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Evidence of Olfactory and Visual Learning in The Asian Citrus Psyllid, Diaphorina citri Kuwayama (Hemiptera: Psyllidae)

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EVIDENCE OF OLFACTORY AND VISUAL LEARNING IN THE ASIAN CITRUS PSYLLID, *DIAPHORINA CITRI* KUWAYAMA (HEMIPTERA: PSYLLIDAE)

A Thesis
by
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ABSTRACT

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Investigation of the mechanisms underlying learning and memory can be achieved through research on neurobiologically simplified invertebrate species. As such, insects have been used for decades as ideal models of olfactory learning. The current study aimed to investigate the mechanisms of chemosensory attraction in an invasive insect, *Diaphorina citri*, the Asian citrus psyllid (ACP), through manipulation of olfactory stimuli. After classical conditioning to a non-innate cue (vanilla extract), psyllids displayed enhanced feeding behavior. There was, however, an inverse relationship between olfactory "noise" and feeding behavior. Preliminary data suggests ACP may also be visual learners, as evidenced by trials attempting to condition ACP to the color blue. The data indicate that while learning is possible in ACP, it is easily disrupted. As a result, innate response to host plant stimuli in oligophagous, selective feeding insects may represent the most adaptive means of locating resources.

DEDICATION

To my entire family, the Stockton-Anderson- Roberts-McIntire-Linder-Derbes clan, who has tirelessly supported me through this 26-year journey, I am truly blessed to have such a brilliant, creative, and loving group of people by my side.

To my parents who listened to me read my manuscript aloud over dinner and pretended not to fall asleep, thank you for your patience. To my brother, who did in fact fall asleep...you keep me honest. To my best friend, Casey, who always makes time for my anxiety, thank you. To my partner, David, thank you for letting me cry at you when Murphy‘s Law fell into effect. You are my rock.

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INTRODUCTION

1.1 Animal Models for Learning and Memory Research

Research in learning and memory has focused largely on mammalian species in an attempt to better understand human cognition. Some of the most feared and debilitating diseases are those that deteriorate memory, such as Alzheimer's disease. For this reason a significant portion of federal and private funding is directed towards psychologists, neuroscientists, and biomedical researchers seeking learn more about how human memory is acquired, maintained, and ultimately retrieved. To carry out this work rats are often used due to their relative anatomical similarity to humans, low cost to procure, and the affinity of the animals to learn simple tasks useful for such research. However, scientists have recognized for well over a century the benefits of studying animals lower on the phylogenetic scale, such as invertebrates. In fact a large number of pivotal studies in psychology have used invertebrates as subjects. The reasons for working with invertebrates are plentiful. They are cheaper, reproduce faster, and are easier to maintain than mammals, but the most significant reason is that lower phyla tend to have simplified nervous systems. Simplicity allows the study of the entire system to occur much more easily. It is analogous to the difference between attempting to untangle a large bundle of wires versus untying a shoelace.

While some insects, like the honey bee, are adept learners, others seem much more limited. To predict whether and to what extent learning is demonstrated by a species, theorists

have proposed certain environmental and biological criteria for learning, such as the spatial and temporal availability of resources and the degree of mobility the animal requires to locate those resources (Dunlap & Stephens, 2009; Hollis et al. 2011). In migratory birds, for example, the functional adaptability of long-distance mobility requires complex neurobiological processes such as place memory (Sherry et al., 1992).

The Asian citrus psyllid (ACP) (*Diaphorina citri* Kuwayama) (Hemiptera: Psyllidae) is an oligophagous (selective feeding) insect appropriate for the study of learning. ACPs feed on phloem sap and vector the causal agent that causes Huanglongbing (HLB), also known as citrus greening disease, which threatens citrus production world-wide. ACPs feed and reproduce only on *Citrus* and closely related genera in the plant family Rutacae. While it is oligophagous, the immature feed only on young foliage, called flush. Since flushing within its host plants may vary both spatially and temporally, learning to recognize stimuli associated with flush may be adaptive to ACP. The following study investigated the ability of ACPs to learn associations between novel non-innate stimuli (odor) and sugar water, thus encouraging feeding on scented mediums otherwise found unfavorable. The major goals of this study were to determine whether adult ACPs are capable of learning to recognize novel olfactory and visual stimuli.

1.2 Research Aims

The present study aimed to address learning in the Asian citrus psyllid by classically conditioning feeding response on non-innate chemical and visual stimuli. Dose response tests measured innate attraction and repulsion to individual volatiles. Response was measured by rate of probing. Odors that did not elicit an innate response were considered ideal candidates for learning. All dose response tests and related behavior analysis used a method described in Patt

& Setamou (2011) through quantification of feeding behavior, measured as stained saliva spots, on a synthetic wax medium (Specialized Pheromone and Lure Application Technology – SPLAT). The stained probe spots are visible under a microscope and can be manually counted, resulting in a directly observable indication of psyllid response to different volatiles.

Ten odors were tested for innate response and were potential candidates for ACP learning trials. Vanilla (vanillin) and menthol were chosen because they represent traditionally neutral odors in insect learning literature (Watanabe & Mizunami, 2007). Banana (isoamyl acetate), which is primarily a combination of fruit esters, was selected because of its molecularly distinct shape. There are no existing data on ACP response to this class of chemicals. Almond (benzaldehyde) was tested because although ACPs have a known receptor for this chemical, there are no data describing a behavioral response. Three chemical relatives of vanilla – eugenol, anisole, and anisaldehyde - were tested as well. Finally, limonene, a common citrus monoterpene was tested. There are existing data supporting the innate attraction of ACP to limonene and this study attempted to examine the effect of conditioning on augmenting the naturally high ACP response.

Classical conditioning trials were performed with a selection of volatiles from the innate response tests using sucrose solution as the unconditioned stimulus. Asian citrus psyllids were allowed to feed for 24 hours on scented sucrose solution. We hypothesized that the combination of sucrose, the biologically significant stimulus (unconditioned stimulus), and the odor (conditioned stimulus) would be sufficient to induce learning, as measured by the number of probe spots (reflexive unconditioned response) visible on the SPLAT.

In addition, we examined the effect of introducing a compound stimulus after conditioning to a single odorant. This was to establish the delicacy of the learning process in

ACP. After identifying an odor that produced an increase in response (after conditioning) relative to the unscented control, we attempted to interrupt the learning process by introducing an odor that produced an innate repellent effect.

The final experiment expanded the conditioning trials from olfactory to visual stimuli. We tested ACP response to a non-innate visual stimulus (blue) after exposure to a blue synthetic food source. The color blue was chosen after a review of a recent study which showed a decline in captures associated with blue sticky traps (Sandoval, 2010).

The results of this study were intended to demonstrate whether learning is possible in the Asian citrus psyllid and allow inferences to be drawn about what constitutes important molecular structures for Asian citrus psyllid olfaction and olfactory learning.

CHAPTER II

LITERATURE REVIEW

2.1 The Relevance of the Asian Citrus Psyllid

Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama, became an insect of great interest when, in 1998, researchers from Florida discovered it on local crops, alarming the agricultural community (Halbert, 1998). ACP are known hosts of the bacteria, *Candidatus* Lieberibacter, that is thought to cause a disease known as huanglongbing (HLB), or citrus greening disease, which harms citrus and citrus related plants considerably (Capoor et al., 1967; Catling, 1970). Eventually, infection leads to plant death. Signs of infection by *C.* Liberibacter closely resemble a number of benign diseases and/or mineral deficiencies, including malformations such as leaf mottling, yellowing of the veins and midribs of the leaves, as well as misshapen and discolored fruit (Boina et al., 2011; Bove, 2006). As a result, it is often difficult to determine that a tree is infected without expensive DNA testing and many infections are left undetected.

C. Liberibacter is a gram negative, phloem-restricted bacteria carried by the Asian citrus psyllid, and may occur as either the asiaticus (Las) or americanus (Lam) variety. Bacterial transmission appears to occur by both nymph and adult ACP through continuous inoculation by the sap-feeding hemipteran (Inoue et al., 2009). In turn, adult ACP may acquire *C.* Liberibacter

from feeding on previously infected trees (Roistacher, 1991). Because of how easily spread the bacteria is, there has been a large recruitment of scientists to hurriedly address HLB and its potential to destroy American citrus. Unfortunately there is limited work being done of ACP ecology that could significantly help this effort.

HLB is considered by many to be the worst disease to ever affect the global citrus industry, threatening to eradicate whole orchards in many parts of the world. This is partly due to the rapid spread of the disease despite large scale ACP population control initiatives using broad spectrum pesticides (Boina et al., 2011). Certainly, the known presence of HLB infected trees in 30 citrus growing counties in Florida is a serious financial concern for the $9.1 billion Florida citrus industry. California and Texas citrus growers are following suit with similar concerns. In 2012, HLB was detected for the first time in South Texas orchards, increasing the need for further study on the Asian citrus psyllid.

