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USING LEARNING PRINCIPLES IN PREDICTIVE  
RISK ASSESSMENT: A PESTICIDE  
TOXICOLOGY ASSAY FOR  
INSECTS USING THE  
HONEY BEE (*APIS*  
*MELLIFERA*) AS  
AN INDICATOR  
ORGANISM

By

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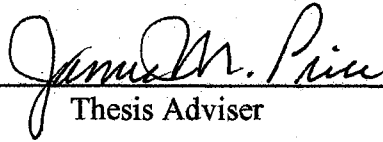
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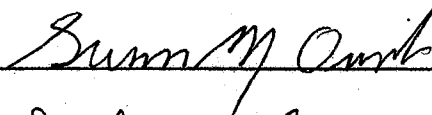
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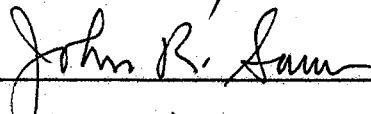
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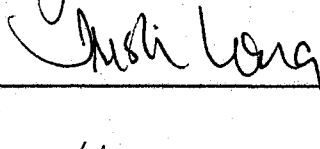
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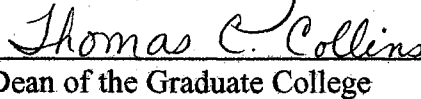
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## NOMENCLATURE

PER	Proboscis Extension Response
DDT	Dichlorodiphenyltrichloroethane
CS	Conditioned Stimulus
US	Unconditioned Stimulus
CR	Conditioned Response
UR	Unconditioned Response
OP	Organophosphate
Low	Low Dose Treatment Group-375ppm
Medium	Medium Dose Treatment Group-750ppm
High	High Dose Treatment Group-1500ppm
ITI	Intertrial Interval
MAPs	Modal Action Patterns

## CHAPTER I.

### INTRODUCTION

#### Background and Significance

*"Animal sentinel systems-systems in which data on animals exposed to contaminants in the environment are regularly and systematically collected and analyzed-can be used to identify potential health hazards to other animals and humans."*

(Committee on Animals as Monitors of Environmental Hazards, National Research Council, 1991).

#### Rationale

The goal of this research was to test the efficacy of using existing principles of learning in the realm of predictive risk assessment in pesticide toxicology. Here, the honey bee (*Apis mellifera*) is suggested as an appropriate sentinel animal to determine *a-priori* any behavioral disturbances that may result from chemical control procedures. Learning paradigms are not currently used in predictive risk assessment toward pesticidal compound approval. However, applied principles of learning are suggested here as the methods through which risks to beneficial insects can be determined, particularly those risks attributable to deficits in learning ability which, because of injudicious chemical use, have indirectly contributed to honey bee mortality. Additionally, behavioral measures may also indicate when non lethal amounts of pesticides create a greater risk for insect vectored human diseases by sensitizing the behavior of vector species.

The broad hypothesis tested in this research was that the introduction of sublethal chemical compounds in the environment creates behavioral disturbances in non-target insects. These disturbances may be assessed through existing laboratory learning paradigms. Current measures mandated by the Environmental Protection Agency (EPA) do not provide for behavioral assessment. In this research, it was believed that even chemical compounds with acute toxicity levels indicating that they are relatively non-toxic to bees would create sublethal effects on honey bee behavior measurable through learning paradigms. Because most pesticides are nerve poisons (Matsumura, 1985) and affect some part of the nervous system involved in signal transduction (e.g., neurotransmitters, enzymes, reuptake mechanisms, axonic conduction), and because it is known that these mechanisms play an important role in mediating learning at the physiological level in invertebrates (Kandel & Hawkins, 1993), and more specifically, in honey bees (Hammer, 1993; Menzel, Hammer, Braun, Mauerlshagen & Sugawa, 1991), it is logical to assume that nerve poisons will ultimately impact performance on learning tasks. In addition to showing how learning principles may be included in standard risk assessment protocols, suggestions are made here as to means of generalizing learning principles across species. Further, selection criteria of other appropriate sentinel species is discussed.

### The Problem

The use of animals as measures of local or global environmental health is not new. Historically, aquatic animals, household pets, birds, and other wildlife and domestic species have served humans as advanced warning systems for environmental

threats from contaminants. However, few examples exist wherein sentinel systems have been used for the benefit of non-human terrestrial fauna, and insects have only rarely served as sentinel animals in either scenario. Further, methods in place to gauge the potential risk from contaminants in the environment have typically used only opportunistic observations of behavior. A complete system would make use of behavioral toxicity research in conjunction with currently-accepted practices for pesticidal compound approval, and be implemented in appropriate sentinel species.

Sentinel animals are those whose physiology, biochemistry, and behavior are used in a systematic fashion to reveal potential risks to other animal species and humans (National Research Council, 1991). Sentinel animals also are referred to as indicator organisms (Thompson & Greig-Smith, 1991); the terms are used interchangeably. For the purposes of determining the effects of pesticides on terrestrial fauna, insects are natural choices for sentinel animals.

This review addresses the current measures used to assess pesticidal risk; discusses the economic importance of insects in society; addresses the problems of pest insect control; assesses the ecological and economic contributions of beneficial insects; and suggests a means to systematically apply well-established learning paradigms to predictive risk assessment methodology. Further, the European honey bee (*A. mellifera*) is suggested as the ideal animal for initial use as an organism through which to assess *a-priori* the behavioral toxicity of pesticides.

## Characterizing Risk Assessment

Neely (1994) outlined 20th-century attitudes toward chemical control in the following way: He characterized the years 1945-1960 as the "First Period" of chemical control. During this period, new technologies allowing for chemical control were implemented, with little regard to the potential mutagenic, teratogenic, and carcinogenic effects of toxic compounds. Dichlorodiphenyltrichloroethane (DDT) was synthesized and introduced as a "miracle bug killer" during this period.

The "Pessimistic Period," according to Neely, was during the years 1960-1969, when drastic measures, fueled by Rachel Carson's (1962) popular press book *Silent Spring*, warned of impending disaster to the environment from the injudicious use of chemicals. Chlorinated hydrocarbons such as DDT were Carson's primary target for criticism. This period was punctuated by congressional moves to ban the use of chemicals in the United States (U.S.) in addition to creating strict tolerance limits for residue levels in the environment.

However, dichotomous opinion eras often are followed by a move toward the center. Neely characterized the years since 1969 as the "Age of Realism." Since this time, rather than banning all chemicals, government entities have implemented strict risk assessment protocols for chemicals, recognizing that banning is not the answer; rather, preventing the misuse of chemicals is necessary in order to protect animals, humans, and the environment in which they live.

From these governmental activities came a systematic means of characterizing risk. In 1983, the National Academy of Science outlined the steps necessary in performing risk assessment. These include "1) Identification of the hazard; 2)

Establishment of dose-related effects; 3) Consideration of exposure assessment; and 4) Characterization of the actual risk" (in Neely, 1994, p. 7). A behavioral toxicity assay using established learning paradigms meets these criteria for risk characterization.

Ecotoxicology (the consideration of the environmental impact of toxins) is in its infancy where risk assessment models are concerned. Suter (1990) reviewed the existing models for characterizing the potential impact of pesticides, noting that current techniques rely merely on hazard assessment. Hazard assessment is based on the assumption that broad distinctions between "safe" and "unsafe" chemicals are sufficient. However, such broad distinctions are insufficient in estimating the safety of pesticides to non-target organisms, particularly in terms of sublethal exposure, as the measures are mortality-based. Moreover, these crude estimates force pre-registration testing procedures into the ultimately costly assessment tool of field testing, an approach that is often relied upon to fill in the broad gaps in safety indices. Suter further mentions that better laboratory methods of pesticide risk assessment must be initiated in order to either 1) Eliminate in some cases the necessity for costly field testing or 2) Improve the validity of risk models from field testing when it is deemed necessary.

The EPA mandates extensive testing of pesticides, both before and after registration (Hooper, Brewer, Cobb, & Kendall, 1990). This testing is particularly rigorous for chemicals that pose potential hazards to non-target species. In early stages of registration testing, a pesticide is characterized by its biochemical and toxicological effects on a small number of test species. Some efforts have been made to characterize poisoning-induced behavior at this stage. For example, in a study of organophosphate (OP) poisoning in quail, brain and plasma acetylcholinesterase levels were compared

with evasion behavior. The quails' evasion behaviors were determined by their ability to evade a domesticated cat following exposure to the OP (Galindo, Kendall, Driver, & Lacher, 1984). Control animals were much better at evading the domesticated cat than were the OP-treated quail. However, this attempt at characterizing behavioral toxicity is not standardized (i.e., within the rubric of standardized learning paradigms), and only has limited application for other species. Further, this work was not conducted within the context of EPA-mandated measures to supplement biochemical and physiological response measures.

Moreover, Johansen (1979) characterized behavioral endpoints associated with poisoning by organophosphate and carbamate insecticides with honey bees. He noted that aggregation at the entrance to the hive signals impending death from slow-acting chemical agents. Bees die at the entrance to the hive, a wet and sticky mass due to regurgitated nectar from their honey sacs. Johansen further noted a *post-hoc* behavioral indicator of organophosphate poisoning; bees die with their proboscis extended. A particular difficulty for the apiculturalist occurs when the poisoning agent is fast-acting, as many bees do not make it back to the hive, die in the field, and the apiculturalist often has little indication of poisoning until the entire colony succumbs to the poisoning.

Pre-mortality observations of poisoning-induced behavior in bees include "stupefaction, paralysis and abnormal jerky or spinning movements" Johansen (1979, p. 109), and "...bees behaving as if they are chilled, crawling around in front of the hive is almost a sure sign of carbaryl (Sevin) poisoning.....(bees) quickly lose their ability to fly; they slow down, and may take as long as 2-3 days to die" (p. 110). These observations, as well as those from Galindo, et al., (1984), while informative to some extent, do little to

promote inclusion of behavioral variables into pesticide registration procedures. Galindo, et al.'s (1984) procedures are not widely applicable to other species, and Johansen's (1979) observations lend themselves only to *post-hoc* identification of poisoning by a chemical agent. No standard learning paradigms are used systematically in any evaluative efforts toward registering pesticides in the U.S.

Current EPA mandates for characterizing risk, while standardized, are designed ultimately to predict mammalian/human risk potential. Testing moves through a series of time-consuming stages. First-stage testing includes mammalian acute toxicity estimates (Riley, 1990). Human toxicity estimates then are extrapolated based on these tests by subsequently mathematically adjusting for differences in human/test species body weight (Neely, 1994). Subchronic and chronic toxicity estimates are second-stage testing methods, with the resulting physiological and biochemical effects of pesticide exposure studied in durations from a single day to two years (Neely, 1994; Riley, 1990).

Some non-target species rate individual assessment, as dietary toxicity studies are frequently conducted on avian species. Birds are chosen for individual assessment because of their particular sensitivity to many compounds, as evidenced by the historical effects of DDT (Carson, 1962; Riley, 1990). Small-scale to large-scale field trials may follow, depending on the species being tested and the chemical being used. These studies may take up to 20 years to complete, and are quite costly (Riley, 1990).

Only a small number of species are used in the laboratory to determine potential toxicity to other fauna, and yet extrapolation of laboratory-generated data to other species is discouraged (Suter, 1990). Clearly, a need exists to broaden the ability to study a



multiplicity of species with a minimum of cost. Learning paradigms with appropriate indicator species such as honey bees should provide this highly needed methodology.

