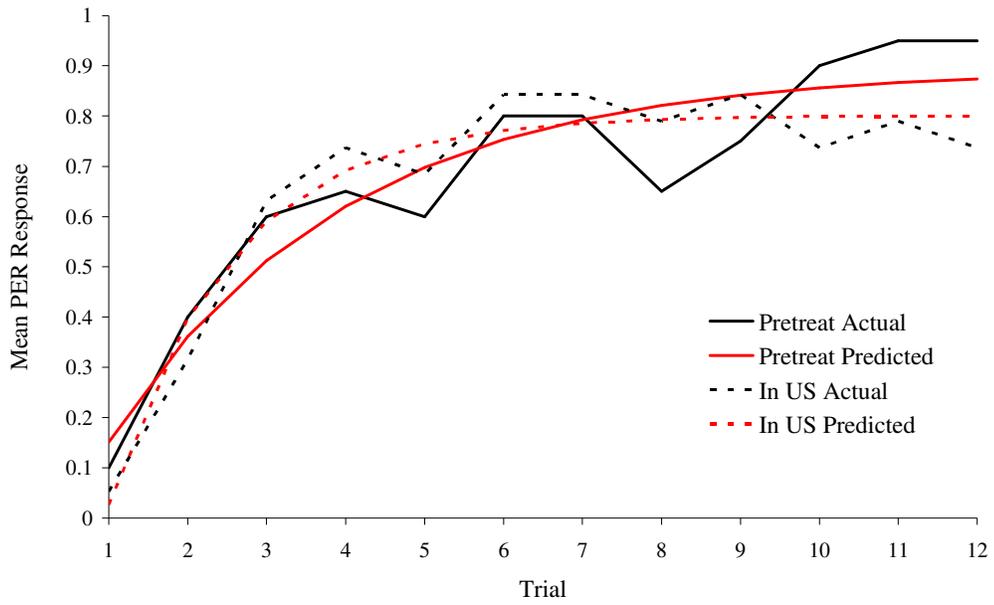
**Fig. 38.** CS responses for 69 µg/bee diflubenzuron, treated in US.



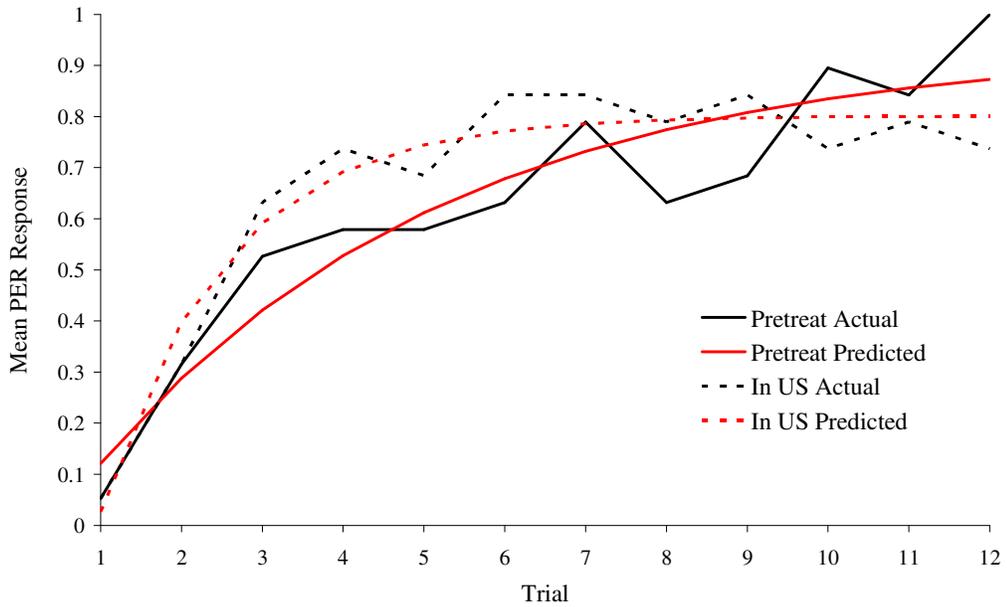
**Fig. 39.** Comparison of CS responses for 0 µg/bee tebufenozide.



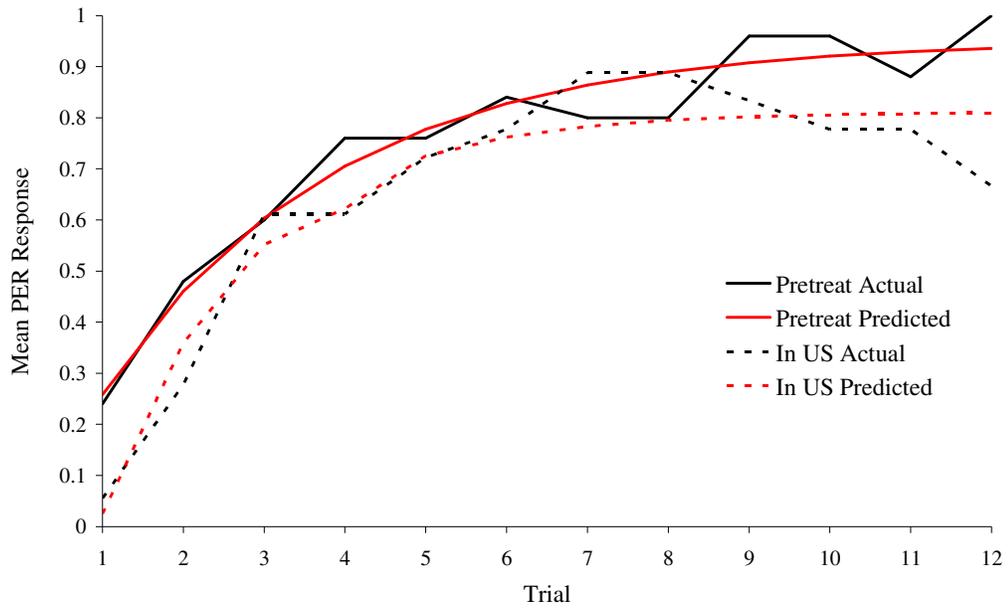
**Fig. 40.** Comparison of CS responses for 16 µg/bee tebufenozide.