Currently, research in HLB prevention is leaning in the direction of ACP eradication. While insecticides are used presently to attempt to slow the spread of the disease, long-term solutions are being developed such as genetically modified plants and ACP that are resistant to *Candidatus* Liberibacter. However, two scientists from Weslaco, Texas, which is approximately 10 miles from the spot where HLB was detected in South Texas, have focused on ACP chemical ecology with the hope of understanding what directs ACP host plant preferences, what sensory cues are responsible for such preferences, and how those preferences can be manipulated. So far, their work has led to the potential for non-pesticide, trap and kill methods, which is ideal for homeowner citrus. These tremendous strides towards addressing the Asian citrus psyllid problem demonstrate the importance of knowledge in the biology and ecology of an insect of interest.

While pesticides and bait-and-kill techniques have their place in pest management within in the agricultural community, it is often not enough to control problem insects in this manner (Thomas, 1999). There is a historical precedent for incorporating knowledge about insect adaptation and learning in the process of bio-control (Prokopy & Lewis, 1993). Work in chemical ecology helps direct efforts towards understanding the nuance of insect behavior so that pests can be managed even to the extent that they modify their behavior to fit the increasingly hostile environment created by bio-control attempts. In a sense, researchers must explore the effect of learning on pest species so as to stay one step ahead of individual organismic and species-wide adaptations.

2.2 ACP Feeding Behavior

To study learning in a species, the natural behaviors of that species must be understood. In insect learning, one of the most important behaviors to investigate is feeding behavior. This is because associative learning, or classical conditioning, relies on the pairing of an innate, biologically significant stimulus (the unconditioned stimulus) with the non-innate stimulus (the conditioned stimulus). In order for conditioning to occur, the stimuli must be carefully selected to accommodate the naturally occurring food preferences and behaviors of the species under investigation.

2.2.1 Significant Feeding Mechanics

Asian citrus psyllids are sap-sucking herbivores found on plants in the family Rutacae. To extract nutrients from the plant, ACPs insert their mouth parts, known as the proboscis, into the leaf of the plant. The proboscis acts like a straw, allowing the ACP to ingest phloem sap, a sugar-rich nutrient solution. For this reason, the researchers involved in this study chose to use

sucrose solution as the unconditioned stimulus. The concentration of the sucrose solution was based on previous data showing 12g cane sugar per 40ml H_2O acts as a suitable, short-term phloem sap substitute on which ACP can be reared (Hall et al., 2010). In addition, the sap-sucking method of feeding is important to the experiment design. The method of stimuli introduction used in this study encourages natural ACP feeding habits and was developed specifically for the Asian citrus psyllid (Patt & Setamou, 2010).

2.2.2 Host Plant Preferences

Asian citrus psyllids feed on Rutacae, a large family of plants, which includes citrus. ACP feed and reproduce on all varieties citrus including Meyer lemon (*Citrus* × *meyeri)*, Mexican lime (*Citrus aurantifolia*), sweet orange (*Citrus sinensis* (L.) Osbeck), and grapefruit trees (*Citrus* × *paradisi*), as well as several species of murraya, a related genus of Rutacae, such as orange jasmine (*Murraya paniculata*) and the curry leaf tree (*Murraya koenigii*). However, the ACP host plant range is limited to these plants specifically. They cannot reproduce on any other type of plant.

A considerable amount of gas chromatography-mass spectrometry (GC-MS) data has been collected about citrus volatiles (Sandoval, 2010). These data have shown what appears to be a fairly consistent list of volatiles that occur in most citrus species and can be ordered in terms of relative prevalence within each variety of citrus. Some of the most common volatiles are limonene, β-caryophyllene, β-ocimene, geraniol, and linalool.

In an attempt to explore the range of limits of ACP, some researchers have intentionally tried to rear ACP on plants from other families. Pena et al., (2006) attempted to rear ACP on jackfruit, *Artocarpus heterophylus* Lamarck, without success. He even placed psyllids in cages with jackfruit and tracked their development for two months. At no time did he record the

presence of eggs or nymphs. For this reason, ACPs are designated as semi-selective, oligophagous insects.

Within Rutacae there are notable examples of host plant preference, particularly for orange jasmine, *M. paniculata*. In fact, the discovery of ACP in Florida in 1998 was on orange jasmine plants, and three years later, a shipment of orange jasmine to the Rio Grande Valley, brought ACP to South Texas. Bové (2006) describes a plant preference order strongest with orange jasmine, sour orange, and the curry leaf tree. Certainly others have noticed the affinity of ACP for orange jasmine. As such, most ACP colonies are reared on orange jasmine.
Tsai (2002) published a study on ACP population shifts in Florida orange jasmine groves. He suggests that preference for murraya is due to the high turnover of flush orange jasmine creates year round.

Other studies, however, have suggested grapefruit may be more preferred than orange jasmine. Tsai (2000) compared colonies of ACP reared on four types of plants including rough lemon, sour orange, grapefruit, and orange jasmine. While egg incubation and nymph development time were constant, the rate of egg laying and nymph survival on grapefruit was significantly greater.

GC-MS data from preferred citrus species helped to establish which volatiles to use in the olfactory learning portions of the present study. In addition, host-plant preference data helped guide the selection of orange jasmine as the primary plant used for ACP rearing. To understand why these host plant preferences occur, it is necessary to look at chemical ecology and sensory mechanisms of host plant selection.

2.3 Mechanisms of Host Plant Selection and Chemical Ecology

Vision is important for host plant selection for a variety of reasons including locating mates for reproduction, identifying appropriate sources of food, and oviposition. Experiments with male psyllids have suggested that males may use vision to identify citrus host plants, and orient towards females by specifically targeting flushing leaves (Wenninger et al., 2008). Other evidence for the importance of vision comes from the effective use of yellow sticky traps in population monitoring efforts (Hall, 2007). In experimental conditions, Asian citrus psyllids appear most attracted to colors with reflectance values approximately 500nm in wavelength, on the border between blue and green. Discovered through colored sticky trap collections, these colors occur in the green-yellow area of the visible spectrum and are similar to the color of flushing citrus leaves, the exclusive site of psyllid oviposition (Sanchez, 2008; Wenninger et al., 2009). It is possible that ACP preference for orange jasmine and lime trees, such as *C. aurantifolia*, is related to the bright, light-green color of their leaves, which reflect light in the 500nm range.

Other species of hemipertans are documented to have keen visual abilities, such as aphids (Doring & Chitka, 2007). In most species, however, color alone is thought to be insufficient for identifying host plants (Prokopy & Owens, 1983). Visual stimuli, in combination with olfactory cues have been found to enhance the response of the glassy-winged sharpshooter *Homalodisca vitripennis* (Patt & Setamou, 2007). Wenninger (2009) found that while ACPs failed to display attraction to isolated olfactory cues, it did respond to isolated visual stimuli, suggesting that even without multimodal stimulation, the effect of color may be significant enough in psyllid host plant detection mechanisms to elicit attraction. This finding may not be surprising considering that not only are the primary volatiles emitted by citrus (terpenes) are

found in virtually all other flora, but overall volatile plant compounds are highly generalized across species (Eisner & Grant, 1981).

Despite these findings, the role of olfaction in guiding host plant detection cannot be ignored, having extensive support in entomological literature showing the importance of volatiles in the foraging behavior of herbivorous insects such as *D. citri* (Thorsteinson, 1960; Visser & Thiery, 1986; Schoonhoven, 1968; Bruce et al., 2005). The first documented case of behavior driven by olfactory cues was described by the French entomologist working with moths (Fabre, 1911). Plant volatiles can both attract and repel herbivores (Visser & Thiery, 1986; Foster & Hams, 1997). They can help insects do the same things visual cues assist – locate food, mates, and oviposition sites – as well as cue insects about non-hosts and the presence of potentially harmful substances (Szendrei & Rodriguez-Saona, 2010). Such volatiles from non-hosts might play an important role in host plant detection. Psyllids may use adaptive avoidance of non-host stimuli in conjunction with enhanced host discrimination based on complex multimodal cues, such as was described by Schroeder (1992) as the method used by conifer colonizing beetles to avoid angiosperm bark. In this way ACP may use a great deal of olfactory information, as a kind of secondary orienteer, to fine-tune its selection process.

Lately more research has been directed towards multimodal cues, including those derived from gustation. There is evidence that cues in combination may interact synergistically to improve host plant detection and selection (Campbell, 2009). Synergism is well documented to occur between pheromones and host plant volatiles in conjunction with visual stimuli in the ambrosia beetle (Borden & Borden., 1982). A recent study on Asian citrus psyllid attraction found that visual, olfactory, and gustatory cues, when combined produced a strong increase in probing behavior (Patt et al., 2011).

In order to study olfaction at the behavioral level, an understanding of the anatomy of the insect involved is important. Olfaction allows animals to detect a wide range of environmental chemicals, discriminate between them, and respond accordingly. Each species has an olfactory system that has been, over the course of evolution, tweaked to benefit its specific survival needs (Pellegrino & Nakagawa, 2009).

For an insect, or any other animal to perceive an odor, a chain of events must occur at the cellular and molecular level. The following is an overview of those mechanisms in the insect brain (Pellegrino & Nakagawa, 2009). First, volatiles in the air make contact with olfactory receptors located in the sensilla of the insect antennae. Stimulation of these olfactory sensory neuron receptors, through the activation of a series of G-coupled protein linked and ligand-gated channels, results in cellular depolarization of the neuron. The bi-polar olfactory sensory neuron then fires a signal to the glomeruli in the antennal lobe. If antennal lobe neurons are sufficiently stimulated, they fire via projection neurons to the mushroom body, which is the insect homologue to cerebral cortex or forebrain structures. The mushroom body is responsible for complex sensory integration and information modulation, resulting in phenomena such as learning and memory.