Insects and Society: Pests, Beneficials, and Economic and Human Health Considerations

Destructive Insects. Destructive insects (pests) create havoc in the environment through many different methods of injury. These injuries include destruction of crops and valuable plants, injury through venomous stings to animals and humans, injury of parasitic pests to host animals as either internal or external parasites, and most important, dissemination of diseases. The economic losses in the U.S. as a result of insect injury exceeded fourteen billion dollars in 1988 alone (Metcalf & Metcalf, 1993). These losses include forest products, fruit crops, greenhouse plants, and staple crops.

In addition to economic concerns, pest insect species constitute a significant human health risk. Insect-vectored diseases, though fairly well-controlled in the U.S., are not yet eradicated globally. Some of the diseases vectored by insects include yellow fever, malaria, Chagas' disease, encephalitides, and African sleeping sickness (Metcalf & Metcalf, 1993). While these are clear human health risks, it is ironic that the elimination of most insect-vectored disease threats in the U.S. has come from chemical control, also considered a human health risk. Third-world countries, however, continue to use insecticides banned in the U.S. in order to gain control over some of these insect-disseminated diseases, as the risks from insect-vectored diseases in these countries far exceed the health risk from the chemical agents.

Beneficial insects. While pest insects exist and have been historically treated with chemical control agents, there are many species of insects that perform ecological

services. Their benefits to humans include the production and collection of products of commerce; pollination to aid in propagation of fruits, seeds, vegetables, and flowers; destruction of injurious insects; destruction of weeds; soil conditioning; and scavenging (Metcalf & Metcalf, 1993).

Many beneficial insect species have provided natural means of pest insect control, and when introduced into a pest-laden ecosystem, these beneficial species allow for economic recovery from sustained agricultural losses resulting from pest species injury. For example, the parasitic wasps *Cotesia marginiventris* and *Microplitis croceioes* currently serve an important role in attacking pests in the *Heliothis/Helicoverpa* groups of insects. Chemical control costs for these pests exceed two billion dollars per year, a cost drastically reduced by use of beneficial parasitic insects (Adams, 1993). The use of parasitic wasps around cotton crops has decreased costs per acre for boll weevil control from \$300.00 to just \$30.00 (Raloff, 1994).

However, the natural efficacy of beneficial species is undermined when they fall under the same chemical attack from pesticides as do the target pest species. The injudicious use of broad-spectrum insecticides has killed many non-target beneficial insects. Elimination of natural enemies through accidental kills is a large reason for insect resurgence following insecticide application (Metcalf & Metcalf, 1993). Further, long-term use of broad-spectrum chemicals creates an ecological imbalance that may be eliminated through conservative use of chemicals. Such is within the purview of Integrated Pest Management (IPM), an approach designed to reduce losses to crops and animals in a manner which causes minimal environmental damage and reduces risks to human health (Hernberry, Glass, Gilbert, King, Miller, & Whitten, 1991).

### Insect Learning: Potential for use in Risk Assessment

Prokopy and Lewis (1993) called for a systematic application of learning theories to pest management strategies. They suggest "...learning as a component of the informational state of the insect, ought to be integrated with knowledge of the physiological and genetical state of the insect if we are to have a robust understanding of the nature of insect behavioral response to environmental stimuli" (p. 332). However, most of the suggestions forwarded by Prokopy and Lewis deal with direct application of principles of learning to insect control. Methods suggested include population density estimates, cultural control, entomophage performance control, and general biological control strategies, many of which are already in use in IPM.

Insect learning, in addition to applied use in pest management, as suggested by Prokopy and Lewis (1993), should also figure prominently as a predictive risk assessment tool. In addition to being low-cost methods, learning paradigms may provide behavioral toxicity data not revealed in EPA-mandated acute toxicity research. Insects do exhibit standard forms of learning, and systematic testing is possible through existing learning paradigms.

Abramson (1994) outlined standard paradigms useful for learning research with invertebrate species. Learning paradigms fall under the rubric of either non-associative learning (in which innate mechanisms are in place for rapid adaptation to unimportant environmental stimuli; Gould, 1993) and associative learning (where associations between environmental cues and behavioral consequences take place by means of contiguity or contingency of stimuli).

Under the definition of non-associative learning are two forms: Habituation and sensitization. Abramson (1994) defined them as follows:

"Habituation refers to the decrease in amplitude, probability, or a change in the topography of a response to a monotonously repeated stimulus. Stimuli that no longer transmit any significant information, such as the presence of a predator or the location of a food source, tend, over time, to be ignored. The reduction in response strength to stimuli that initially elicited a host of reactions is the fundamental characteristic of habituation..." (p. 105).

"Sensitization is, in essence, the opposite of habituation and refers to an increase in frequency or probability of a response and is often accompanied by a decrease in latency and a lower threshold following the monotonous presentation of a usually strong stimulus. In addition to counteracting the influence of habituation, sensitization is important because it gives the animal an ability to increase the frequency of innate reaction "(p. 105).

Habituation and sensitization have two sub-qualities; short-term and long-term.

In terms of behavioral toxicity, short-term behavioral effects are those likely to indicate acute toxicity effects. Long-term events may result from long-term sublethal exposure, and may indicate neuronal, physiological, or even morphological necrosis of nervous tissues important in non-associative forms of learning.

Associative forms of learning include classical conditioning and instrumental/operant conditioning. In classical conditioning paradigms, neutral stimuli are paired with biologically significant stimuli that elicit reflexive behaviors. The stimuli are presented in a contiguous fashion such that a previously neutral stimulus gains predictive ability. The standard classical conditioning paradigm is as follows:

Unconditioned stimulus (US) = Stimulus eliciting a reflexive response.

Unconditioned response (UR) = The reflexive response elicited by the US.

Conditioned stimulus (CS) = Behaviorally neutral stimulus.

Conditioned response (CR) = Response resulting from CS-US pairing over trials  
in the following manner:

Early in training: CS+ US => UR.

Pairing CS + US over trials until CS => CR.

The CR is a response within the constellation of the UR, though it may not be as strong as the UR. The CR is the result of a learned association between the CS and the US. The CS gains predictive power through the repeated association between the CS and US (see Kimble, 1961; Pavlov, 1927).

For analysis of more complex forms of learning, species may be subjected to instrumental or operant paradigms. In these, the delivery of a stimulus with motivational properties becomes contingent upon specific behaviors required by the experimenter (Abramson, 1994).

Insects exhibit the ability to learn in all of the paradigms mentioned (Abramson, 1994). Habituation and sensitization have been demonstrated in many insect species (Dethier, Solomon, & Turner, 1965), and are considered evolutionary precursors of associative forms of learning, representative of simpler learning abilities (Gould, 1993). Associative forms of learning have been demonstrated in houseflies, fruit flies, bees, blow flies, cockroaches, and locusts (Abramson, 1994; Fukuski, 1979; Hirsch & Holiday, 1988; McGuire, 1984).

Associative learning abilities appear to exist in insect species where associative capacity plays a functional role in species survival. In social insects and in foragers, particularly those that feed on several food sources, associative learning is an adaptive feature. However, there are some species in which non-associative learning is

paramount. For this reason, there are a number of variables that must be considered when determining which paradigm variation should be used to test the learning abilities of different insect species (see Abramson, 1994 for variables important in designing non-associative and associative learning experiments in invertebrate species).

Learning paradigms can clearly provide important tools for risk assessment measures in pesticide toxicology. The current use of sentinel animals in incidence-monitoring underscores the need for the employment of standardized behavioral testing. For example, in a comprehensive study of accidental bee kills in the United Kingdom from 1981-1991, Greig-Smith, Thompson, Hardy, Bew, Findlay, and Stevenson (1994) lament that mortality data from incidents of honey bee poisoning are merely *post-hoc* indications of environmental health. They further mention that no systematic measures exist for the use of honey bees as a predictive risk tool, but acknowledge that mortality data on honey bees may be used in risk assessment for other species. The notion that bees are valuable in risk monitoring only in their mortality, along with recent infestations of tracheal and varroa mites, has led to dramatically decreasing numbers of both wild honey bees and domesticated honey bees kept by apiculturalists. Further, a recent move to underscore the importance of natural pollinators (those indigenous to a geographical area; honey bees are typically imports) comes as a direct result of the decreasing numbers of honey bees for pollination (Buchman & Nabham, 1996). Buchman and Nabham (1996) also point out that these natural pollinators are also subject to risk from chemical compounds.

Behavioral responses are generally not included in systematic risk assessment methods for pesticides. Behavior is mentioned anecdotally, however. It is well-known,

for example, that a certain chain of behaviors precedes death by acetylcholinesterase-inhibiting compounds (Matsumura, 1985; Thompson & Greig-Smith, 1991). These behaviors include, in order of appearance, excitability, convulsions, paralysis, and death.

Additionally, behavioral changes are often noted in a single magnitude of concentration away from acute dose amounts (Thompson & Greig-Smith, 1991). Behavioral measures, then, are good indicators of the sublethal effects of a compound on an animal, and provide sensitive analysis of the animal's health at both non-toxic and near-toxic sublethal doses.

In a typical predictive risk assessment situation, the measurable response is biological, as exposure to toxic chemicals sets up a detectable series of biological events. However, behavioral events often are evident before any biological measures are taken. If applied in a systematic fashion, the tools for behavioral toxicity in pesticide toxicology assessment may be a more conservative estimate of toxicity than traditional acute toxicity estimates, and may also yield a quicker measurable response than is afforded by biological measures. In short, behavioral measures may narrow the gaps inherent in EPA-mandated hazard assessment methods.

#### The European Honey Bee as an Indicator Organism in Behavioral Toxicity Measures

The first criterion for classification as a sentinel animal is that the animal should have a measurable response (National Research Council, 1991). Additionally, it should be easily captured and enumerated, with sufficient population density to aid in enumeration. Thompson & Greig-Smith (1991) broadened the defining criteria for indicator species. Their criteria included the following:

1. Species that are particularly likely to be exposed.
2. Species thought to be particularly sensitive to a given exposure.
3. Species for which an adverse impact would be especially damaging.
4. Species which closely resemble the typical characteristics of a large number of others in the community.
5. Abundant species permitting large sample sizes for data collection.
6. Species whose ecology and behaviour provide easy opportunities to measure effects (Thompson & Greig-Smith, 1991, p. 82).

While these criteria may be met by other beneficial species, they are particularly significant for both wild and domesticated honey bees. Because of their positioning in fields for pollination, honey bees are particularly likely to be exposed to pesticidal compounds. Additionally, because honey bees are particularly sensitive to exposure levels, often acute toxicity data are reported for honey bees (e.g., Johansen, 1979; Stevenson, 1968, and product labels for commercial insecticides carry warnings regarding bee toxicity). Further, the nature of honey bee colonies, wild or domestic, allows for easy capture of numerous individuals. Finally, because of the wealth of learning literature on honey bees, behavior under controlled conditions is easily established, measured, and interpreted (Abramson, Edwards, Buckbee, & Bowe, 1996; Batson, Hoban, & Bitterman, 1992; Bitterman, 1996; Bitterman, Menzel, Fietz, & Shafer, 1983; Hammer, Braun, & Mauelshagen, 1994; Menzel & Bitterman, 1983).