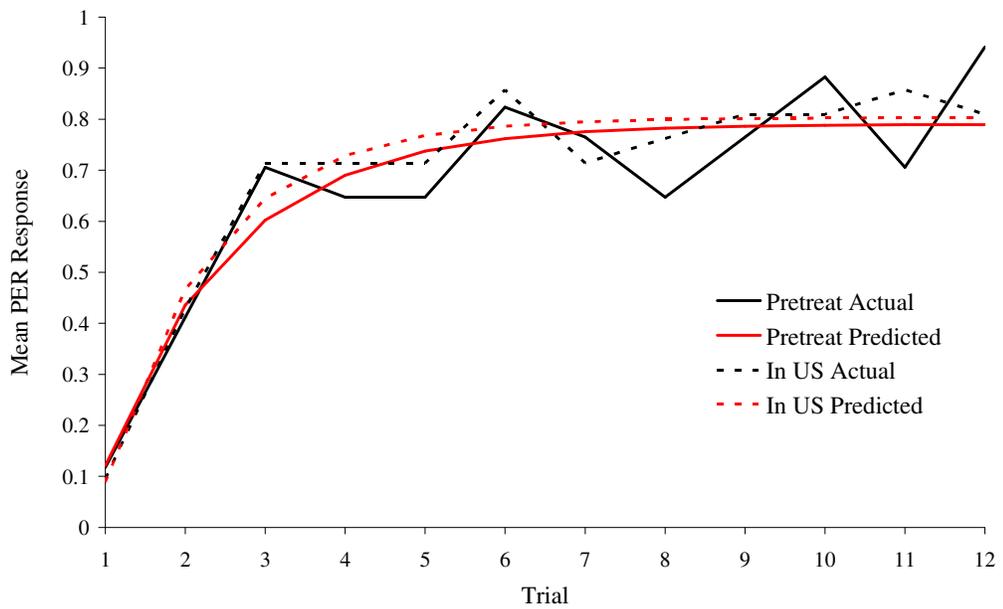
**Fig. 41.** Comparison of CS responses for 24 µg/bee tebufenozide.



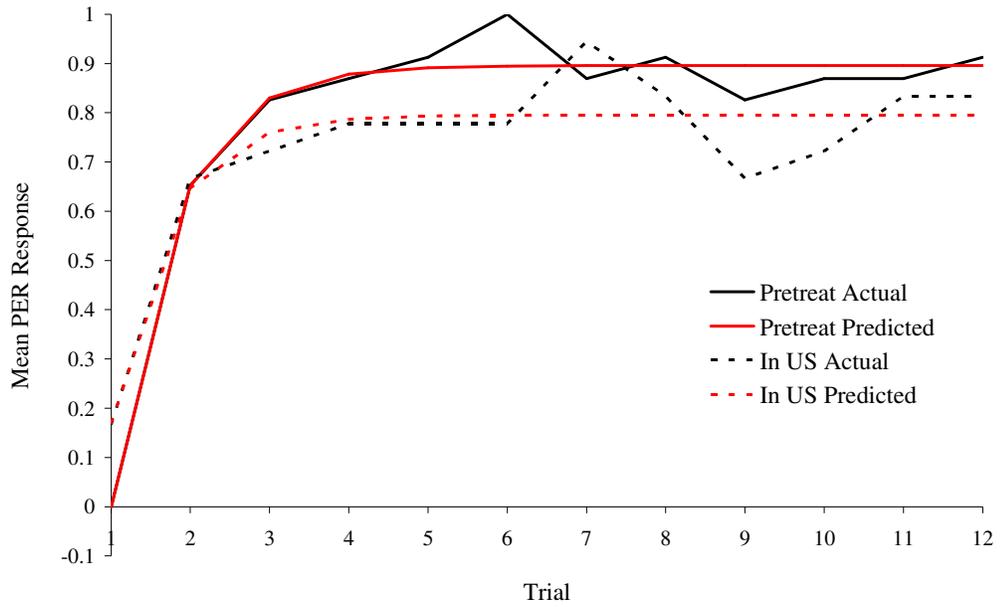
**Fig. 42.** Comparison of CS responses for 32 µg/bee tebufenozide.