One result of higher level processing of olfactory information is the qualitative variable, perception. It is known that depending on the volatile concentration, insects and other animals may perceive dramatically different substances (Gross-Isseroff & Lancet, 1988).
Wright et al., (2005) found this to be true while working with honeybees. Following conditioning to an odor, the same odor presented at novel concentrations were perceived as *more* different than novel odors with concentrations similar to the conditioned odor. Wright suggested that this occurs because the evolutionary cost of concentration invariant coding is too high,

meaning it was adaptive to develop olfactory organs capable of detecting small changes in concentration. In an environment with a large number of plants emitting similar volatiles, without concentration coding, insects might fail to distinguish between potential host plants.

Volatile ratios within mixtures are also important. Ratio variation can produce perceptually distinct substances which elicit a wide variety of behaviors (Laska & Hudson, 1992). Interestingly, numerous studies have shown that within a mixture, olfactory systems are capable of distinguishing between individual components (Smith, 1998; Giannaris et al., 2002).

2.4. Invertebrate Learning

In most known animal species learning is not only present but required for survival. This higher-order processing ability, once thought to be unique to vertebrates, is now being re-evaluated as a much more ancient, and possibly basic, evolutionary adaptation.

Learning, from a biological perspective, is the acquisition of neuronal representation of new information. Memory, on the other hand, is the retention of learned information over a period of time. The information can be spatial, auditory, olfactory, visual, gustatory, or motor, and is represented as morphological changes such as increased dendritic arbor. Because we do not currently have a means of accurately monitoring such tiny and numerous anatomical changes *in vivo*, learning can only be studied indirectly, through its effect on behavior (Dukas, 2008).

Early work on invertebrate learning was done on mollusks. Neurobiologists utilized mollusks in a wide variety of studies due to their simplified neuroanatomy. Much of what was learned about action potentials, for example, was gleaned from work on the giant squid (*Loligo vulgaris*) axon (Hodgkin & Huxley, 1939). However, organisms are usually chosen for their small rather than large size. Small size, structural simplicity, and small numbers of cells provide

the opportunity to study neuronal pathways in direct cell-to-cell interactions that is not possible in more complex systems.

Early invertebrate models include leg-position learning in the locust (Horridge, 1962), odor aversion learning in *Limax* (Sahley et al., 1981 a, b), phototaxis based associative learning in *Hermissenda* (Alkon, 1974); Lederhendler et al., 1986), and gill and siphon withdrawal reflex in *Aplysia* (Carew et al., 1981; 1983). Work on the marine snail, *Aplysia*, which is famous for habituation and sensitization studies, represents decades of experiments by psychologists and neurobiologists alike. Walters et al., (1979) found that *Aplysia* not only responded to aversive conditioning, but that the conditioned stimulus was capable of modulating behaviors not included in the conditioning procedure, such as escape. This modulatory effect is likely due to changes in the internal state of the animal, reflecting the existence of what behaviorists would call motivating operations, such as appetitiveness. Work with *Aplysia* demonstrated the extent to which some mammalian cognitive constructs can apply to other taxonomic classes.

Associative learning with the marine snail *Hermissenda crassicornis* revealed a considerable list of criteria by which most psychologists assess vertebrate conditioning to be applicable to invertebrate species as well. Invertebrate animals display long-term retention and saving (Crow & Alkon, 1978), extinction (Richards et al., 1984), and stimulus specificity (Crow & Offenbach, 1983).

Invertebrates can also learn quickly. Studies in *Limax* show odor/taste associations after just one trial (Sahley et al., 1981 a, b). In fact this species was so adept at learning and its physiological simplicity so ideal, *Limax* has become an excellent and often used candidate for studying the underlying anatomical mechanisms of olfactory associative learning (Kimura et al., 1998).

Physiological conditions usually associated with learned phenomena have also been documented in invertebrates. Conditioned taste aversion training in the pond snail *Lymnaea stagnalis* showed that sucrose (the conditioned stimulus), after repeated pairings with KCl solution (the aversive unconditioned stimulus), evoked conditioned avoidance of sucrose, as demonstrated by retracting into its shell (Kita et al., 2011). What is interesting is that this was not the only response recorded. After conditioning, the snails also displayed a skipped heartbeat when presented with sucrose. Cardiac alterations were previously detected in crab species following aversive conditioning (Hermitte & Maldonado, 2006). Changes in cardiac activity associated with aversive stimuli have traditionally been regarded as a solely mammalian occurrence, and when discussing such mammalian phenomena, this reaction often labeled as fear. However, fear is a conscious experience; a cognition (Panksepp, 2005; Kita 2010). Work such as this opens doors to new avenues of the philosophical debate on animal consciousness. Some argue that mammalian-like consciousness might be present in invertebrate species such as the octopus (Edelman et al., 2005). However, the problem might be that when we discuss higher level associative reactions, we are only capable of human-relevant language such as "fear" that suggest a level of consciousness usually reserved for humans. In addition to renaming certain behavioral phenomena, neuroscience must further refine its criteria for the neural basis of consciousness.

Applewhite & Morowitz (1966) demonstrated several learned phenomenon in multiple species of "micrometazoa," mostly aquatic worms measuring less than 1mm in length. The copepod *Paracyclops fimbriatus poppei* (0.8mm) as well as the ostracod *Cyclocypris forbesi* (0.6mm) successfully navigated a 12 chamber aquatic maze, representing some of the smallest organisms in a documented maze learning trial. *Cyclopris* was also found capable of avoidance

learning. When paired with a mild shock, ambient illumination produced a shell closing response. Other species such as tiny flatworms were successfully habituated to noxious stimuli.

As with mollusks, learning and memory research on several species of insect as well as has been extremely valuable. The four key species under investigation are the fruit fly *Drosophila melanogaster*, grasshoppers such as *Locusta migratoria*, parasitoid wasps like *Microplitis croceipes,* and the honeybee *Apis mellifera*. Each taxa provides their own uniquely valuable glimpse into the mechanisms underlying cognition.

Fruit flies can associate neutral odor, color, and visual patterns with both appetitive and aversive conditions. For instance, they will prefer odors paired with sucrose and avoid odors paired with electric shock (Temple et al., 1983; Quinn et at., 1974). *D. melanogaster* in larval stages can do the same (Aceves-Pina & Quinn, 1979). Fruit flies will also avoid sources of illumination when paired with an aversive stimuli, as well as visual patterns paired with a harmful heat source (Folkers, 1982; Lui et al., 2006). Other studies have shown the importance of learning in reproductive viability. Males can draw associations between unsuccessful mating attempts and the female pheromones emitted during those failed attempts (Ejima et al., 2005). When females, who normally mate with larger males, are only provided with the opportunity to be courted by smaller males, those females shift their preference toward smaller mates (Dukas, 2005). Males also learn appropriate contexts for aggression when competing for female courtship, being less likely to show aggressive behavior towards another male that has previously won a match (Yurkovic et al., 2006).

Grasshoppers have the sophisticated ability to learn to visit nutritionally valuable food. This behavior involves learning. If grasshoppers are deprived of some key nutrients and given an excess of others, each associated with a particular color, the grasshoppers will choose to

consume the food color of which they have been deprived (Simpson & Raubenheimer, 2000). There are clear evolutionary advantages to this. The ability to learn what foods are most and least available is directly tied to overall health, and ultimately reproductive success. One study found that rapid learning in grasshoppers was correlated with a 20% greater growth rate (Dukas & Bernays, 2000). This also may represent the presence of motivating operations, like in *Aplysia*, as there are clearly shifts occurring in satiety and deprivation at the nutritional level.

Parasitoid wasps require the ability to detect their hosts on a variety of environmental substrates. As a result, these wasps are more likely to prefer stimuli, such as odors or visual cues, associated with previously detected hosts. A key example of this effect was an experiment in which wasps were provided with hosts in combination with different odors such as chocolate, a non-innate attractant. After pairing, the wasps were significantly more likely to seek out hosts in chocolate scented areas (Lewis & Takasu, 1990).

Finally, the honeybee probably exhibits the keenest insect learning of all, being capable of learning and interpreting complex waggle dances to encode information about flower location (Dryer, 2002). They also use spatial learning to recall the location of previously visited plants, as well as to recall how to efficiently navigate complex flowers (Carter, 2004; Laverty, 1994).

However, there has been a long standing argument that learning is characteristic only of mobile animals. This comes from the idea that mobility, for foraging etc., forces an organism to depend on learning skills, such as place memory, that are unnecessary in sedentary insects. Certainly all the animals listed above are mobile compared to the psyllid, but the psyllid is far more mobile than true sedentary insects like the antlion. If sedentary insects such as the antlion can learn, maybe psyllids can too.