The European honey bee is not only an excellent choice for a sentinel animal based on field responses to chemicals, it also has demonstrated both non-associative and associative learning ability in laboratory situations (Bitterman, 1996; Bitterman, et al., 1983; Buckbee, 1995; Hammer, Braun, & Mauelshagen, 1994; Menzel & Bitterman, 1983; Stone, Abramson, & Price, 1997). In addition, honey bees have served as an *in-situ* assessment species on a random basis as estimators of air pollution (National Research Council, 1991). Several points of interest make the honey bee the logical



candidate for this systematic application of learning principles as a predictive risk assessment tool. These include their economic importance as commercial product producers (honey and beeswax) and as pollinators of agriculturally significant crops (e.g., alfalfa in the arid southwestern U.S.). Additionally, honey bees are easily worked with in the laboratory in controlled learning scenarios. Further, it is well-known that physiological and biochemical information derived from honey bees is easily extrapolated to other bee species, and bees are good overall indicators of the general health of the environment (Greig-Smith, et al., 1994).

In 1988, the income from honey and beeswax exceeded 283 million dollars (Metcalf & Metcalf, 1993). Additionally, more than 100 species of bees are pollinators of significant crops in the U.S. Introduction of honey bee colonies into alfalfa fields has been known to more than double the seed yield. To further underscore the economic importance of bee pollinating behavior, Metcalf and Metcalf (1993) estimate that for every five dollars made in honey from honey bees, 100 dollars of seed or fruits are made. They further estimate the total value of honey bee pollination was \$9.3 billion dollars in 1988 alone. Honey production is also a major economic venture in the United States. Production values from 1991 to 1993 went from \$121 million to \$125 million dollars (Hoff, 1995).

Honey bees are capable of all forms of learning described earlier. They exhibit habituation and sensitization appropriately to monotonously-presented stimuli (Hammer, et al., 1994). Honey bees exhibit classical conditioning of the proboscis extension reflex (PER) in laboratory olfactory conditioning paradigms (Batson, et al., 1992; Bitterman, et al., 1983; Buckbee, 1995; Stone, et al., 1997). They easily adapt to semi-natural learning

situations, methods which may eventually serve as good field study measures of learning (e.g., Abramson, et al, 1996). And, honey bees exhibit instrumental/operant learning in a laboratory leg-lift paradigm (Stone, Abramson, & Buckbee, 1996). Further, learning is mediated by biochemical and neuronal activity that has been outlined for a number of behavioral responses in great detail (Burrell & Smith, 1995; Hammer, 1993; Menzel, et al., 1991; Mercer & Menzel, 1982).

Because these activities are mediated by biochemical and neuronal events, learning is subject to disruption by exogenous chemicals. Sublethal doses of parathion, methyl-parathion, and formulated methoprene disrupted foraging behavior at doses far below the recommended application dose (Barker & Waller, 1978). Oral and contact applications of deltamethrin disturbed homing-flight activities in honey bees (Vandame, Meled, Colin, & Belzunces, 1994). Dicofol prevented acquisition of the PER in a classical conditioning paradigm (Stone, et al., 1997). Further, learning capacities were found to be temporarily disrupted by a low-level, single permethrin dose (Mamood & Waller, 1990). Even "bee-safe" insecticides have been implicated in behavioral deficits and in poisoning incidents (Greig-Smith, et al., 1994). Further, behavioral peculiarities have been seen even when homogenates of honey bees showed no detectable traces of insecticide residue (Vandame, et al., 1994) Clearly, many behavioral responses can be altered through the application of pesticides. However, because no single assay exists through which pesticide toxicity can be measured in insects at the behavioral level, a need is present to develop a means to do so, and this is the focus of the following experiments.

Two experiments were performed in order to generate behavioral data for analysis within the rubric of predictive risk assessment. Experiment 1 utilized the European honey bee as an indicator organism through measuring sublethal effects of the insecticide methoxychlor, a commercially-approved insecticide, on classical conditioning performance. Experiment 2, also using honey bees, examined the effects of methoxychlor on habituation/sensitization performance. Both experiments were designed to apply established learning paradigms to predictive risk assessment methodology, and to view the ensuing behavioral results within the context of risk characterization.

## CHAPTER II

### EXPERIMENT 1

#### Classical Conditioning

##### Classical Conditioning of the PER

Using a paradigm first designed by Menzel & Bitterman (1983) and revised by Buckbee (1995), honey bees exposed to three doses of the insecticide methoxychlor were assessed for behavioral changes hypothesized to differ as a function of insecticide application and dose. While not all insects are capable of associative learning (of which classical conditioning is the most rudimentary form), honey bees have repeatedly demonstrated the ability to form associative relationships among a number of stimuli (see Batson, et al., 1992, Bitterman, 1996; Bitterman, et al., 1983). It was important to include this paradigm, as it has been demonstrated that not only may the insecticide create behavioral disturbances in honey bees, this effect may differ as a function of learning paradigm (Stone, et al., 1997).

This experiment contained a repeated-measures design that is inherent in the classical conditioning methodology for honey bees. This design allowed for the assessment of any potential changes in learning ability that may have varied as a function of time and amount of training as well as a function of insecticide dose. Beginning with the commercially recommended dose for methoxychlor (1500ppm), and reducing the concentration of solutes of the insecticide per solvent by 50% twice, it was expected that, should behavioral disturbances be evident, they would be most strongly noted in the animals receiving the highest dose, as compared with an untreated control group. This

effect was expected to interact with the manner of preparation, as orally ingested insecticides generally carry a lower acute toxicity value than do contact poisons for honey bees (see Johansen, 1979; Metcalf & Metcalf, 1993; Stevenson, 1968).

### Method

Subjects. Subjects were 128 honey bees (*A. mellifera*) captured from a local hive within the city limits of Stillwater, Oklahoma. All bees were captured and trained during the first two weeks of October, 1996. The colony from which the subjects were collected is owned by a local commercial honey producer and had been treated with permethrin strips as a miticide to control for varroa mites. The colony had been cleared as tracheal and varroa mite-free by a local Entomologist, according to the owner.

Apparatus and Materials. Bees were mounted in brass tubes designed to harness the free movement of the bees and to enhance proboscis extension (see Batson, et al., 1992). During the conditioning procedure, bees were mounted at the opening of a laboratory-designed exhaust system. The exhaust system was designed to diffuse olfactory stimuli and to exhaust these directly into the laboratory fume hood.

Insecticides were applied either by contact or orally through the use of a micropipette pump with disposable tips. CS delivery was performed by means of a 20 cc. syringe. Sucrose solution US delivery was completed with a 1 cc. syringe.

Chemical control agent. The chemical control agent selected for this experiment was the DDT analogue methoxychlor, or 2, 2,-bis(4-chlorophenyl)-1, 1, 1-trichloroethane. Methoxychlor is known to have a fast knock-down in houseflies, and is essentially considered nontoxic to mammals with acute toxicity estimates for rats in

excess of 6000 mg/kg. For honey bees, lethal doses fall in the range of 20-100 mg/kg (Metcalf & Metcalf, 1993). This agent does not bear the problems of DDT and other banned chlorinated hydrocarbon insecticides because it is not stored in fatty tissue, nor is it excreted in animal milk (Matsumura, 1985). It retains a soil persistence half-life of one to four years (Metcalf & Metcalf, 1993).

Metabolically, methoxychlor is degraded by the mixed-function oxidation (MFO) system to phenols which are rapidly excreted after conjugation (Matsumura, 1985; Metcalf & Metcalf, 1993). Its mode of action, like other DDT analogues, is axonic, causing a volley of axonic impulses in the insect peripheral sensory organ system and an increase in negative afterpotential. Axonic transmission disruption has been indicated in both vertebrate and invertebrate species as the impetus for abnormal behavior (Kandel & Hawkins, 1993). When lethal, death is attributed to metabolic exhaustion brought on by the violent trains of afferent neural impulses. The site of action for methoxychlor is thought to be related to the sodium channels of the insect nerve axon through inhibition of calcium ATPase (Metcalf & Metcalf, 1993).

Methoxychlor was further selected for this research to establish a relevancy criterion because it is recommended for treatment of leafhoppers, lygus bugs, grasshoppers, defoliating caterpillars, pea aphids, and spotted alfalfa aphids, all insects likely to damage alfalfa crops in the southern U.S. (Oklahoma Cooperative Extension Service, 1996). Additionally, the likely exposure from methoxychlor to bees is high, as bees serve as pollinators of alfalfa in the arid areas of the southern U.S. (Buchman & Nabham, 1996; Metcalf & Metcalf, 1993). Finally, in addition to its applied relevancy in this case, methoxychlor is selected because it is considered relatively non-toxic to bees as

based on acute toxicity estimates (Johansen, 1979; Oklahoma Cooperative Extension Service, 1996).

Design and Procedure. In this classical conditioning experiment, the manipulations included the preparation of the insecticide (oral vs. contact), the dosage of the agent (1500ppm, 750ppm, and 375ppm, and solvent-only control), and length of training (1-12 Trials). Preparation and dose were between-subjects factors, while length of training (trials) was a within-subjects factor. Subjects was the only random factor in the design, while preparation and dose were treated as fixed factors.

The oral and contact preparations were necessary for two reasons. First, analysis of the effects of insecticides generally includes both variables (Matsumura, 1985). Additionally, because of the nature of chemical control application in the field, the likelihood is high that insects might come into contact with the agent through either of these means. Through pollination behavior, bees may come into contact with the agent orally through proboscis extension into treated flowering plants. Additionally, bees may also receive contact exposure through mere tactile contact with the treated plant or through airborne residues (Davis & Williams, 1990).

The selection of the three dosage amounts was based on the manufacturer's recommendations for commercial-grade 25% methoxychlor. The most conservative recommended dose is 1500ppm as directed by the label of the commercial product. The other two doses were successive 50% reductions from the commercially recommended dose, since reduction by a percentage has been shown to be a better indicator of dose-response effects than merely reducing the dose by a fixed amount (Neely, 1994). The selection of 12 trials for training was based on the required number of trials to reach

asymptotic performance in untreated honey bees in a classical conditioning experiment (Batson, et al., 1992; Buckbee, 1995).

The treated groups in the oral conditions were compared with a control group that had received only a sucrose oral treatment. For the contact conditions, a group treated by contact with a lipophilic acetone (1% v/v) solvent served as the control (solvent selected based on the methods of Murray, 1985; Vandame, et al., 1994).

The honey bees were captured from the local hive in small glass vials, then brought to the laboratory on the campus of Oklahoma State University. After immersing the vials in an ice-water bath to cool the bees to dormancy, the bees were mounted in brass tubes. The bees were placed in the tubes so that the head region was positioned above the top of the tubes, with a small piece of tape affixed to the back of the head to keep the bee in position.

Bees were captured four hours prior to the experimental treatment to allow them to habituate to the conditioning apparatus, and allowed to feed to satiation from 50% (w/v) sucrose solution before the apparatus-habituation period commenced (sucrose solution concentration selected from methods of Murray, 1985; Vandame, et al., 1994). One hour prior to the classical conditioning training, the bees were given the respective insecticide or control treatments. Five hours after the initial capture, the bees were given classical conditioning training.