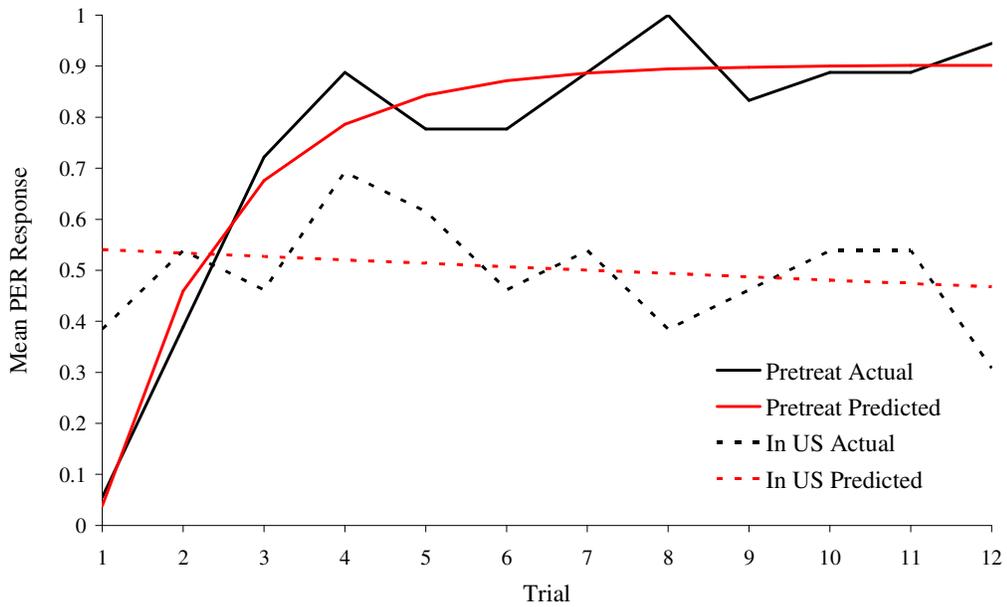
**Fig. 43.** Comparison of CS responses for 69 µg/bee tebufenozide.



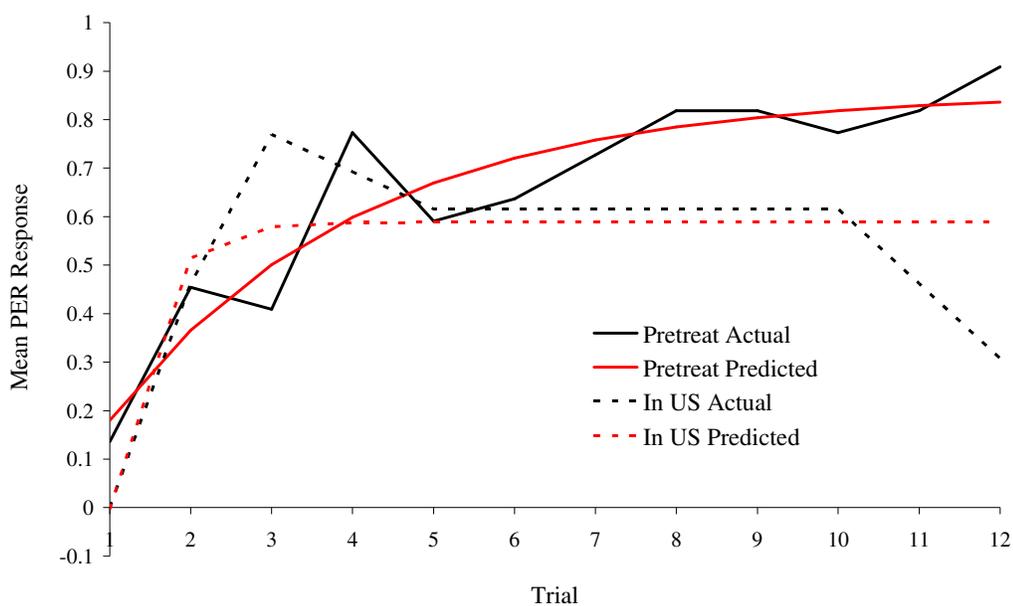
**Fig. 44.** Comparison of CS responses for 131 µg/bee tebufenozide.