Studies on the antlion (*Myrmeleon crudelis)* have shown, unequivocally that even sedentary insects can learn (Guillette et al., 2009). Antlion larvae hunt for food by digging "funnel-shaped pits," into which prey fall and are then captured. Researches extended the range of normal antlion attack by 20-40% by pairing food with a vibrational signal set far from the pit. This was accomplished in only two training sessions. Antlions were also trained to build bigger pits and hunt more efficiently, leading to faster molt time for trained antlions compared to antlions without training. In a follow-up study (Hollis et al., 2011) the evolutionary advantage of antlion learning was confirmed by showing that faster molt time decreased the vulnerable larval phase time and thus increased the chance of reproduction as an adult. Because of studies like this, more scientists are arguing that learning is an emergent property of the nervous system itself – that all organisms can and do learn, not by choice, but because their bodies are innately designed to make associations between stimuli as they occur in nature.

2.5 Learning and Evolution

Evolutionary theory states that for a trait such as learning to be maintained within a species that trait must pose a reproductive advantage (Darwin, 1859). The advantages of learning are enormous, allowing an organism to adapt, on the individual level, to the constant changes in its environment (Johnston, 1982). In doing so, the organism can more readily acquire resources that extend its life, and theoretically, increase the likelihood of reproduction. It has been proposed that the evolutionary pressure insects experienced to develop greater spatial navigation skills coincided with enlargement of the mushroom bodies, the structures responsible for learning processes in insects (Farris & Schulmeister, 2011). This is analogous to speculation about the concurrent evolution of dexterous hands and a large hominid brain.

In recent years, new theories of the evolution of learning have come forth that augment the more classical approach through a combination of computer modeling and the latest in behavioral testing. As a result a new and controversial topic has emerged – what is the advantage of non-learning in insect species?

The one theory addressing this question was proposed by Dunlap and Stephens (2009) who developed a mathematical model to predict whether or not an insect species will evolve the ability to learn. They used two predictors, the reliability of a behavior in producing food, and the predictable, steady supply of food sources in the environment. This is based on a popular view amongst evolutionary theorists, which states that environmental stability, or the continuous and uninterrupted supply of food sources, is seen as the single most important predictor of whether learning evolves in a species (Dukas, 1998). They found that learning emerges in a species only if the environment is variable. If the environment steadily produces food and the existing insect behavior successfully locates and uses this food, then learning does not evolve because there is no evolutionary pressure to do so.

However, another theory directly addresses the first, purporting that Dunlap's approach is too simplistic and does not encompass the complexities of evolutionary process (Hollis et al., 2011). In Dunlap's model, behavioral rigidity is the default and learning is an adaptive reform of the basic system. Hollis argues that it might be an incorrect assumption that what is occurring is the evolution of learning. Rather, learning may be the default operating system which is overridden when environmental conditions favor the "evolution of behavioral inflexibility." Simply, insects evolve not to learn.

The following study aimed to address associative learning in a semi-sedentary insect, the Asian citrus psyllid, in two sensory modalities.

CHAPTER III

METHOD

3.1 Psyllid Colonization

D. citri were housed in an indoor colonization chamber at 26 ± 2°C. The chamber was set on a 14hr light / 10hr dark cycle. Light sources included full spectrum (Intertek® energy saving lamp; 120V 60Hz 6400K; SuZhouHongSheng Lighting Products Co., Ltd) and infrared grow lights (Flower Accelerator®; 90W Illuminator UFO; Prosource Worldwide, Inc.) for maximum plant sustainability. Psyllids were reared on a combination of orange jasmine (*Murraya paniculata)*, provided by USDA-APHIS-CPHST, Mission, Texas), and curry leaf (*Murraya koenigii)*, grown from seeds (Accession No PI539745) acquired from the USDA-ARS National Clonal Germplasm Repository, Riverside, California. Orange jasmines were regularly pruned to promote flush. Prior to exposure to *D. citri*, all plants were housed in a secure greenhouse free of pest contaminants and maintained with reverse osmosis water and M-Pede safer soap (Mycogen Corporation) to remove pest contaminates. Every two weeks, plants were rotated to ensure plant health.

3.2 Innate Response Tests

3.2.1 Behavior Platform

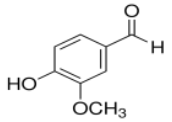
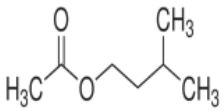
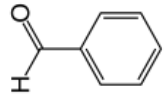
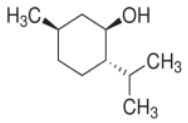
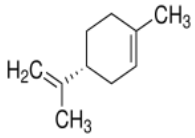
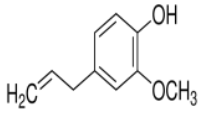
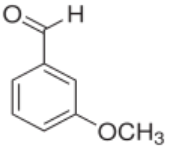
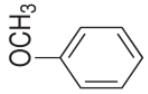
To identify compounds that did not elicit an innate response, a series of initial dose response tests were performed. Odors were mixed with SPLAT (Specialized Pheromone and Lure Application Technology), a viscous, white wax developed to hold semiochemicals for slow release (ISCA Technologies., Inc.). It was utilized in this study to deliver the odor to the ACP, and acted as the medium for reading ACP response. The apparatus consisted of 55mm plastic petri dish covers wrapped in Parafilm (Pechiney Plastic Packaging Company) to create a membrane. Using a syringe and a 20G needle, two intersecting lines of SPLAT were applied to the Parafilm membrane covering the dish (see figure 1.1). Late in the study, the two line technique was modified by applying only one line of SPLAT and was incorporated into the dose response protocol for vanilla, limonene, eugenol, anisaldehyde, and anisole (see table 1 & 2).

Table 1. List of odorants evaluated with dose response tests.

Odor	Lines of SPLAT	% Purity	CAS No.	Manufacturer Information
Vanilla Extract	1	---------	---------	Adams Extract Co.
Banana Extract	2	---------	---------	McCormick & Co., Inc.
Almond Extract	2	---------	---------	McCormick & Co., Inc.
L-Menthol	2	≥99.0%, FCC	2216-51-5	Sigma-Aldrich; W266590
(R)-(+)-Limonene	1	≥99.0% (GC)	5989-27-5	Fluka; 62118
Eugenol	1	98+% natural	97-53-0	SAFC; W246719
Anisole	1	97%	591-31-1	Aldrich; 129658
Anisaldehyde	1	≥99.0% (GC)	100-66-3	Fluka; 10520

Psyllids were exposed to SPLAT mixed with vanilla extract, banana extract, almond extract, menthol in EtOh solution, limonene, eugenol, anisaldehyde, or anisole at four different concentrations based on a half-log scale: 3µl scent/10ml SPLAT; 9µl scent/10ml SPLAT; 30µl scent/10ml SPLAT; 90µl scent/10ml SPLAT. Psyllids in the control treatment were exposed to unscented SPLAT.

Table 2. Volatile information for the odors tested in experiments 1-3 showing the chemical names of each odorant and its corresponding molecular structure. In the case of the three extracts used in this study, the information provided is relevant to the primary compound of each extract.

Odor	IUPAC Name	Chemical Class	Structure
Vanilla Extract (Vanillin)	4-Hydroxy-3-methoxybenzaldehyde	aromatic aldehyde	
Banana Extract (Isoamyl acetate)	3-methylbut-1-yl ethanoate	fruit ester	
Almond Extract (Benzaldehyde)	benzaldehyde	aromatic aldehyde	
L-Menthol	(1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexanol	alcohol	
(R)-(+)-Limonene	1-methyl-4-(1-methylethenyl) - cyclohexene	monoterpene	
Eugenol	4-Allyl-2-methoxyphenol	phenylpropene	
Anisaldehyde	4-Methoxy benzaldehyde	aromatic aldehyde	
Anisole	methoxybenzene	aromatic ether	

Menthol is in solid state at room temperature; therefore it was dissolved in ethanol, 55g/800ml EtOH. The control treatment for the menthol dose response test was prepared with 90µl EtOh/10ml SPLAT, to compensate for the presence of EtOH in the menthol solution.

All SPLAT preparations included the use of neon green food coloring (McCormick & Co., Inc.) 6µl/10ml SPLAT. For all treatments SPLAT was mixed with a vortex a minimum of two minutes, until the mixture was evenly tinted. Five psyllids were released onto each dish, which was placed inside a larger 10 cm plastic petri dish, and was left in isolation in an incubator for 2 hours at 26 ± 2°C.

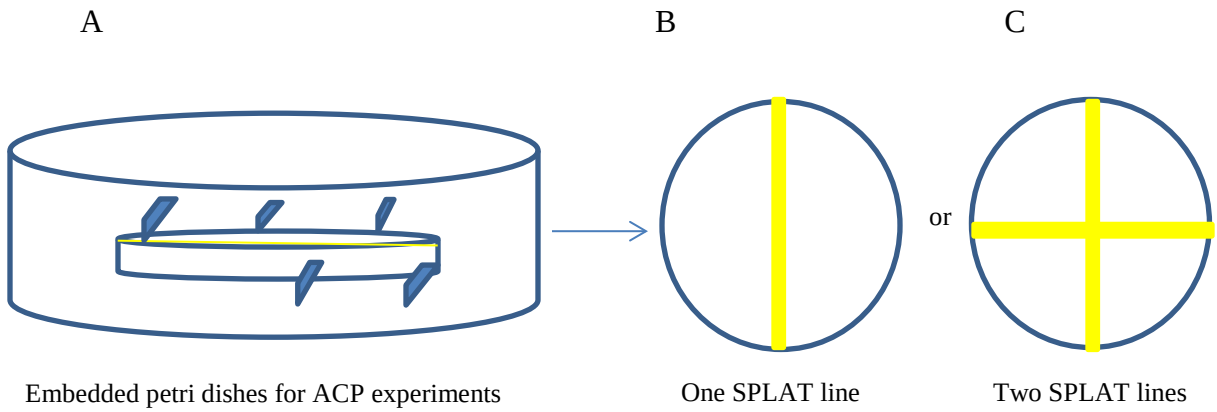


Figure 1.1 Visual description of the behavior platform. Diagram (A) shows how two petri dishes are used together to create the conditioning apparatus or behavior platform. The general apparatus is the same for both phases of the experiment. A smaller dish is placed within a larger dish, into which the ACPs are released. Diagram (B) shows behavior platform preparation using one line of SPLAT. Diagram (C) shows behavior platform preparation using two lines of SPLAT.