Bees in the four contact conditions received one of the methoxychlor treatments (1500ppm; 750ppm; 375ppm; Control) in the 1% (v/v) acetone solvent. Two microliters of the solution were applied to the dorsal side of the thorax (amount of solution and location for application replicated from the methods of Murray, 1985). Bees in the four



oral conditions received one of the methoxychlor treatments (1500ppm; 750ppm; 375ppm; Control) in the 50% (w/v) sucrose solution. Two microliters of the sucrose-insecticide solution were delivered to the proboscis for feeding (amount of solution and means of insecticide delivery replicated from the methods of Murray, 1985). Bees in the control conditions received either the contact acetone solvent or oral sucrose solution only. One hour after treatment, the bees were brought to the conditioning situation. The order in which the groups were trained was controlled for calendar variables through variation in the day of training across preparation and doses through random selection.

Bees were trained in squads of 16 animals in a sequential fashion. Bees in their individual tubes were placed at the entrance of the exhaust ducts designed to diffuse the olfactory stimuli once a subject was given a conditioning trial. After the first bee was placed in front of the exhaust fan and received a stimulus presentation sequence (CS-US), it was returned to a general staging area while the second bee was placed in front of the exhaust fan for training. This continued sequentially, so that all 16 bees received stimulus presentations for a single trial before the first bee received the stimuli for the next trial.

The odor of cinnamon oil (the CS) was delivered to the antennal area through an air puff delivered by a 20 cc. syringe. Cinnamon oil has been determined to be a neutral stimulus (Buckbee, 1995) in favor over typically employed stimuli such as Geraniol. Geraniol is a major chemical constituent of honey bee Nasonov pheromones, and is also a fairly abundant plant kairomone (Metcalf & Metcalf, 1983; Winston, 1987). Because of the inherent attractant properties in Geraniol, its neutrality as a CS is questionable.

Additionally, cinnamon oil has shown success as a CS in classical conditioning of the PER in honeybees in laboratory olfactory paradigms (Buckbee, 1995).

The 20 cc. syringe to deliver the CS was prepared by soaking cinnamon oil onto filter paper and affixing the filter paper to the rubber plunger of the syringe. When the syringe plunger was depressed manually, it delivered an air puff of the cinnamon oil. The US (50% sucrose solution) was delivered to the proboscis by means of a 1 cc. syringe. The single US method (oral US delivered to proboscis) was used in favor of the compound US method (tactile US delivered to the antenna followed by oral US to the proboscis) because of the inherent difficulties in interpretation of the PER whenever a compound US is utilized in training (Buckbee, 1995).

The intertrial interval (ITI) employed was 10 minutes. This length of ITI is necessary to control for central excitation, which can occur due to feeding on the sucrose. Longer ITIs allow the excitatory after-effects of the US to dissipate before the next trial commences. Additionally, the 10-minute ITI has become a standard for classical conditioning of the PER in honey bees (Batson, et al., 1992; Bitterman, et al, 1983; Buckbee, 1995). The training period consisted of 12 trials. When classical conditioning paradigms are employed with neutral stimuli, full performance is reached by the twelfth trial (Buckbee, 1995).

The olfactory CS was delivered to the head area in the manner of Smith, Abramson, & Tobin (1991) for a duration of six seconds, followed by a single sucrose US delivery to the proboscis three seconds after the presentation of the CS commenced. The CS and US overlapped by three seconds. Bees were allowed to drink from the drop of the sucrose solution US for three seconds.

An effort was made during training to keep the experimenters blind to the conditions in the experiment. Vials containing the solvents were given a letter code, rather than labelled with the actual treatment preparation and dose. The data sheets from which the experimenters worked contained matching codes. It must be noted, however, that though these efforts were made, the experiment was not wholly blind to the experimenters. First, the application of the acetone solvent on the dorsal side of the thorax created a sheen on the thorax which not evident in animals that received oral treatments. In addition, the insecticide methoxychlor has an unpleasant aroma that, even at the very low doses employed here, is unmistakable. However, experimenters did not know which dosage amount was being used when training the animals.

The dependent measure used for analysis was the probability of response as measured for the entire groups of 16 bees on 12 individual trials. On a single conditioning trial, a zero was recorded if the PER was not evident to the presentation of the CS. A one was recorded if the PER occurred to the presentation of the CS. Experimenters were conservative in their estimation; if unclear if the PER actually occurred to the CS, a zero was recorded. All three experimenters were trained to recognize full PER to the presentation of stimuli, and had a combined experience of seven years working with honey bees in conditioning scenarios. The probability of response measure is standard in honey bee conditioning research (e.g., Batson, et al., 1992; Bitterman, et al., 1983, Buckbee, 1995; Menzel & Bitterman, 1983). Training focussed on acquisition only, as learning disturbances as a function of insecticide application will manifest themselves during acquisition training (Stone, et al., 1997).

## Results

To analyze for behavioral disturbances in the classical conditioning experiments, a 2 X 4 X 12 (Preparation X Dose X Trials) repeated measures Analysis of Variance (ANOVA) was conducted.

The three-way interaction (Preparation X Dose X Trial) was significant [ $F(33, 1320) = 1.46, p=.0460$ ]. Collapsing across preparations, the two-way interaction between dose and trials was also significant [ $F(33, 1320) = 2.80, p=.0001$ ]. Table 1 shows the ANOVA source table for all effects tested in Experiment 1. Figures 1 and 2 graphically depict the three-way interaction; graphed separately for each preparation, while Figure 3 graphically depicts the Dose X Trials interaction when collapsed across preparation.

Simple effects tests were conducted to isolate which of the groups were exhibiting learning. Collapsing across preparation, simple-effects tests were conducted for the 375ppm, 750ppm, 1500ppm, and control groups across trials. Table 2 illustrates the findings of each simple-effects test, and the results indicate that all groups except for the 750ppm showed a significant effect. However, these tests were incomplete in explaining how and why these contributions took place. Therefore, Scheffé *post-hoc* comparisons were conducted on pairwise and complex comparisons, first on the between-subjects effects from the overall ANOVA, and then on the simple-effects tests. All comparisons were selected based on the graphical depiction of the data (evident in Figure 1) as being potentially the most informative.

Scheffé *post-hoc* comparisons of between-subjects effects indicated that, on Trial 1, there were no significant differences in performance across dosage amounts. While

Table 1  
 Analysis of Variance Source Table  
 Experiment 1 - Classical Conditioning

<u>Source</u>	<u>SS</u>	<u>df</u>	<u>MS</u>	<u>F</u>	<u>p</u>
<u>Between Subjects</u>					
Preparation	.00260	1	.00260	0.00	.9531
Dose	20.91667	3	6.97222	9.28	.0001
Prep. X Dose	12.92447	3	4.30815	5.73	.0011
Btw. Subj. Error	90.14583	120	.75121		
<u>Within Subjects</u>					
Trial	18.92187	11	1.72017	10.56	.0001
Dose X Trial	15.06770	33	.45659	2.80	.0001
Prep. X Trial	2.23177	11	.20288	1.25	.2517
Prep. X Dose X Trial	7.84114	33	.23761	1.46	.0460
Within Subj. Error	215.10416	1,320	0.16295		
Total	383.16250	1,535			