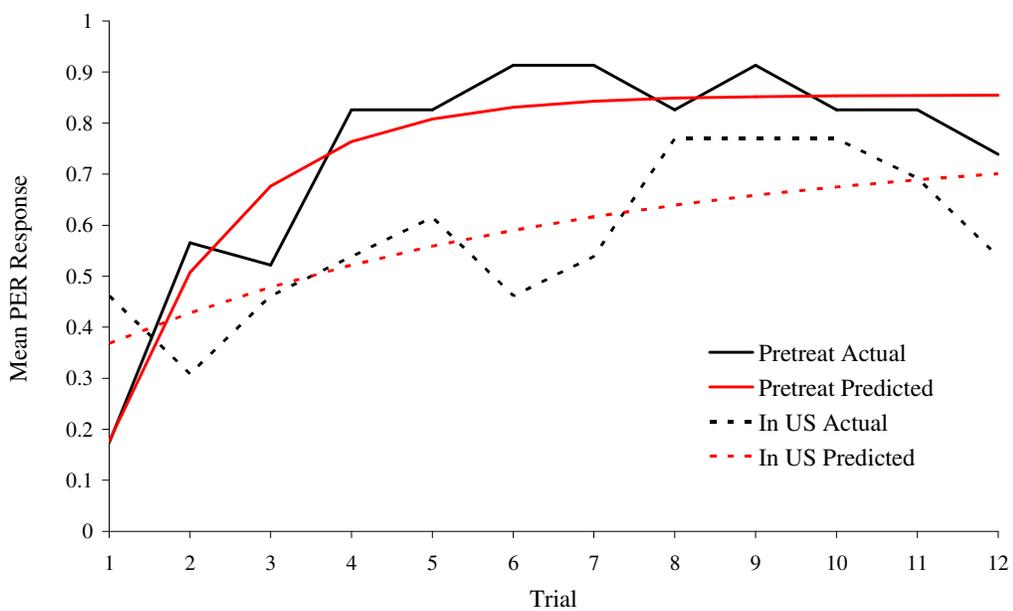
**Fig. 45.** Comparison of CS responses for 0 µg/bee diflubenzuron.



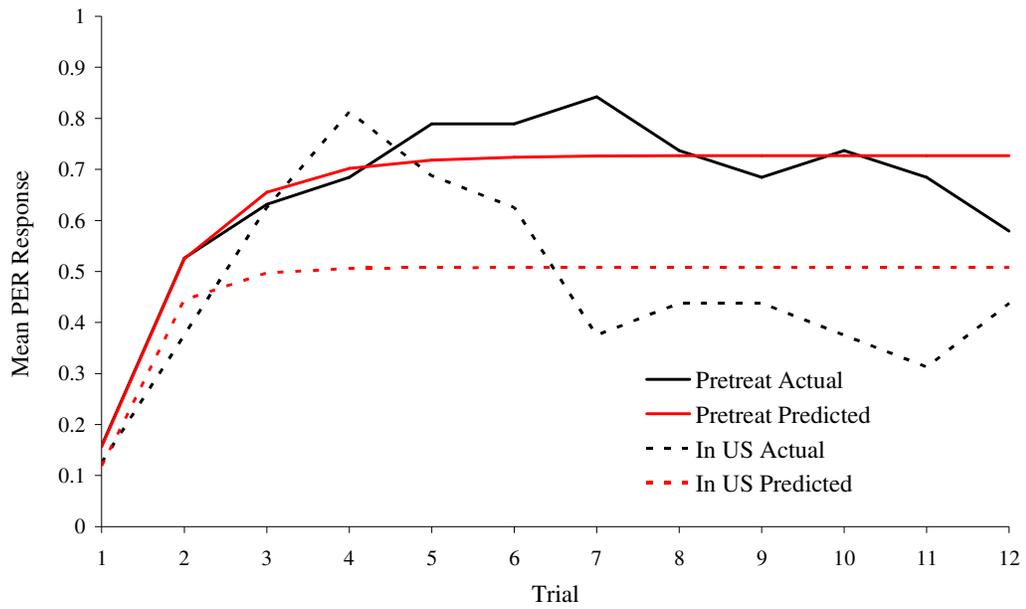
**Fig. 46.** Comparison of CS responses for 3 µg/bee diflubenzuron.



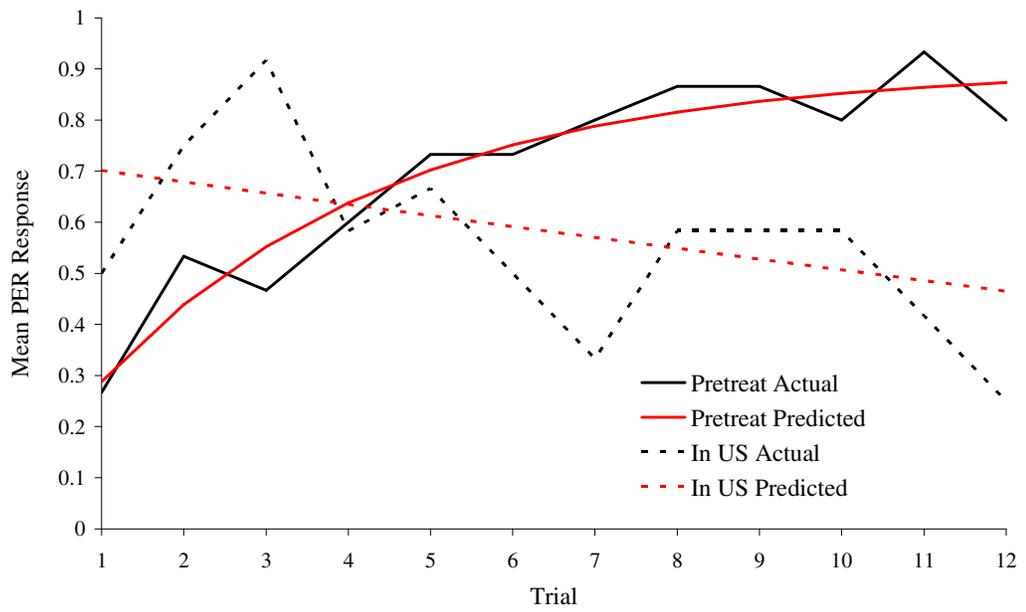
**Fig. 47.** Comparison of CS responses for 9 µg/bee diflubenzuron.