3.2.2 Evaluation of Probing Behavior

Following removal of the psyllids by manual aspiration, all dishes used in the behavior platform were stained for 2 minutes with Coomassie blue R350 (C.I. 42660;

CAS Number 6104-59-2; Sigma-Aldrich Chemical Co.), rinsed with reverse osmosis water twice, and dried with a fan. Dye concentration was 0.1% Coomassie blue R250, 20% (v/v) methanol, and 10% (v/v) acetic acid.

Dishes were then evaluated under a microscope for evidence of probing on the lines of SPLAT (see Image 1). Probes were quantified manually with a stereomicroscope at 4X magnification level. In the portion of the study on conditioning to vanilla, evaluation was performed in sets of two 5mm sample sections along each arm of the dish. All other dishes in the study were evaluated by counting the total number of probes on the entire length of each arm.

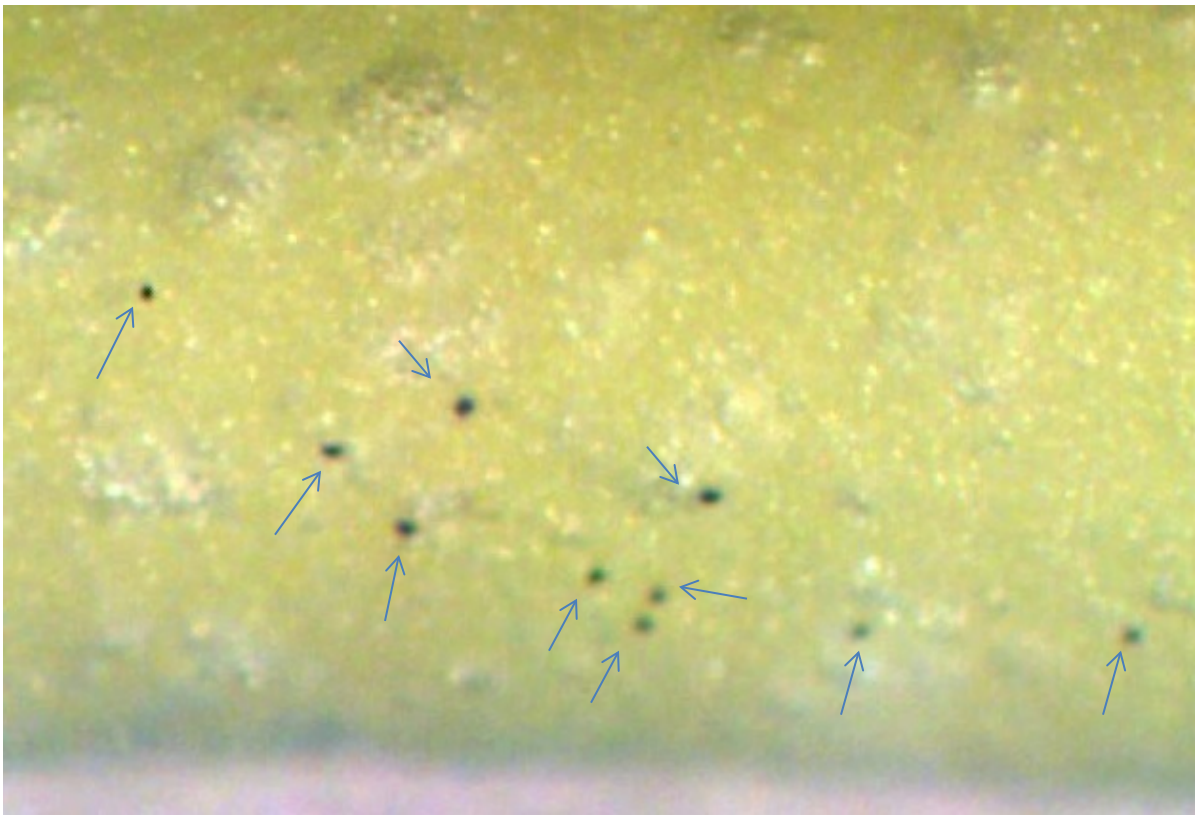


Figure 1.2 Stained probe spots on SPLAT left behind by ACP feed attempts. This image has been magnified 4X with a stereomicroscope. The probes are visible as dark dots on the background of yellow-green. There are ten probe spots in this image.

3.3 Conditioning Tests

3.3.1 Conditioning Phase

Conditioning required a two phase protocol, consisting of a conditioning phase, in which the insects were exposed to unscented (B) or scented (S) sucrose solution, and a behavior testing phase, which provided the opportunity for insects to probe unscented (B) or scented (S) wax. The wax was then stained for salivary enzymes and probing behavior was quantified.

The four possible treatments were described using two-letter abbreviations, BB, BS, SB, or SS (see figure 1.3). The first letter represents the conditioning solution which is either unscented (B) or scented (S). The second letter represents the SPLAT, which is also either unscented (B) or scented (S).

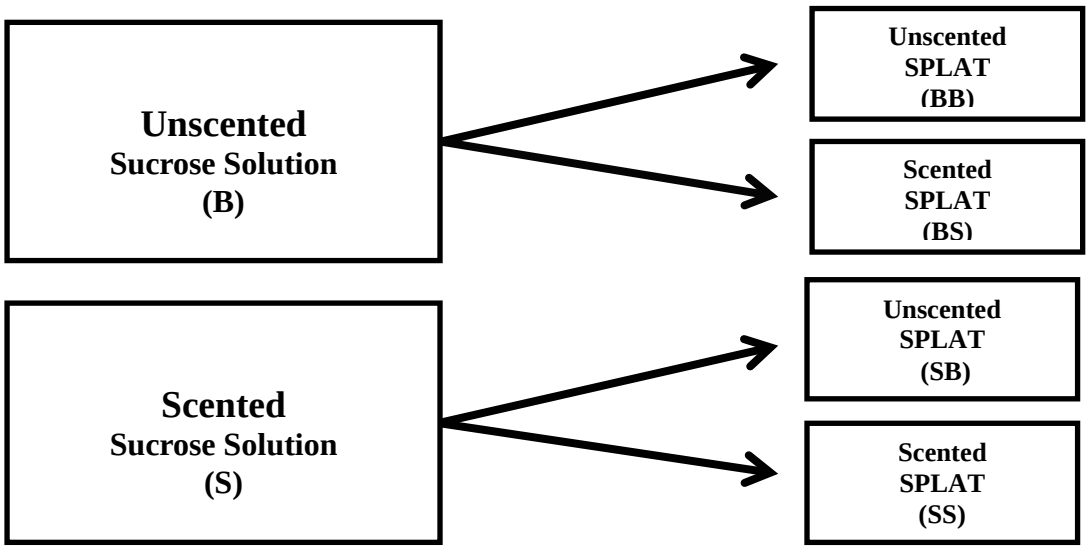


Figure 1.3 The conditioning and behavior testing protocol used in conditioning tests. The first phase divides the treatment into two groups, those ACP on either plain sugar water or scented sugar water. Phase two breaks the first two groups in half again, resulting in a total of four treatment groups.

The conditioning apparatus consisted of 5cm plastic petri dish covers, wrapped in Parafilm (Pechiney Plastic Packaging Company) to create a membrane. The dishes were then filled, using a 20G needle and syringe, with green tinted sucrose solution, 40ml H_20 (reverse osmosis) / 12g cane sugar / 50ml neon green food coloring (McCormick & Co., Inc.) / x µl scent, and sealed with melted paraffin wax. Scent concentration varied between treatments due to differences in odor intensity (see table 3).

Table 3. List of odors used in conditioning experiments with differences in applied odor concentration for both the conditioning solution and the SPLAT. These differences reflect adjustments for odor intensity. The extracts are less intense than the isolated volatiles and are set at higher concentrations.

Odor	Lines of SPLAT	Solution Odor Concentration	SPLAT Odor Concentration
Vanilla Extract	2	100µl	40µl
Banana Extract	2	100µl	20µl
Almond Extract	2	100µl	20µl & 3µl
Anisole	1	20µl	20µl
Anisaldehyde	1	3µl	20µl

Between eight and fifteen psyllids were released onto each dish, which was placed inside a larger 10 cm plastic petri dish, and was left in isolation in an incubator for 24 hours at 26 ± 2°C, with a 14hr light/10hr dark cycle. The number of psyllids per dish in this phase was not kept exact because it was an overestimation of the numbers actually needed in phase two.