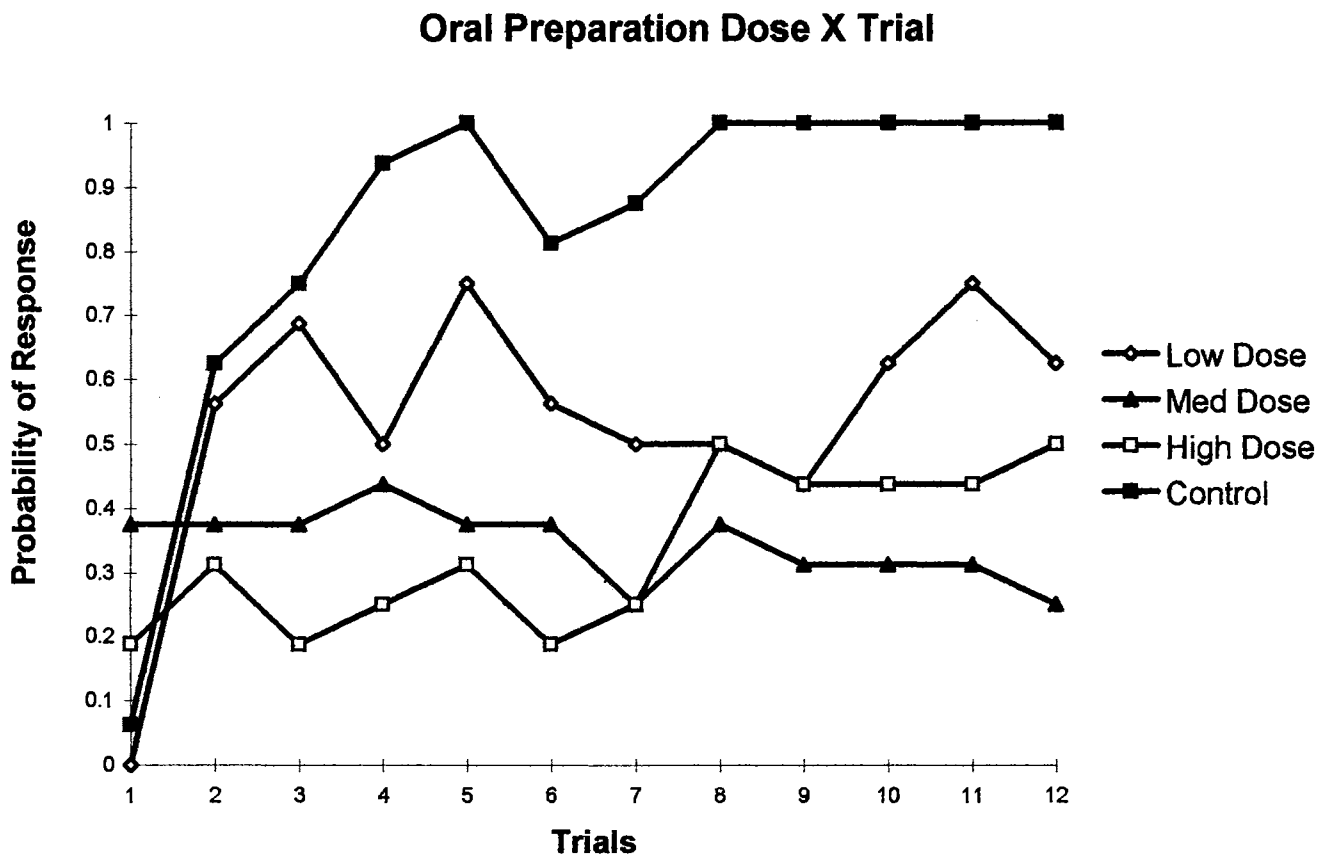


Figure 1. Oral Preparation Dose X Trial Interaction

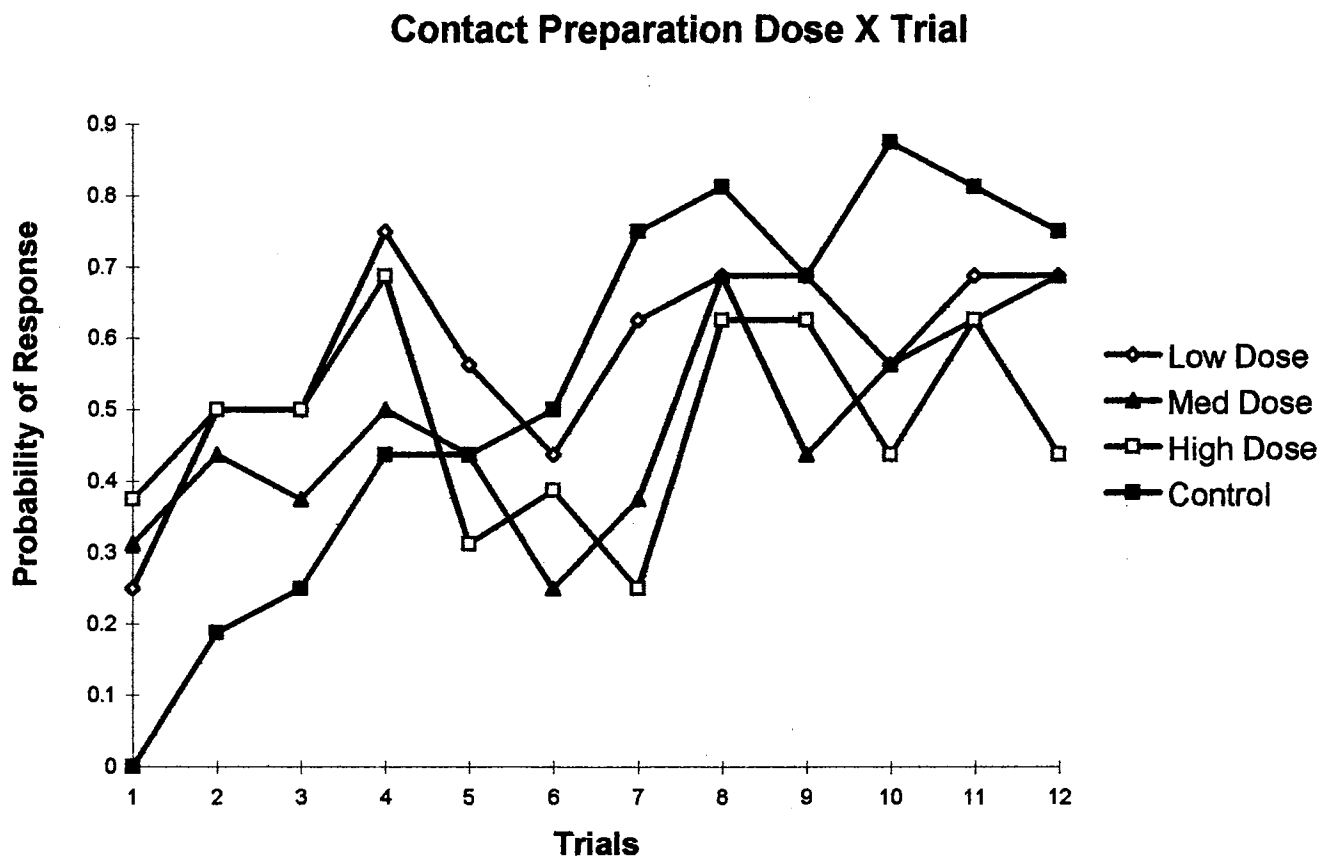


Figure 2. Contact Preparation Dose X Trial Interaction

### Dose X Trial Across Preparation

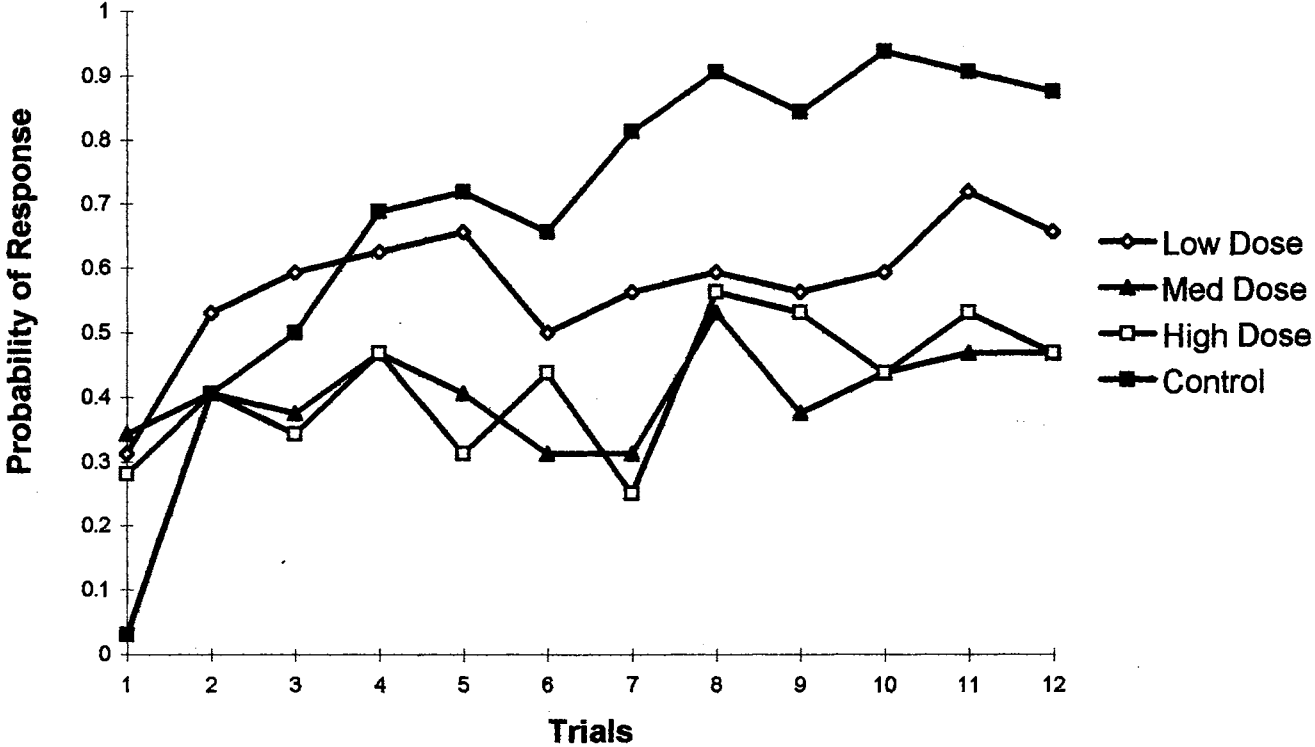


Figure 3. Dose X Trial Interaction collapsed across preparation



Table 2  
Simple Effects Tests  
Classical Conditioning - Experiment 1

<u>Source</u>	<u>SS</u>	<u>df</u>	<u>MS</u>	<u>F</u>	<u>p</u>
Trials@Low Dose	3.654	11	0.330	2.038	0.022
Trials@Med Dose	1.654	11	.150	.923	0.52
Trials@High Dose	3.716	11	.338	2.073	.020
Trials@Control	24.966	11	2.270	13.928	<.0001
Error (Error for Dose X Trials Interaction)	215.104	1,320	.163		

upon visual inspection (See Figure 3), it appears that the treatment groups on Trial 1 were sensitized in comparison with the control groups, this effect was not statistically significant [ $F(3, 120) = .8424, p > .05$ ]. Additionally, it appeared that, on Trial 12, there might be differences in the final training trial across groups. Three separate Scheffé comparisons were conducted on combinations of Trial 12 data. The complex comparison between the 375ppm (low) group and the combined 750ppm (medium) and 1500ppm (high) groups revealed that the combined low group was not significantly different from the combined medium and high groups. Further, two other comparisons were conducted with Scheffé's formula to see if there were statistically significant differences in first the pairwise low vs. control groups, and separately, the medium and high groups combined vs. the control group. Neither effect proved to be statistically significant at the .05 rejection level. Table 3 illustrates the findings of the between-subject Scheffé comparisons conducted for Experiment 1.

Next, Scheffé comparisons were conducted on the simple-effects tests to ascertain where in the training process each group was contributing to the Dose X Trial significant interaction. Of primary interest here was whether, for each group, there were significant differences in the probability of response at Trial 1 vs. Trial 12. Pairwise comparisons of Trial 1 vs. Trial 12 were carried out separately for each group. Only the control group's performance [ $F(11, 1320) = 6.35, p < .01$ ] proved significant. Alpha levels for these tests were set at .05 for one-tailed tests, as it was expected that Trial 1 to Trial 12 would show an increase in response rate had learning occurred. Refer to Table 3 to view results of the simple-effects-based Scheffé comparisons for Experiment 1.

Table 3  
 Sheffe *post-hoc* comparisons  
 Experiment 1 - Classical Conditioning

<u>Test</u>	<u>Result</u>
<u>Between-subjects comparisons</u>	
Treatment groups vs. Control group-Trial 1	[F(3, 120) = .8424, p>.05]
Low vs (Medium-High)-Trial 12	[F(3, 120) = .3326, p>.05]
Low vs. Control-Trial 12	[F(3, 120) = .3399, p>.05]
(Medium-High) vs. Control-Trial 12	[F(3, 120) = 1.5619, p>.05]
<u>Comparisons within simple effects tests</u>	
Low Dose-Trial 1 vs. Trial 12	[F(11, 1320) = 1.0544, p>.05]
Medium Dose-Trial 1 vs. Trial 12	[F(11, 1320) = .13947, p>.05]
High Dose-Trial 1 vs. Trial 12	[F(11, 1320) = .31398, p>.05]
Control-Trial 1 vs. Trial 12	[F(11, 1320) = 6.35, p<.01]

The three-way interaction (Preparation X Dose X Trial) was also significant [ $F(33, 1320) = 1.46, p=.0460$ ]. This difference was expected due to typical differences in oral and contact toxicity estimates, though the results were in the opposite direction; the contact preparation group showed less in the way of associative capacity than the oral groups. However, when separate ANOVAs were conducted for the Dose X Trial interaction for each level of preparation, both the oral groups [ $F(33, 1320)=2.133, p<.0001$ ] and the contact groups [ $F(33, 1320)=2.127, p<.0001$ ] showed significant effects (See Figures 1 and 2). To better isolate information on dose-specific effects, simple effects tests were conducted for each preparation (oral and contact) for each level of dose across trials. In the case of the orally treated subjects, only the control group showed a significant effect across trials [ $F(11, 1320)=7.354, p<.0001$ ]. In the case of the contact preparation, all dose groups showed significant effects across trials. For the low dose group, [ $F(11, 1320)=1.961, p=.029$ ]. For the medium dose group, [ $F(11, 1320)=1.985, p=.027$ ]. For the high dose group, [ $F(11, 1320)=2.124, p=.016$ ]. And for the control group, [ $F(11, 1320)=7.973, p<.0001$ ].

Again, as in the analysis of the overall two-way interaction, the simple effects test don't reveal whether or not learning has occurred. For this reason, Scheffé comparisons were performed for all significant simple effects tests. As was found in the analysis of the data collapsed across preparations, only the control groups for each preparation showed a significant difference from Trial 1 to Trial 12. For the oral control group, [ $F(11, 1320)=3.9227, p<.01$ ]. And for the contact control group, [ $F(11, 1320)=2.511, p<.01$ ]. All Scheffé tests here were conducted using one-tailed

significance levels, as it was hypothesized that Trial 12 would exceed Trial 1 had learning actually taken place.