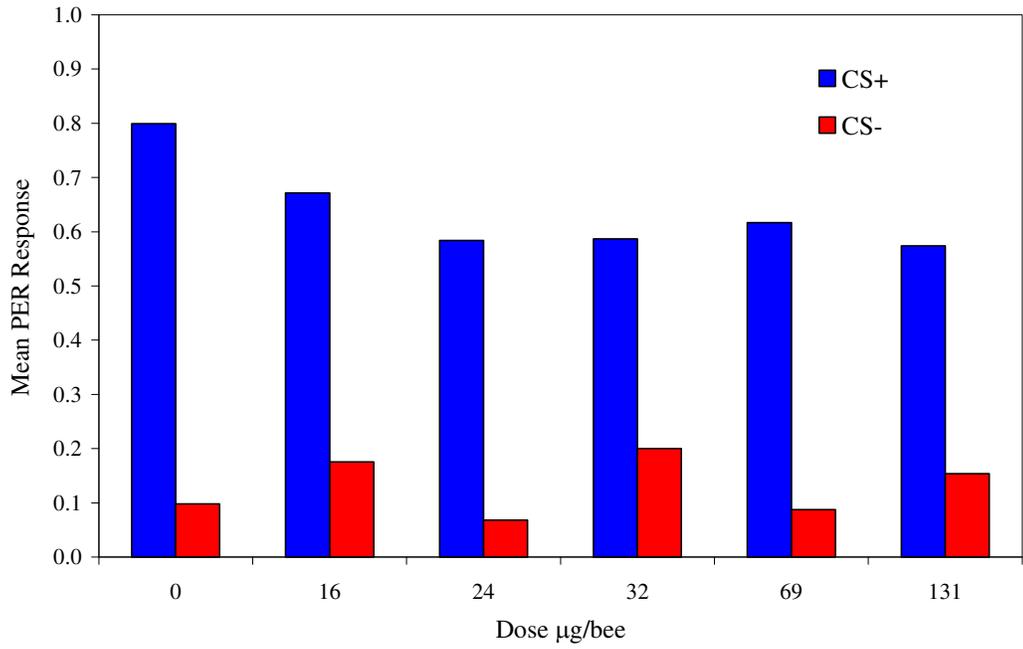
**Fig. 48.** Comparison of CS responses for 16 µg/bee diflubenzuron.



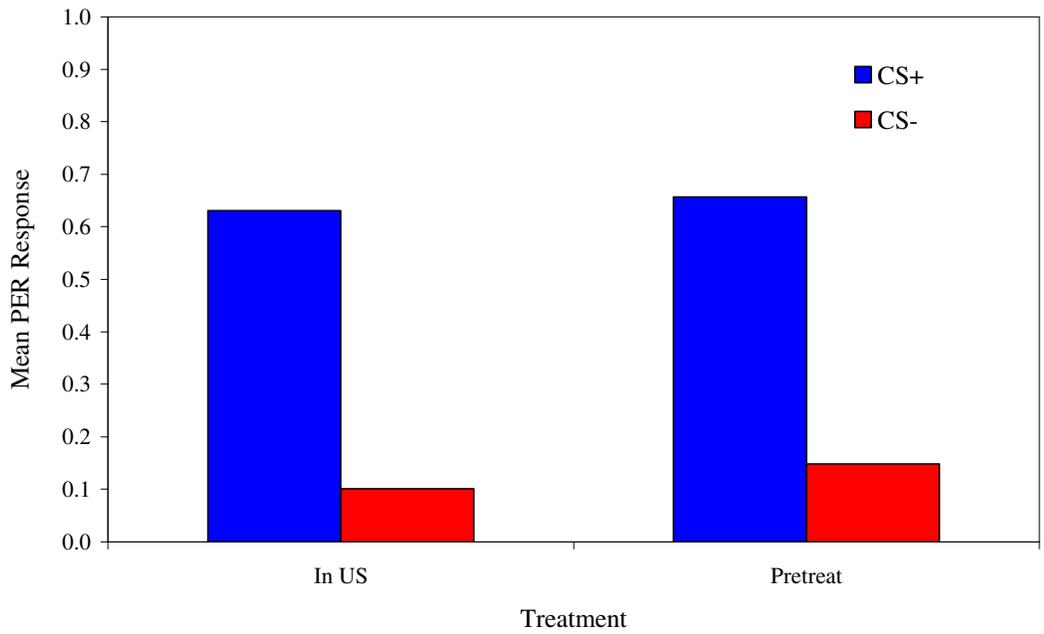
**Fig. 49.** Comparison of CS responses for 32 µg/bee diflubenzuron.