3.3.2 Behavior Testing Phase

The behavioral platform consisted of dishes prepared similarly to the conditioning apparatus; however the membranes were left empty (figure 1.4). Using a syringe and a 20G needle, two intersecting lines of SPLAT were applied to the Parafilm membrane covering the new dish. Vanilla, limonene, eugenol, anisole, and anisaldehyde were prepared using one line of SPLAT. All SPLAT was tinted with neon green food coloring (6µl/10ml SPLAT) and was mixed with a vortex a minimum of two minutes, until the color was evenly dispersed.

Of the original 8-15 psyllids released per dish in the conditioning phase, only five were released onto each dish, which was placed inside a larger 10 cm plastic petri dish, and was left in isolation in an incubator for 2 hours at 26 ± 2°C. Excess psyllids that did not die overnight were rereleased onto outdoor colonies.

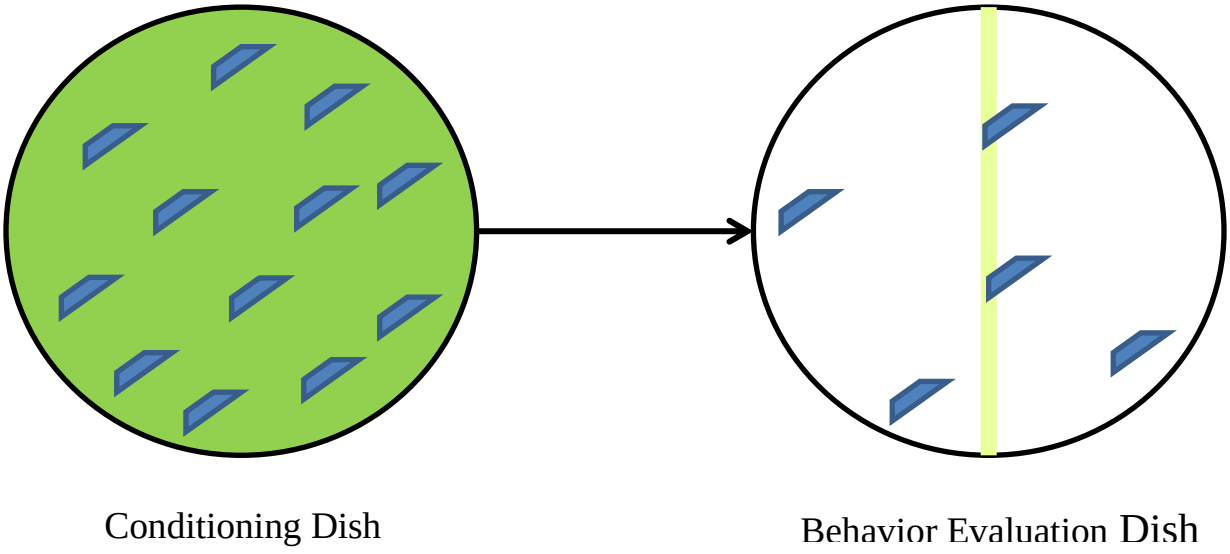


Figure 1.4 Visual description of the transition between conditioning dishes and behavior evaluation dishes. The conditioning dish filled with green solution. There are 8-15 ACP released on each dish in the conditioning phase, as indicated by small blue quadrilaterals. During the transition to the behavior dish, 5 of the original ACP are moved to an empty dish prepared with one line of SPLAT.

The behavior platform was prepared differently when testing almond extract (figure 1.5). Conditioning was tested with two concentrations, 3µl and 20µl / 10ml SPLAT. There were no unscented SPLAT treatments. For all conditioning tests, probing was evaluated using the same method described for the innate response tests.

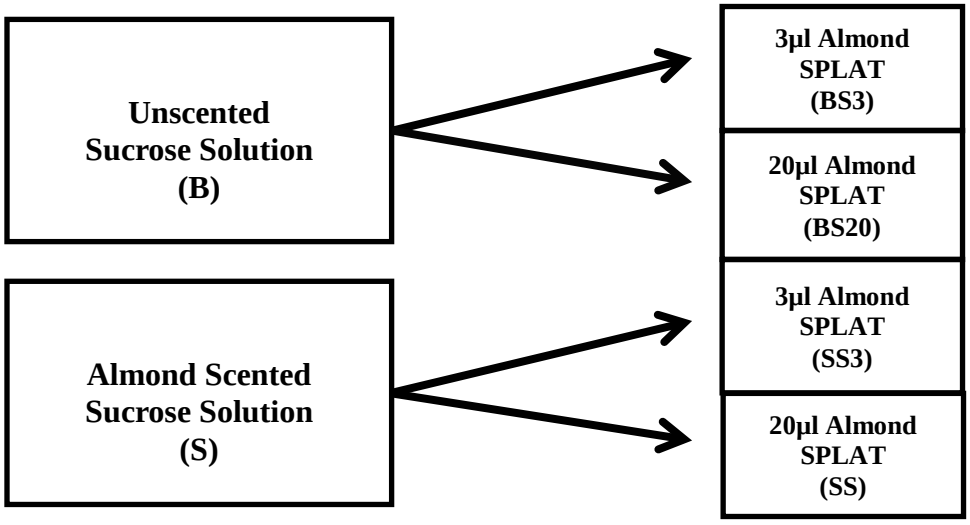


Figure 1.5 Diagram of the conditioning and behavior testing protocol used with almond extract. The conditioning phase is the same as in all other conditioning tests; ACPs are exposed to either unscented sucrose solution or almond scented sucrose solution. The behavior testing phase, however, does not incorporate unscented SPLAT. All ACP are exposed to almond scented SPLAT at either the 3µl or 20µl concentration.

3.4 Compound Stimulus Test

A factorial design was used to analyze interactions between vanilla and banana in eliciting probing behavior. All ACP were exposed to vanilla scented sucrose solution during the condition phase. The procedures were performed as previously described. During the behavior testing phase, ACP were tested on six different SPLAT preparations: blank, 40µl banana, 40µl

vanilla, 40µl 1banana:1vanilla, 40µl 3banana:1vanilla, 40µl 1banana:3vanilla (figure 1.6). All treatments on the behavior platform were set up using one line of SPLAT.

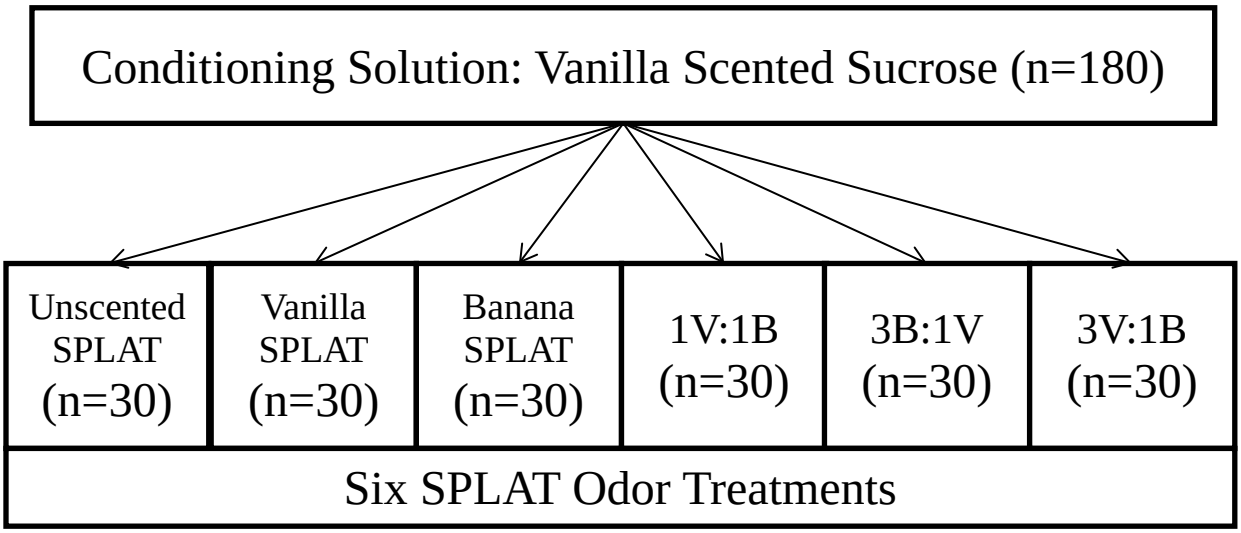


Figure 1.6 Diagram of the conditioning and behavior testing protocol in the environmental noise test. Note that all ACP were exposed to the same conditioning solution. All scented SPLAT treatments were prepared with 40µl scent/ 10ml SPLAT.

3.5 Visual Learning

In an attempt to identify visual learning, ACPs were introduced to a similar but modified conditioning protocol. During the conditioning phase, 6-20 ACP were confined to dishes containing the conditioning solution: blue (McCormick food coloring; 50µl /40ml H_2O), blue with sugar (12g/40ml H_2O), blue with scent (MS 5; 2µl/ 40ml H_2O), or blue with sugar and scent (12g sugar + 2µl scent /40ml H_2O).