Viewed within the context of the simple effects tests, the within-subjects comparisons reveal that only the control group showed associative capacity as demonstrated by its performance in this experiment. The significant simple-effects test for the low and high dose groups across trials, though significant, is not indicative of learning performance. Because there were no significant differences in either of these groups between Trial 1 and Trial 12 as revealed by the Scheffé comparisons, learning cannot be assumed to have taken place. The same holds true for when the groups were analyzed separately within preparation. Only the control groups demonstrated performance indicative of learning (See Figures 1 and 2). The graphical depiction of all the groups' performance as illustrated in Figure 3 shows that the significant effects in the the low and high doses comes from variation in performance across trials during the middle of training. Because learning is assumed to be gradual, and increasing in performance over training (Kimble, 1961), it must be concluded that these groups simply did not learn. Of additional interest in Figure 3 is the observation that the lowest dose, while not statistically similar to the control group, did indeed perform across trials at a higher probability-of-response rate than did the higher-dose groups. This same phenomenon holds true when the preparations are graphed separately. While this observation is counter-intuitive to general thinking about dose-response effects, it may be indicative of sensitization at both the neuronal and behavioral levels due to a non-lethal dose of methoxychlor. This possibility is discussed further in the context of Experiment 2.

Further, greater variability in response patterns are noted in the contact preparation groups. This may have further implications against the use of lipophilic solvents in insecticide spraying, and even have considerations in the realm of herbicide spraying. Herbicides have often been seen to harm bees in some way (Johansen, 1979), but the reasons have been unclear, as herbicides do not contain physiologically significant compounds to insects. Behaviorally, perhaps it is indeed the addition of solvents designed to foster faster absorption by plants, as indicated here with the contact preparation group's performance over trials.

In terms of the classical conditioning paradigm, Experiment 1 showed that there appears to be a deficit in learning performance as a result of insecticide application. None of the dose concentrations in this experiment can be considered behaviorally safe based on this test, and it will require further reduction in dose concentration to determine which, if any, concentration of methoxychlor is indeed behaviorally safe for use near honey bees. It is important to remember that all three doses used in this experiment are considered relatively non-toxic to bees, provided careful spraying methods are used (Oklahoma Cooperative Extension Service, 1996).

The behavioral data generated in this classical conditioning experiment warrant, at the very least, more scrutiny into appropriate dose amounts of methoxychlor for use in areas where honey bees may be located. This reduction in dose, compared with the manufacturer's suggestions, may indeed be the necessary "filling in of the gaps" warranted because of the incomplete information afforded by hazard assessment alone.

## CHAPTER III

### EXPERIMENT 2

#### Habituation/Sensitization

##### Habituation/Sensitization Test on Antennal Stimulation

Although associative capacity plays the greatest role in honey bee learning, Experiment 2 was designed to see if nonassociative learning was affected by the application of methoxychlor at sublethal levels. Additionally, task-specific effects on learning have occurred with honey bees (Stone, et al., 1997), though in this study, both tasks observed were associative in nature. It was thought that dose-dependent effects would be noted in this experiment as well.

##### Method

Subjects. Subjects in this experiment were 128 honey bees collected in the same manner as described for Experiment 1.

Apparatus and Materials. The brass tubes described in Experiment 1 to mount the honey bees for training were also employed in Experiment 2. The exhaust fans described for Experiment 1 were employed in this experiment as well. To apply the monotonous stimulus to the antenna in this experiment, a single, sterile cotton-tipped swap was used. The means of applying the insecticide solutions were the same as described for Experiment 1.

Chemical Control Agent. As in Experiment 1, the DDT analogue methoxychlor was used. The same doses as described in Experiment 1 were employed as well.

Design and Procedure. The methods of capturing, harnessing, habituating, and pre-training treatment with either the insecticide or control application were the same as described in Experiment 1.

In order to induce habituation, 2% (w/v) sucrose solution was soaked onto a sterile cotton swab and applied briefly to one training antenna. This sucrose concentration is salient enough to elicit proboscis extension, but remains low enough to eliminate concerns about sucrose build-up on the training antenna as training progresses. No published precedent exists for the use of this concentration in this manner; rather, a number of different concentrations were evaluated last summer in the laboratory for their appropriateness for use as a habituation stimulus in this experiment. Typically, a 50% (w/v) sucrose solution has been used as an antennal US in classical conditioning experiments employing a compound US (e.g., Bitterman, et al., 1983; Menzel & Bitterman, 1983). However, there are two reasons why this stimulus is impractical for a habituation/sensitization experiment. First, stimuli to be habituated must be of low magnitude in order for habituation to take place within a reasonable amount of time (Abramson, 1994). Additionally, evidence from Buckbee (1995) showed that 50% sucrose applied to a bee's antenna during training creates a build-up of sucrose on the antenna which impacts performance. The trial-and-error investigation in the laboratory of various concentrations of sucrose showed that at the 2% concentration, the PER was reliably elicited, and sucrose build-up also was not a difficulty at this concentration. A binocular microscope was used for post-training analysis of the antenna after 20 applications of the various sucrose concentrations, making the 2% concentration the most effective for use in this experiment.



Bees were individually trained in the exhaust system described in Experiment 1. The sucrose solution stimulus was presented every three seconds to a training antenna. The three-second ITI in this experiment generates the monotonous criterion required to induce habituation, as determined in pilot data collected in the laboratory. Longer ITIs make habituation impossible because they allow for recovery between stimulus presentations, and shorter ITIs overlap the time it takes for the extension and withdrawal of the proboscis, making judgment about proboscis extension to the presentation of the antennal stimulus impossible.

To measure habituation to the sucrose stimulus, each bee was individually trained to criterion before the next bee was trained. The criterion for this experiment was five consecutive trials wherein no proboscis extension occurs. This criterion was also selected based on preliminary data collected in the laboratory. Criteria of fewer than five trials did not adequately show habituation to the stimulus, as measured through dishabituation tests. With the criterion of five non-response trials in untreated animals, dishabituation tests were successful. This trials-to-criterion measure was the dependent variable for this experiment.

Following the meeting of the criterion, the contralateral antenna (opposite to the training antenna) was stimulated with the 2% (w/v) sucrose solution to assess for proboscis extension. This is a dishabituation test which, when proboscis extension occurs, verified that habituation on the training antenna has actually occurred. If proboscis extension did not occur to the dishabituation stimulus, then the animal was eliminated from the experiment because its general health came into question.

Separate neural pathways exist from each antenna into the antennal lobes (Menzel, et al., 1991); stimulation of the right antenna does not impact the left, and vice-versa. Consequently, the bees in each group were counterbalanced for antennal training to eliminate the possibility that the results were antenna-specific.

### Results

In one of the training groups, two bees failed to perform a dishabituation response and had to be eliminated from the experiment. In one other of the training groups, one bee failed the dishabituation test and had to be eliminated. In order to create equal sample size across groups to make the analysis of variance robust with respect to violations of its assumptions, two bees' data out of the remaining groups were randomly discarded. In the group wherein one bee failed the dishabituation test, one bee's data was randomly selected for discard. This created an N of 14 subjects in each group, as opposed to the 16 subjects originally proposed for this experiment.

Because the bees in this experiment were trained individually to criterion, it was important to assess for behavioral effects due simply to the passage of time during the training process. In order to assess for such effects, before other analyses were performed, a Sign Test was performed. The Sign Test showed that only one group (The high dose, oral preparation group) out of the 16 groups in the experiment showed a significant difference between animals trained early and animals trained late to criterion ( $k=7$ ,  $p=.016$ ). Because this one difference could be expected by chance alone, the method employed here, training the animals individually to criterion, did not confound the performance results.

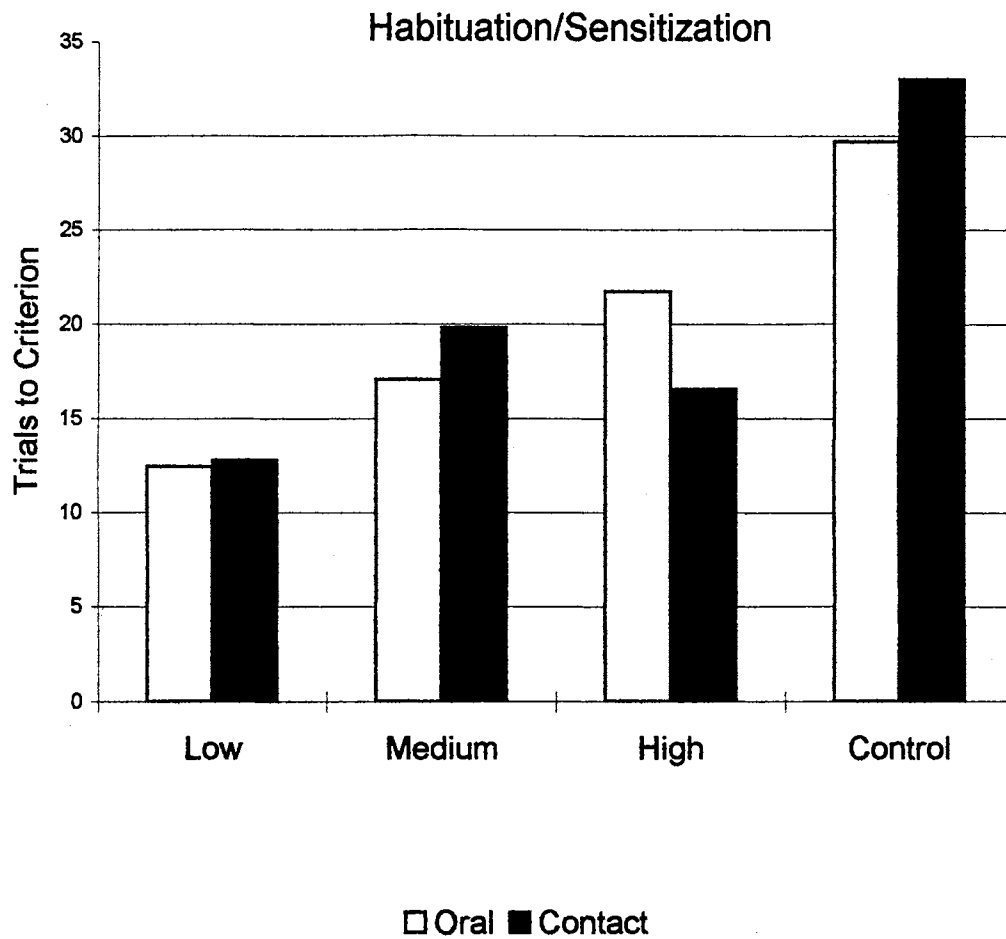
The design of this experiment required a 2 X 4 (Preparation X Dose) between-subjects ANOVA. The main effect for preparation was not significant [ $F(1, 104) = 1.090, p=.299$ ], but the main effect for dose was statistically significant [ $F(3, 104) = 42.137, p<.0001$ ]. The two-way Preparation X Dose interaction was also significant [ $F(3, 104) = 5.064, p=.003$ ]. Table 4 shows the ANOVA source table for the tests conducted for Experiment 2.

In order to determine where the contributions to the interaction took place, a number of Scheffé pairwise and complex comparisons were conducted. The comparisons were selected for their ability to provide the most interpretable information based on the graphical depiction of the results from Experiment 2, which are shown in Figure 4.