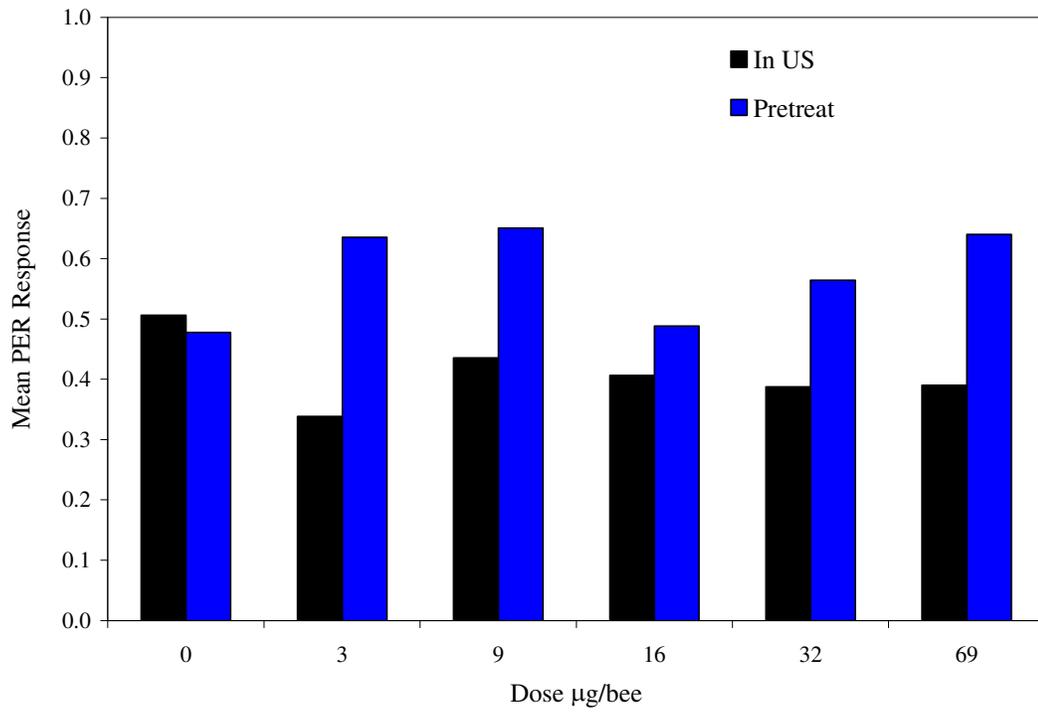
**Fig. 50.** Comparison of CS responses for 69 µg/bee diflubenzuron.



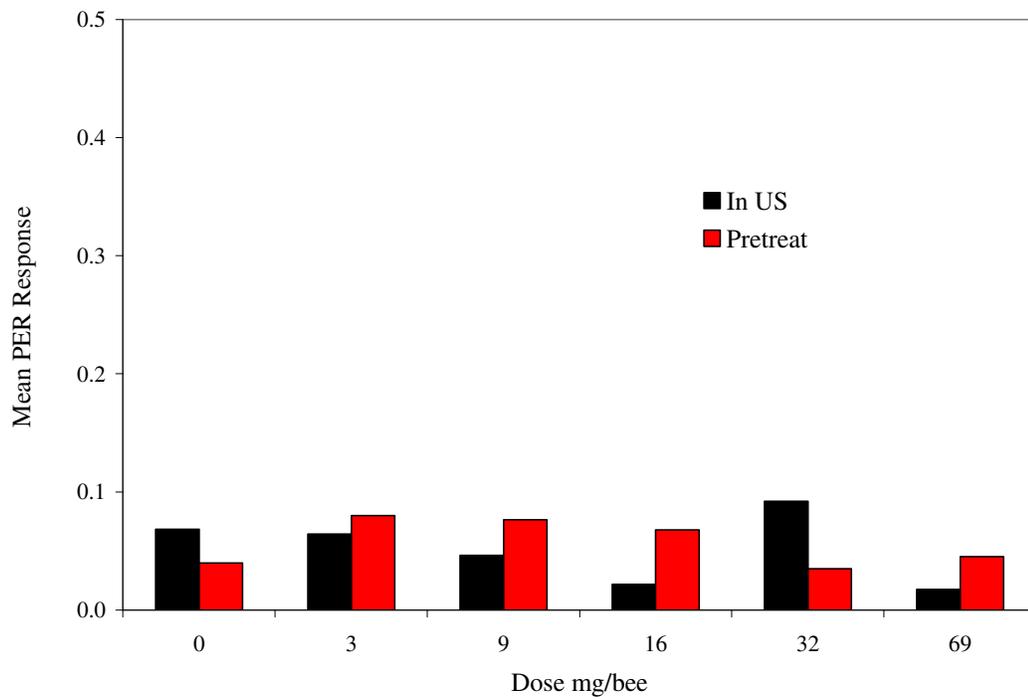
**Fig. 51.** Discrimination: Stimulus\*Dose for tebufenozide.



**Fig. 52.** Discrimination: Stimulus\*Treatment for tebufenozide.



**Fig. 53.** Discrimination: Stimulus\*Dose\*Treatment for diflubenzuron. CS+ responses.



**Fig. 54.** Discrimination: Stimulus\*Dose\*Treatment for diflubenzuron. CS- responses.

**Table 1.** Positive PER responses for tebufenozide acquisition experiments *before* the learning filter was applied. The number of responses per trial/dose is out of a possible 25. CS responses only.