The scent MS5 was a mixture of common citrus volatiles found to be an innate stimulus using the dose response test previously described. The ACPs were exposed to the conditioning solution for 24 hours in an incubation chamber to give the ACP adequate time to feed. After 24hrs, the ACPs were transferred to dishes with SPLAT stained with the same blue food

coloring. All dishes were prepared with one line of SPLAT tinted with 50 µl dye/ 5ml SPLAT. All SPLAT was unscented, isolating color as the only stimulus prompting probing behavior. As a control, 1/3 of the dishes from all four conditioning treatments were prepared with one line of neon green SPLAT (50 µl dye/ 5ml SPLAT). These dishes were distributed randomly in the incubator across all treatments (figure 1.7). Unlike the previous experiments, only one psyllid was transferred per dish due to high overnight mortality.

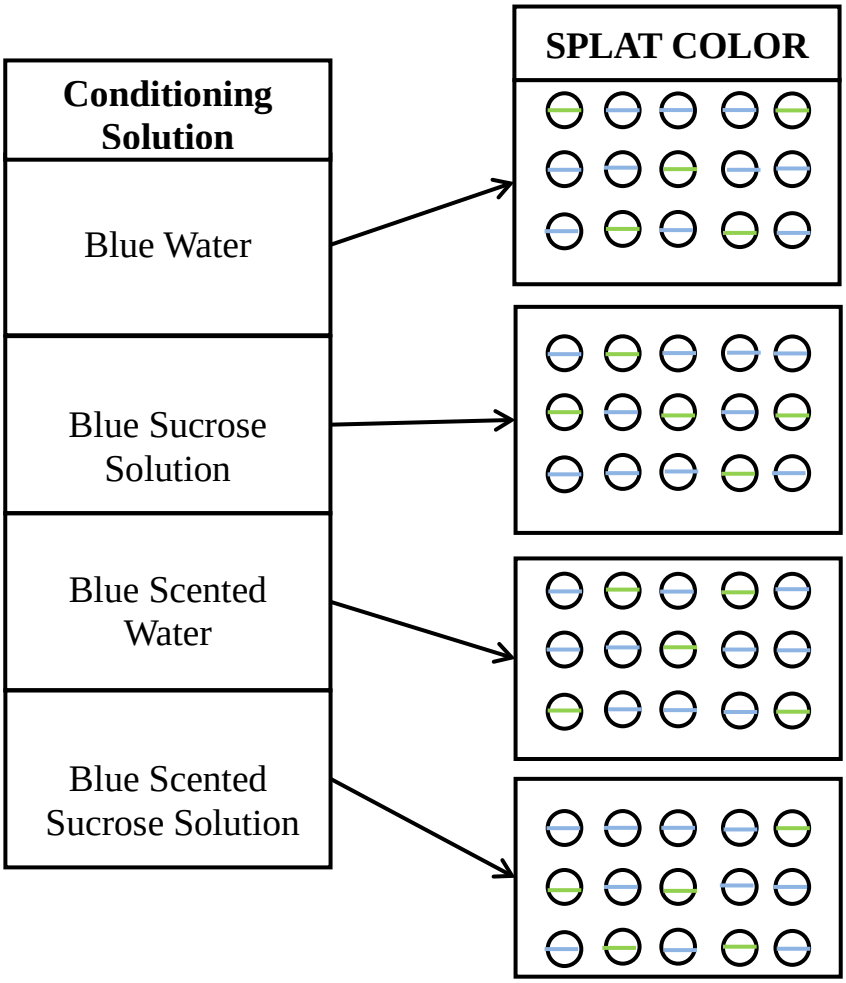


Figure 1.7 Diagram showing the random placement of control dishes relative to the experimental dishes in the visual learning experiment. Experimental dishes are depicted as circles (on the right) with a blue line running across horizontally. Control dishes are depicted as circles with a green line. All SPLAT was left unscented. Only the effect of color was evaluated in the behavior testing phase.

3.6 Statistical Analysis

Analysis of Variance was used to test for differences in the number of probe spots with changing volatile concentration (0µl, 3µl, 9µl, 30µl, 90µl). When ANOVA yielded significant F values, planned comparison t-tests were used to look for significant differences between individual pairs of treatments, where $\alpha = 0.05$ divided by the number of treatments.

Using planned analyses considerably reduces the alpha level, making it more difficult to reach statistical significance (see table 4). Conditioning data was evaluated with paired samples t-tests. Environmental noise test data were evaluated with one-way ANOVA (α: 0.05) and paired samples t-tests (α: 0.05). All statistics were calculated with Microsoft Excel 2007. Graphs were produced with SigmaPlot 10.0.

Table 4. Alpha level categories for paired-samples t-test analysis. Each block has a designated α level derived from (α: 0.05) / number of treatments.

F3: α is 0.025	F1: α is 0.0125	F2: α is 0.008
unscented/scented	unscented/3µl	3µl/9µl
	unscented/9µl	3µl/30µl
	unscented/30µl	3µl/90µl
	unscented/90µl	9µl/30µl
		9µl/90µl
		30µl/90µl

CHAPTER IV

RESULTS

4.1 Innate response

Of the eight odorants evaluated for innate response, three elicited true neutral response, two elicited a decrease in response, and three elicited an increase in response to at least one concentration treatment. The number of probe spots did not differ significantly among treatments in vanilla scented SPLAT, $F(4,145) = 0.05$, $p = .995$ (figure 2.1), anisole scented SPLAT, $F(4,220) = 1.73$, $p = .145$ (figure 2.2), or anisaldehyde scented SPLAT, $F(4,145) = 2.263$, $p = 0.065$ (see figure 2.2).

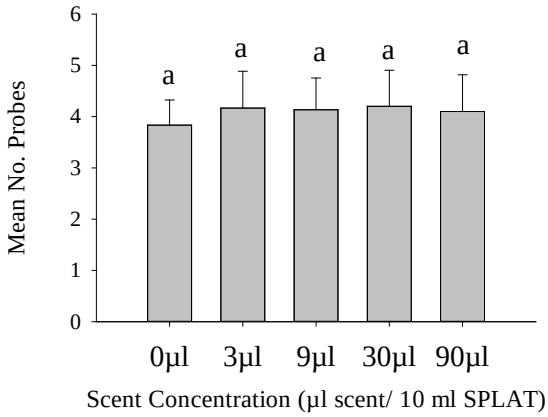


Figure 2.1 Innate probing response to vanilla extract scented SPLAT. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT at each odor concentration (x-axis). Different letters indicate statistically significance differences.

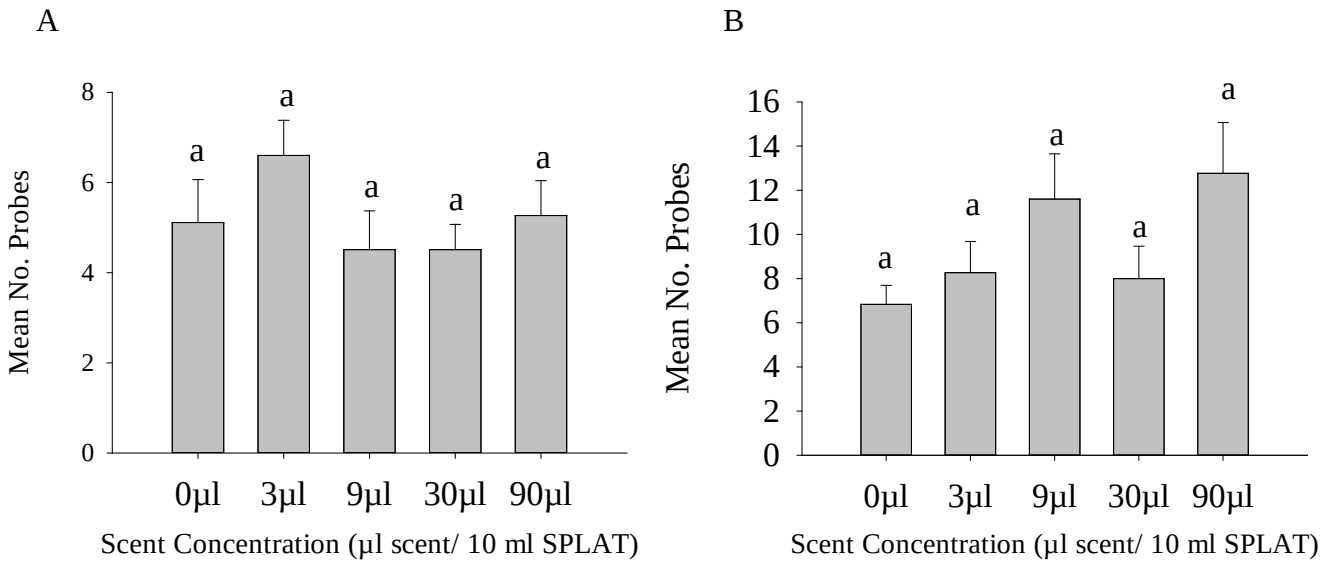


Figure 2.2 Innate probing response to anisole (A) and anisaldehyde (B) scented SPLAT. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT at each odor concentration (x-axis). Different letters indicate statistically significance differences.

There was a significant difference among treatments in almond scented SPLAT, $F(5,174) = 4.045$, $p = 0.002$ (figure 2.3). Planned analyses showed significantly more probes on 3µl scented SPLAT compared to unscented SPLAT ($p < 0.001$).