First, the combined treatment (dose) groups across preparations were assessed for differences from the control groups across preparations. The Scheffé comparison revealed that the treatment groups were statistically different from the control [ $F(3, 104) = 34.0563, p<.01$ ]. Additionally, regardless of preparation, the treatment groups were statistically different from their respective controls. For the oral preparation, the combined treatment groups (Low-Medium-High) were statistically different from the oral control [ $F(3, 104)=3.8535, p<.01$ ]. And for the contact preparation, the treatment groups also differed from their respective control [ $F(3, 104)=12.343, p<.01$ ]. These comparisons, much like the simple-effects comparisons performed in Experiment 1, are not completely informative as to which individual treatment groups contributed to the Dose X Preparation interaction, so a number of pairwise Scheffé comparisons were performed in to delineate this information.

Table 4  
 Analysis of Variance Source Table  
 Experiment 2-Habituation/Sensitization

<u>Source</u>	<u>SS</u>	<u>df</u>	<u>MS</u>	<u>F</u>	<u>p</u>
Preparation	36.571	1	36.571	1.090	.299
Dose	4242.786	3	1414.262	42.137	<.0001
Preparation X Dose	509.929	3	169.976	5.064	.003
Error	3490.571	104	33.563		



**Figure 4.** Habituation/Sensitization Preparation X Dose Interaction

Table 5  
Scheffe post-hoc comparisons  
Experiment 2-Habituation/Sensitization

<u>Test</u>	<u>Results</u>
(Low-Medium-High) vs. Control	[F(3, 104) = 34.5063, p<.01]
Low vs. Medium	[F(3, 104) = 4.7129, p<.01]
Medium vs. High	[F(3, 104) = .0683, p>.05]
Low vs. High	[F(3, 104) = 5.9397, p<.01]
Low vs. Control	[F(3, 104) = 40.5242, p<.01]
(Medium-High) vs. Control	[F(3, 104) = 21.9978, p<.01]
Oral Control vs. Dermal Control	[F(7, 104) = 1.3148, p>.05]
Oral Low vs. Dermal Low	[F(7, 104) = .00379, p>.05]
Oral Medium vs. Dermal Medium	[F(7, 104) = .2253, p>.05]
Oral High vs. Dermal High	[F(7, 104) = .87299, p>.05]
Oral (Low-Medium-High) vs. Oral Control	[F(7, 104) = 3.8535, p<.01]
Dermal (Low-Medium-High) vs. Dermal Control	[F(7, 104) = 12.343, p<.01]

The data revealed by these Scheffé comparisons showed that the low dose group differed significantly from both the medium and the high groups. The combined medium-high group was statistically different from the control, regardless of preparation. And the low group was significantly different from the control, regardless of preparation. No differences were seen in any of the matched groups across preparations (i.e., low-contact vs. low-oral, etc). Table 5 illustrates the findings of all the Scheffé comparisons performed for Experiment 2. The findings of these comparisons are supported graphically in Figure 4.

## GENERAL DISCUSSION AND CONCLUSIONS

### Discussion

#### General conclusions

One of the most obvious findings of these experiments is that the application of the DDT analogue methoxychlor produced task-specific effects that can only be interpreted within the context of the learning paradigm, much like those noted by Stone, et al., (1997). This was not unexpected, as nonassociative and associative learning tasks with invertebrate species are typically not comparable in interpretability (see Abramson, 1994), and for this reason, the experiments were designed separately. However, though the results were task-specific, in terms of physiology and making recommendations about behavioral safety, both experiments yielded similar findings.

In Experiment 1, the honey bees receiving the insecticide treatments simply did not learn. There was nothing in their performance that was even remotely related to creating associations between the stimuli employed in the experiment.

Certainly, Experiment 1 contributes to the notion that broad characterizations of insecticide safety, based on hazard estimates alone, are insufficient in elaborating potential behavioral disturbances. Such behavioral disturbances may lead to eventual death of honey bees, brought on by a reduction in associative capacity, if exposed to higher insecticide concentrations that are based solely on hazard estimates. Death may come from the inability to locate feeding sites or to return to the hive. Such insecticide-induced behavior is consistent with honey bee behavior reported by apiculturalists in Clay, County, Texas, following injudicious spraying last summer



(Clements, 1996). In addition to impacting the ability of bees to forage if associative capacity is absent, these findings have agricultural significance because pollination may also suffer from the bees' inability to associate feeding sites with the hive as well as the inability to create routes to and from each through associative learning.

The analysis of the Experiment 2 data appeared paradoxical, in that a high degree of sensitization appeared evident in the low-dose groups (causing performance least like the controls), while the medium and high dose groups performed in a manner more like the controls, though they were statistically different from the controls. The expectation was that, should dose-response effects be present, the low dose groups would perform more like their respective controls, with more behavioral disturbance noted in the higher dose groups.

However, viewed within the context of known effects of DDT analogues at the level of the neuron, these data may not be paradoxical at all, and appear to support the findings of Experiment 1. First, it was unknown at the beginning of this experiment whether the application of methoxychlor would produce sensitized behavior in the subjects (in which fewer trials to criterion would be noted in comparison with an untreated control), or whether the insecticide would slow the process of habituation (in which treated animals would take longer to reach criterion than the untreated control animals). The results obtained in this experiment appear to correlate with known physiological responses to DDT analogues, of which methoxychlor is one.

At the level of the neuron, DDT analogues create first a lowering of the threshold for response (raising the resting potential of the polarized neuron), increase the negative

afterpotential, and eventually cause a conduction block, which slows down and then abolishes the ability of the neuron to fire in the insect peripheral sensory system (Matsumura, 1985). The refractory periods for neuronal firing disappear due to the changes in the negative afterpotential, causing initial sensitivity to weaker stimuli, followed by eventual shut-down of the neuron.

Given this information, it is logical to assume that the low groups' sensitized responses in this experiment mirror what is happening at the level of the neuron; this lower dose sensitizes the animal (perhaps through lowering of the threshold for response), but not to the degree that the conduction block takes place (which is a result of the increasing negative afterpotential). In the case of the medium and high dose groups, their seemingly paradoxical results, far from indicating behavior that resembled the control, may indeed be indicative of greater neuronal injury, closer to the dose required to create the conduction block which was occurring at the level of the neuron. Interestingly, the one group that showed a significant Sign Test, the oral-preparation, high-dose group, was perhaps not a chance occurrence at all. With this group, the later in training, the longer it took the animals to reach criterion. Given what is known about xenobiotic detoxification in insects, and that for honey bees, oral lethal dose estimates are often lower than for contact, this one group may have indeed been the closest to losing the ability to respond at all.

This interpretation establishes behavioral correlates for known neurophysiological activity that results from poisoning from DDT analogues. What is not revealed through this interpretation is where, perhaps, the neuronal disturbance takes place. There are a number of nervous system structures involved in insect learning. For this experiment,

disturbance could have been the result of neuronal blocks in the antennal sensory sensillae, in the antennal lobes, in the mushroom bodies, or other deutocerebral structures, all of which are involved in insect learning (Menzel, et al., 1991). Because much of the research uncovering neuronal effects of agents is of the *in-vitro* variety (e.g., Burrell & Smith, 1995), behavioral results like those reported in this experiment provide a needed *in-vivo* substantiation of partial preparation analysis.

The results from Experiment 2 add a behavioral substantiation to what is happening at the level of the neuron when DDT analogues are used in pest control. The value of Experiment 2 in this realm was unexpected, and suggests that the application of insecticides, along with subsequent behavioral and physiological research, may also add to the knowledge of the insect nervous system in addition to demonstrating behavioral indices of safety for honey bees.

The results from Experiment 2 also underscore the need to select appropriate behavioral tests for the species. It is important to keep in mind that for honey bees, habituation and sensitization are not thought to play a large role in bees' repertoire of survival-induced behavior (Gould, 1995). Rather, it is believed that associative learning, as shown in Experiment 1, is the form of learning bees utilize in order to feed, to pollinate, and to engage in behaviors necessary for not only survival, but for pollination of products of commerce. For species of less neuronally-complex insects, habituation and sensitization information may be crucial to outlining indices of safety. This in no way jeopardizes the notion of using honey bees as sentinel animals, however.

### Scope of Applicability: The Honey Bee as a Behavioral Indicator Species

Honey bee mortality has long been used as an indicator of environmental health, and data from honey bee kills has been extrapolated to other bee species (Greig-Smith, et al, 1994). Extrapolating the findings from Experiment 1 to other bee species is a simple process, as extrapolations in risk assessment are typically done by body-weight transformations (Neely, 1994), and may be demonstrated in this case. For example, the following 375ppm is extrapolated based on body-weight: Average body-weight for worker honey bees is 128 mg (Johansen, 1979). As the concentrations are reported here, this yields a 2.93ppm per mg of body weight for the worker honey bee. Size of the bee impacts its susceptibility to insecticides (Johansen, 1972), therefore, larger bees would be less susceptible than worker honey bees, while smaller bees would generally be more susceptible to pesticidal effects. A smaller bee, the alkali bee (*Nomia melanderi*), which has an average body weight of 24mg, would only tolerate a concentration of 235ppm, based on the results of Experiment 1. Additionally, the larger bumble bee (*Bombus*, spp.), with a mean body weight of 180mg, could tolerate a higher concentration, 527ppm (average body weight figures from Johansen, 1979). At these concentrations, it is likely that behaviorally, the alkali bee and the bumble bee would perform similarly to the worker honey bee as reported in these experiments.

Because of recent concerns about the "forgotten pollinators," (Buchman & Nabham, 1996), the ability to extrapolate behavioral results from worker honey bees adds to the agricultural significance of behavioral research. Because of the rapid adaptive success of the European honey bee (*A. mellifera*, the species under scrutiny here), and because of injudicious spraying, native pollinators such as the alkali bee are also

decreasing in numbers (Buchman & Nabham, 1996). Behavioral indices of safety as delineated through research with the honey bee may also protect native species of bees, species for which little behavioral data are available because they are not domesticated, as honey bees are, and therefore not as available for laboratory research.

This presents a limitation, however, in this project, and one which must be addressed in order to be absolutely certain that behavioral data adds to what exists in predictive risk assessment methodology. With the exception of bumble bees and some wasp species (Abramson, Shuranova, & Burmistrov, 1996), few bee species other than the honey bee have been subjected to numerous and rigorous applications of standard learning paradigms. While on the surface, extrapolation of behavioral data based on body weight seems appropriate, it would be more complete if data existed on other bee species to support body-weight extrapolation. Additionally, it is unreasonable to assume at this point that behavioral data collected on the honey bee (a Hymenopteran) would extend to Diptera, Coleoptera or to Lepidoptera. For this reason, it would be wise to create a behavioral adaptation of standard learning paradigms for representative species from each phylogenetic Order--species that easily exhibit at least rudimentary forms of learning, and species whose physiology is appropriate for extrapolation. Likely, this will indeed require phylogenetic Order-specific indicator organisms, since many Order-specificities occur in insect physiology (e.g., high midgut pH in Lepidoptera; Metcalf & Metcalf, 1993), though similarities in learning research have been reported among species in the Hymenoptera and Diptera orders (e.g., with *Drosophila melanogaster* (fruit fly), *Phormia regina* (blowfly), *Musca domestica* (house fly), and *Apis mellifera* (honey bee); Fukushi, 1979; Hirsch & Holliday, 1988; McGuire, 1984).