Tebufenozide-Pretreat-CS Responses												
Dose μg/bee	Trial											
	1	2	3	4	5	6	7	8	9	10	11	12
<b>0</b>	<b>3</b>	<b>20</b>	<b>22</b>	<b>23</b>	<b>24</b>	<b>25</b>	<b>23</b>	<b>22</b>	<b>25</b>	<b>23</b>	<b>21</b>	<b>22</b>
16	1	14	21	22	22	23	22	23	23	22	22	22
24	2	8	12	13	12	17	16	13	15	18	19	19
32	1	6	10	11	11	12	17	12	13	17	16	20
69	6	12	15	19	19	21	20	20	24	24	22	25
131	2	8	13	12	12	14	14	11	16	16	13	16

Tebufenozide-Treat in US-CS Responses												
Dose μg/bee	Trial											
	1	2	3	4	5	6	7	8	9	10	11	12
<b>0</b>	<b>1</b>	<b>13</b>	<b>19</b>	<b>23</b>	<b>22</b>	<b>21</b>	<b>20</b>	<b>21</b>	<b>21</b>	<b>21</b>	<b>21</b>	<b>21</b>
16	0	10	16	15	17	20	17	16	16	17	15	16
24	1	7	12	14	13	16	16	15	16	14	15	15
32	2	8	9	13	18	16	22	16	17	16	16	17
69	1	5	11	12	13	14	17	16	16	14	15	14
131	2	9	15	15	15	19	15	17	18	19	18	18

**Table 2.** Positive PER responses for diflubenzuron acquisition experiments *before* the learning filter was applied. The number of responses per trial/dose is out of a possible 25. CS responses only.

Diflubenzuron-Pretreat-CS Responses												
Dose μg/bee	Trial											
	1	2	3	4	5	6	7	8	9	10	11	12
<b>0</b>	<b>0</b>	<b>15</b>	<b>19</b>	<b>20</b>	<b>21</b>	<b>23</b>	<b>20</b>	<b>21</b>	<b>19</b>	<b>20</b>	<b>20</b>	<b>21</b>
3	2	8	13	17	15	14	16	18	15	16	16	17
9	3	11	9	17	13	14	16	18	18	17	18	20
16	4	13	12	19	19	21	21	19	21	19	19	17
32	3	11	12	15	15	18	16	16	13	16	13	12
69	4	9	7	11	11	12	12	13	13	12	14	14

Diflubenzuron-Treat in US-CS Responses												
Dose μg/bee	Trial											
	1	2	3	4	5	6	7	8	9	10	11	12
<b>0</b>	<b>3</b>	<b>12</b>	<b>13</b>	<b>15</b>	<b>14</b>	<b>14</b>	<b>17</b>	<b>15</b>	<b>13</b>	<b>13</b>	<b>15</b>	<b>16</b>
3	5	7	7	9	9	7	8	6	6	8	7	6
9	1	6	12	9	8	9	8	9	8	8	7	4
16	7	4	7	7	11	6	8	10	11	11	10	8
32	2	6	10	13	14	10	6	7	7	6	5	7
69	7	10	14	8	8	6	4	8	7	7	6	4

**Table 3.** Number of bees that “learned” in acquisition experiments out of 25 bees per treatment/dose. These numbers correspond to bees that were used in the final statistical analyses.

Dose mg/bee	Tebufenozide		Dose mg/bee	Diflubenzuron	
	Pretreat	Treat in US		Pretreat	Treat in US
<b>0</b>	<b>25</b>	<b>24</b>	<b>0</b>	<b>23</b>	<b>18</b>
16	23	21	3	18	13
24	25	20	9	22	13
32	19	19	16	23	13
69	25	18	32	19	16
131	16	21	69	15	12

## References Cited

- Abramson, C. I. 1994.** A primer of invertebrate learning: the behavioral perspective. American Psychological Association, Washington D.C.
- Abramson, C. I. 1997.** Where have I heard it all before: some neglected issues of invertebrate learning, pp.55-78. *In* G. Greenberg and E. Tobach (Eds.), Comparative Psychology of Invertebrates: The Field and Laboratory Study of Insect Behavior. Garland Publishing, New York.
- Abramson, C. I., D. A. Buckbee, S. Edwards, and K. Bowe. 1996.** A demonstration of virtual reality in free-flying honey bees: *Apis mellifera*. *Physiol. & Behav.* **59**: 39-43.
- Brandes, C. H., B. Frisch, and R. Menzel. 1988.** Time-course memory formation differs in honey bee lines selected for good and poor learning. *Anim. Behav.* **36**: 981-985.
- Decourtye, A. and M. H. Pham-Delègue. 2002.** The proboscis extension response: assessing the sub-lethal effects of insecticides on the honey bee, pp.67-84. *In* J. Devillers and M. H. Pham-Delègue (Eds.), Honey Bees: Estimating the Environmental Impact of Chemicals. Taylor and Francis, London.
- Decourtye, A., J. Devillers, E. Genecque, K. Le Menach, H. Budzinski, S. Cluzeau, and M.H. Pham-Delègue. 2005.** Comparative sub-lethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Arch. Environ. Contam. Toxicol.* **48**: 242-250.
- Dhadialla, T. S., G. R. Carlson, and D. P. Le. 1998.** New insecticides with ecdysteroidal and juvenile hormone activity. *Ann. Rev. Entomol.* **43**: 545-569.