### Extension to Other Potential Indicator Species

In order to determine which species other than the honey bee would be appropriate for such research, appropriate responses should be used in the conditioning process. Applying learning principles as a predictive risk assessment tool to multiple species will require procedural control. Additionally, selection of the behavior must be a function of the natural behavior of the insect under scrutiny. For example, the PER in honey bees is a logical choice because of its role in pollination. Smith (1993) offers some insight that will aid in determining systematic variation (Bitterman, 1965) of learning paradigms to different species. Smith uses the notion of modal action patterns (MAPs) to indicate behaviors innately important to a species. These MAPs may be behavioral responses to pheromones, kairomones, to visual stimuli, or to other olfactory stimuli. MAPs are innate behavioral motor routines evolved in species to have an adaptive function. In assessing the delivery of standard learning paradigms, then, it is important to know the species' MAPs. Drawing from this knowledge, the following are considerations I have determined must be met in order to deliver behavioral paradigms to various species for the purposes of predictive risk assessment. These considerations, while general, include criteria set forth by the National Academy of Sciences (1983, as cited in Neely, 1994), the species selection requirements outlined by Thompson & Greig-Smith (1991), and are also couched in the context of the experiments performed here:

1. *Is the species of insect a beneficial or pest species?*

--The honey bee is a beneficial insect species.

2. *What functional role does the species play in the environment, or what is the specific risk to human health that results from the species' behavior?*
  - The honey bee functions as a pollinator of agriculturally significant crops and contributes to the agricultural economic base through its honey production as well (Metcalf & Metcalf, 1993).
3. *What are the specific stimuli that cause MAPs in the species?*
  - In the honey bee, both visual and olfactory stimuli stimulate modal action patterns (Bitterman, et al., 1983). Additionally, polarized light may create modal action patterns (Abramson, et al., 1996), and tactile stimuli also elicit MAPs. Further, pheromones also stimulate sexual behaviors, aggregation behaviors, and caste-appropriate behaviors (Metcalf & Metcalf, 1993).
4. *How can the stimuli that cause MAPs be simulated in the laboratory?*
  - For the honey bee, in this experiment, the olfactory stimuli were simulated in the laboratory through delivery of odor via airpuff. This is a common CS delivery means (e.g., Batson, et al., 1992; Bitterman, et al., 1983; Menzel & Bitterman, 1983).
5. *How can the reflexive behavior of the species be elicited in the laboratory?*
  - Reflexive behaviors, those involved in MAPs, were elicited in this and other experiments in learning with honey bees through simulation of flower nectar by using 50% (w/v) sucrose solution. This has been determined to be the most effective US in a number of experiments (e.g.,

Batson, et al., 1992; Bitterman, et al., 1983; Lee & Bitterman, 1992; Menzel & Bitterman, 1983).

6. *What geographic locations are likely to support the species?*

--Domesticated honey bees were brought to the United States with the first European settlers at Jamestown (Buchman & Nabham, 1996). They currently successfully inhabit all regions of the United States, some regions of Canada, Mexico, England, and Continental Europe (Buchman & Nabham, 1996; Greig-Smith, et al., 1994).

7. *What chemical agents are typically employed in these geographical locations?*

--Different countries have different rules for legal use of pesticides (Greig-Smith, et al., 1994), yet in the United States, methoxychlor and other insecticides are used in the arid southwest for control of pests on alfalfa crops (Oklahoma Cooperative Extension Service, 1996).

8. *What are the known physiological mechanisms of the chemical to be studied (e.g., mode of action, metabolic considerations, etc.)?*

--The DDT analogue methoxychlor, used in the experiments here, has an axonic mode of action, as opposed to synaptic, as is seen with organophosphate and carbamate poisonings. It is metabolized through the MFO system to phenols for excretion after conjugation (Matsumura, 1985; Metcalf & Metcalf, 1993).

9. *What measures of behavioral efficiency are to be used? (e.g., Is there a precedent for the species' ability to achieve associative learning? Is the species more suited to habituation/sensitization tests?)*



--The honey bee uses associative learning skills in its every-day activities (Bitterman, et al., 1983), but is also capable of demonstrating habituation and sensitization, an evolutionary precursor to associative learning (Gould, 1993).

10. *Is there a precedent for extrapolation of biochemical and physiological results to other species?*

--Insecticide effects on honey bees have been extrapolated to other bees species (Johansen, 1972), and information on honey bee mortality has been extrapolated to other bee species as well (Greig-Smith, et al., 1994)

Just as these questions were considered for the purposes of designing the two experiments reported here, so must they be answered effectively in the design of any experiments aimed at using behavioral measures in predictive risk assessment with other indicator species. After these are answered effectively, then behavioral testing of the species may commence. Abramson (1994) gives detailed considerations of factors involved in habituation/sensitization, instrumental, and operant conditioning experiments. These include controls that must be implemented, apparatus to be employed, etc. Once these are employed, then the reliability of the learning experiment is high, and can be used as a predictive tool for expected behavioral effects of chemical agents.

### Applied Significance of Behavioral Measures to Pest Species

Not only should beneficial insects such as honey bees be included in behavioral toxicity measures, but some pest species, particularly those that are disease vectors, should have systematic behavioral principles applied. Envision a disease vector not killed by chemical control. Many chemical agents sensitize animals, causing a greater incidence of response than is normal. If the *Anopheles* mosquito is sensitized by a chemical agent, the sensitization may increase the likelihood that it will feed on humans, even when satiated from a recent blood meal. If the Africanized honey bee becomes sensitized due to a chemical agent, then it may be more aggressive than normal, and more likely to sting humans. The neurohormone octopamine, subject to disruption by some classes of insecticides, is involved in the sting reflex (Burrell & Smith, 1995), so sensitization resulting from sublethal doses of octopamine-impacting agents is reasonable. The threat from Africanized bees is not from the potency of their venom, rather, from the increased likelihood of mass stings, a potential problem if the bees are sensitized by chemical intervention (Winston, 1987).

Habituation effects in insect species are also a concern. Consider beneficial insects such as lacewings or parasitoid wasps. If a chemical agent causes down-regulation of sensory receptors involved in locating their hosts, the problems are two-fold. First, the insects will be unable to sense plant kairomones that signal the presence of the host. Additionally, the host insect will likely grow in population because it is no longer under attack by the beneficial insects. Additionally, inability to locate a food source as a result of pesticide-induced behavioral deficits may cause death in

beneficial species through starvation or desiccation, mortality reasons noted in Texas and other southern U.S. states (Clements, 1996).

### Recommendations and Final Conclusions

The goals of the two experiments reported here were to test the efficacy of using standard learning paradigms in predictive risk assessment. Based on behavioral data alone, these experiments showed that significant behavioral disturbances occur in honey bees even when an agent is employed that is considered relatively non-toxic to bees. While mandates for Ultra-Low-Volume (ULV) spraying methods and taking care to spray insecticides at appropriate times have both reduced direct risks to honey bees in the field (Johansen, 1980), bee species continue to fall under attack from injudicious spraying of agricultural chemicals (Clements, 1996).

Because EPA mandates for pesticide approval call for mere hazard assessment, huge gaps exist between safe and unsafe labels on pesticidal compounds. Broad characterizations of hazard are incomplete in determining risks to non-target insect species (Suter, 1990). The experiments here reveal that performing behavioral research using standard paradigms, and paradigms appropriate to the natural behavioral repertoire of the insect, appear to be more conservative means for characterizing risk.

Recommendations include continued testing of existing chemicals used in the field to which honey bees may become exposed, This will require time and effort, though it will not be a costly endeavor, since raising and training honey bees in the laboratory in learning scenarios is a frugal undertaking. Further, behavioral measures should be a part of chemical registration procedures as well. Predictive risk assessment

measures currently focus on specific physiological, biochemical, carcinogenic and teratogenic research, with eventual epidemiological studies as a compound reaches the end-process toward commercial approval (Neely, 1994). No systematic behavioral measures exist in predictive risk assessment, though to some degree they have found application in IPM (Prokopy & Lewis, 1993). To some extent, the lack of behavioral measures in current predictive risk assessment protocols may be due to what Sapolsky (1997) calls "physics envy." Physics envy means that, given a behavioral explanation for a scientific phenomenon versus one forwarded by a "harder" science, the "harder" science generally wins because of its implied experimental rigor (Buckbee, 1997). This idea is not new; it was noted in an early study aimed at inclusion of classical conditioning methodology as a means for uncovering insecticidal effects by Russian researchers in the 1960's (Medved, Spynu, & Kagan, 1964), though their efforts went largely unnoticed. Standard learning paradigms, implemented correctly, provide the experimental rigor and control necessary for giving credence to behavioral explanations.

Further, use of learning paradigms in predictive risk assessment protocol meets with guidelines established for predictive risk (National Academy of Sciences, 1983, as cited in Neely, 1994), which include 1) Identifying the Hazard; 2) Establishment of dose-related affects; 3) Consideration of Exposure Assessment; and 4) Characterizing the actual risk. Other factors supporting behavioral toxicity measures in predictive risk assessment include the low cost of implementing the measures, and the existence of standard research paradigms for testing the behavioral effects of chemical compounds. Additionally, behavioral measures are likely to be of service in identifying unexpected non-inductive synergists.

Some limitations of this research include the lack of truly blind experimenters. Although not standard, it might add rigor to the PER conditioning process if the training procedures were videotaped so that a blind researcher could make judgments about the extension of the proboscis. Further, this research was conducted with only one of several classes of insecticides. A complete picture would include major classes such as carbamates, organophosphates, and other less prominently used insecticide classes. Additionally, as pointed out earlier, data collected from the honey bee is likely of use in similar species, warranting order-specific data collection for accurate risk assessment. And finally, this research was designed to look at acute effects only; a complete picture would include analysis of time-based training.

Behavioral measures cannot stand alone in predicting risk from pesticidal compounds in indicator species. Appropriate protocols should be multidisciplinary in nature, and inclusion of behavioral research as a part of risk assessment protocols would make the existing multidisciplinary picture complete.

The goals of this research included identification of the honey bee as an *a-priori* indicator organism for pesticide toxicology risk assessment research. Honey bees meet the criteria as sentinel animals, since they can be studied in the laboratory, in the field, and in *in-situ* studies (National Research Council, 1991). Additionally, there is a precedent for honey bee behavioral and physiological data extrapolation to other bee species (Greig-Smith, et al., 1994). This is important, since more than 100 bee species are involved in pollination in the United States (Metcalf & Metcalf, 1993). The success of behavioral toxicity in honey bee research has the potential for use in predictive risk assessment (Greig-Smith, et al., 1994), as has been demonstrated here.

Finally, the notion of animals as sentinels of environmental health has typically focussed on mortality (National Research Council, 1991). A better sentinel will "warn us ahead of time" of impending environmental threats. The honey bee, through applied behavioral measures, will demonstrate that sentinel animals need not also be martyrs.

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VITA

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Doctor of Philosophy

**Thesis:** USING LEARNING PRINCIPLES IN PREDICTIVE RISK ASSESSMENT: A PESTICIDE TOXICOLOGY ASSAY FOR INSECTS USING THE HONEY BEE (*APIS MELLIFERA*) AS AN INDICATOR ORGANISM

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