- Erber, J. 1975.** The dynamics of learning in the honey bee (*Apis mellifera carnica*) I. The time dependence of the choice reaction. *J. Comp. Physiol.* **99**: 231-242.
- Gerber, B., N. Geberzahn, F. Hellstern, J. Klein, O. Kowalsky, D. Wüstenberg and R. Menzel. 1996.** Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Anim. Behav.* **52**: 1079-1085.
- Information Ventures, Inc. 1995.** Diflubenzuron pesticide fact sheet.  
<http://infoventures.com/e-hlth/pesticide/difluben.html>
- International Programme on Chemical Safety (IPSC). 1995.** Diflubenzuron health and safety guide No. 99. <http://www.inchem.org/documents/hsg/hsg/hsg099.htm>
- Mamood, A. N. and G.D. Waller. 1990.** Recovery of learning responses by honeybees following a sub-lethal exposure to permethrin. *Phys. Entomol.* **15**: 55-60.
- Marfaing, B., J. Rouault, and P. Laffort. 1989.** Effect of the concentration and nature of olfactory stimuli on the proboscis extension of conditioned honey bees *Apis mellifica Ligustica*. *J. Insect Physiol.* **35**(12): 949-955.
- Menzel, R. 2001.** Searching for the memory trace in a mini-brain, the honeybee. *Learn. Mem.* **8**: 53-62.
- SAS Institute, Inc. version 9.1,** Cary, NC, USA.
- Stepanov, I. and C. I. Abramson. 2005.** A new mathematical model for assessment of memorization dynamics. *Span. J. Psych.* **8**(2): 142-156.
- Stone, J. C., C. I. Abramson, and J. M. Price. 1997.** Task dependent effects of dicofol (Kelthane) on learning in the honey bee (*Apis mellifera*). *Bull. Environ. Contam. Toxicol.* **58**: 177-183.

- Taylor, K. S., G. D. Waller, and L. A. Crowder. 1987.** Impairment of a classical conditioning response of the honey bee (*Apis mellifera* L.) by sub-lethal doses of synthetic pyrethroid insecticides. *Apidologie*. **18**: 243-252.
- Thompson, H. M. 2003.** Behavioural effects of pesticides in bees – their potential for use in risk assessment. *Ecotoxicol.* **12**: 317-330.
- Tripathi, R. 1996.** Information sheet on Dimilin. Virginia Department of Health: Bureau of Toxic Substances.
- Walgenbach, J. F. 1999.** Attributes of Confirm and Spintor. North Carolina Cooperative Extension Service. Apple Insect News July 13.  
<http://fletcher.ces.state.nc.us/staff/cpalmer/july991.html>.
- Weick, J. and R. S. Thorn. 2002.** Effects of acute sub-lethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **9**: 227-236.

## CONCLUSIONS

It is interesting that both of the studies in this thesis produced results that favor further investigation of each respective area of honey bee behavioral research. In review, the results from the experiment in Chapter II validated self-administration of ethanol solutions by free-flying foragers, even when bees were given a choice of straight sucrose. There were significant differences in the choice of 1% over the choice of 5% ethanol solution when target-switching was evaluated, but an overall solution preference was not evident between the control and two levels of ethanol. The lack of an observable solution preference indicated that the “flower constancy” behavior predominated throughout the experiment, proving that ethanol at the 1% and 5% levels is not aversive to bees. The insecticide study presented in Chapter III revealed that the two insect growth regulators, tebufenozide and diflubenzuron, can alter a normal learning curve in both a single-exposure and repeated-exposure scenario. The degree of learning disruption did not appear to be dose-dependent for either insecticide, but all levels of insecticides produced significantly fewer responses than sucrose. The only cases in which a normal learning curve (according to the model proposed by Stepanov and Abramson, 2005) was not generated were when diflubenzuron was administered in the US at levels of 3, 16, and 69 $\mu$ g/bee. An accurate comparison of dose effects was not possible in this study because of the large differences in sample sizes. This problem would therefore need to be addressed in subsequent experiments.

This thesis has presented the reader with two important applications of honey bee behavioral research that contribute to agro-economics and human wellness. It is not common that a single invertebrate species promises to have such a profound impact on both agricultural and medical aspects of research, and even less common that the impact on both industries is a benign one. Honey bees are one of few introduced species that have provided more positive rather than negative contributions to the United States economy. Genome mapping and further investigations of bee physiology and behavior may uncover other salient applications of economic import.

For the naturalist, honey bee research and observation has unlocked the door to the “mind” of social insects. The complexity of hive infrastructure and the altruistic behavior of individuals in a colony contradict the general principle of self-preservation held by most insect species. The morphologist has also benefited from the study of the honey bees’ anatomy and physiology, particularly in how the bees’ eusocial behavior is regulated by specialized glands and structures not present in other species.

As to my personal gleanings from studying the honey bee, they have served to quicken my interest in all aspects of this little member of the Hymenoptera. I have gone on to produce another PER study investigating fungicides used on soybeans, and have just embarked on a Small Hive Beetle biological control endeavor. There is no end to possibility in honey bee research!

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VITA

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Master of Science

Thesis: STUDIES ON HONEY BEE (*APIS MELLIFERA* L.) BEHAVIOR:  
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