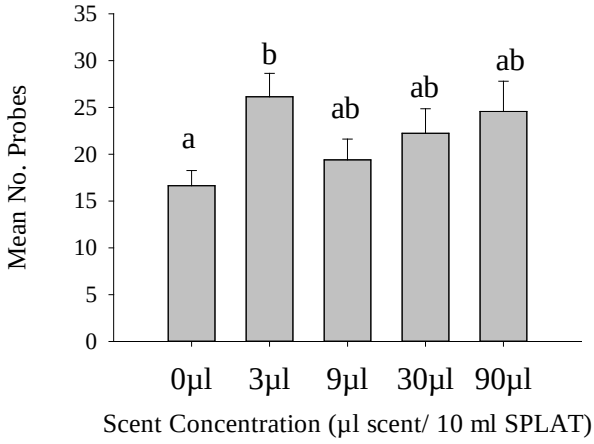


Figure 2.3 Innate probing response to almond extract scented SPLAT. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT at each odor concentration (x-axis). Different letters indicate statistically significance differences.

There was a significant difference among treatments in banana scented SPLAT, F (4,143) =3.821, p=0.006 (figure 2.4). Planned analyses showed significantly fewer probes on 9µl and 30µl scented SPLAT compared to unscented SPLAT (p=0.001).

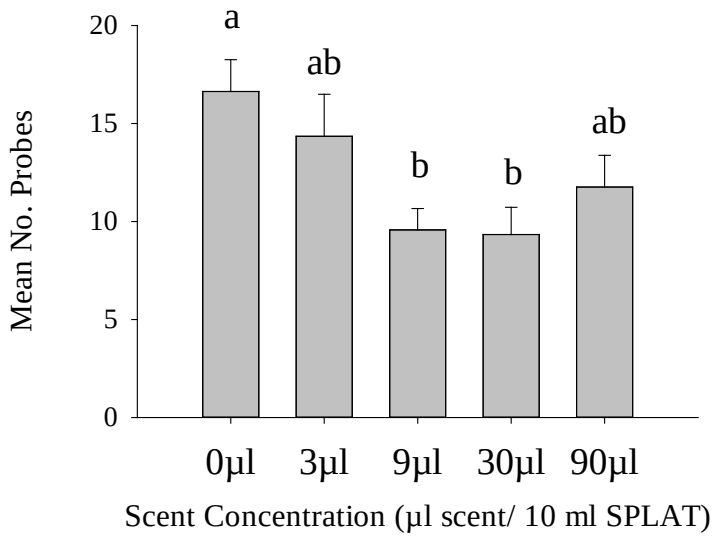


Figure 2.4 Psyllid probing response to banana extract scented SPLAT. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT at each odor concentration (x-axis). Different letters indicate statistically significance differences.

There was a significant difference among treatments in limonene scented SPLAT, F (4, 70) =4.952, p=0.001 (figure 2.5, A). Planned analyses showed significantly more probes on 9µl (p=.0004), 30µl (p=.008), and 90µl (p=.002) scented SPLAT compared to unscented SPLAT. Limonene scented SPLAT at 9µl and 90µl concentrations had significantly more probes than 3µl limonene scented SPLAT ($p<0.01$).

There was also a significant difference among treatments in menthol scented SPLAT, F (4,145) =10.296, p=0.0001) (figure 2.5, B). Planned analyses showed significantly more probes on 9µl (p=.001) and 30µl (p=.006) scented SPLAT compared to unscented SPLAT. There were

significantly fewer probes on 3µl and 90µl scented SPLAT compared to 9µl and 30µl scented SPLAT ($p<0.008$).

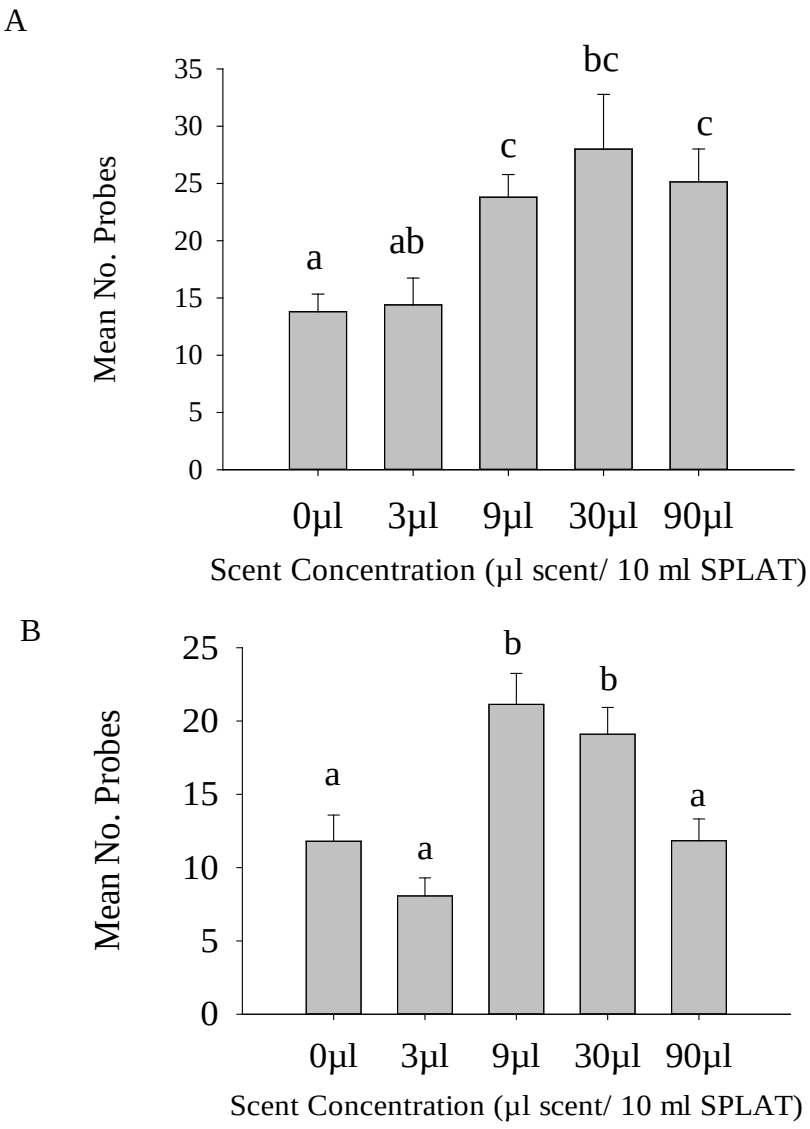


Figure 2.5 Psyllid probing response to limonene (A) and menthol (B) scented SPLAT. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT at each odor concentration (x-axis). Different letters indicate statistically significance differences.

There was a significant difference among treatments in eugenol scented SPLAT, $F(4, 70) = 7.805$, $p=2.92E-05$) (figure 2.5). Planned analyses showed significantly fewer probes on 9µl

(p=.001) and 30µl (p=.00003) scented SPLAT compared to unscented SPLAT. There were significantly more probes on 3µl (p=0.002) and 90µl (p=0.001) scented SPLAT compared to 9µl and 30µl scented SPLAT.

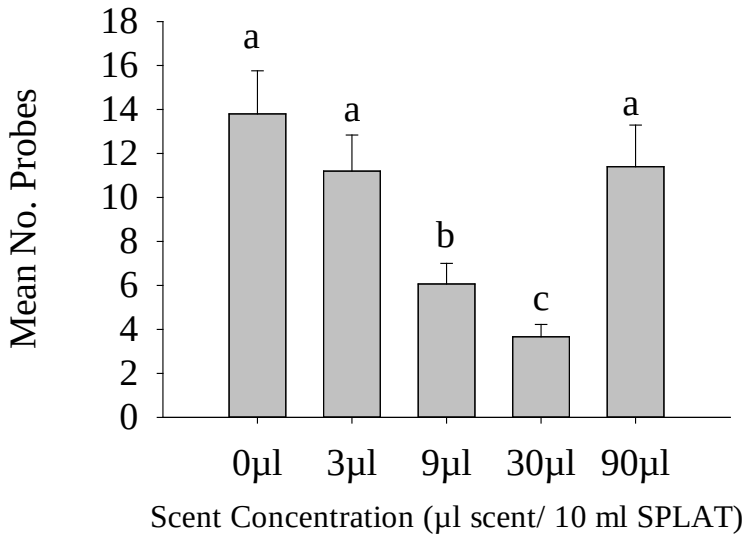


Figure 2.6 Psyllid probing response to eugenol scented SPLAT. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT at each odor concentration (x-axis). Different letters indicate statistically significance differences.

4.2 Classical Conditioning

Five odors were investigated for conditioning using a two-day protocol. The first day the insects were exposed to a sucrose + odor solution. On the second day, conditioning was evaluated by quantifying the insects' probing response to scented SPLAT. Odors were tested for conditioning if they failed to yield a positive innate response in previous testing. There was no difference among treatments after conditioning to banana extract ($p>0.39$; α: 0.025), anisole ($p>0.18$; α: 0.025), anisaldehyde ($p>0.87$; α: 0.025), or limonene ($p>0.05$; α: 0.025); (figure 2.7).

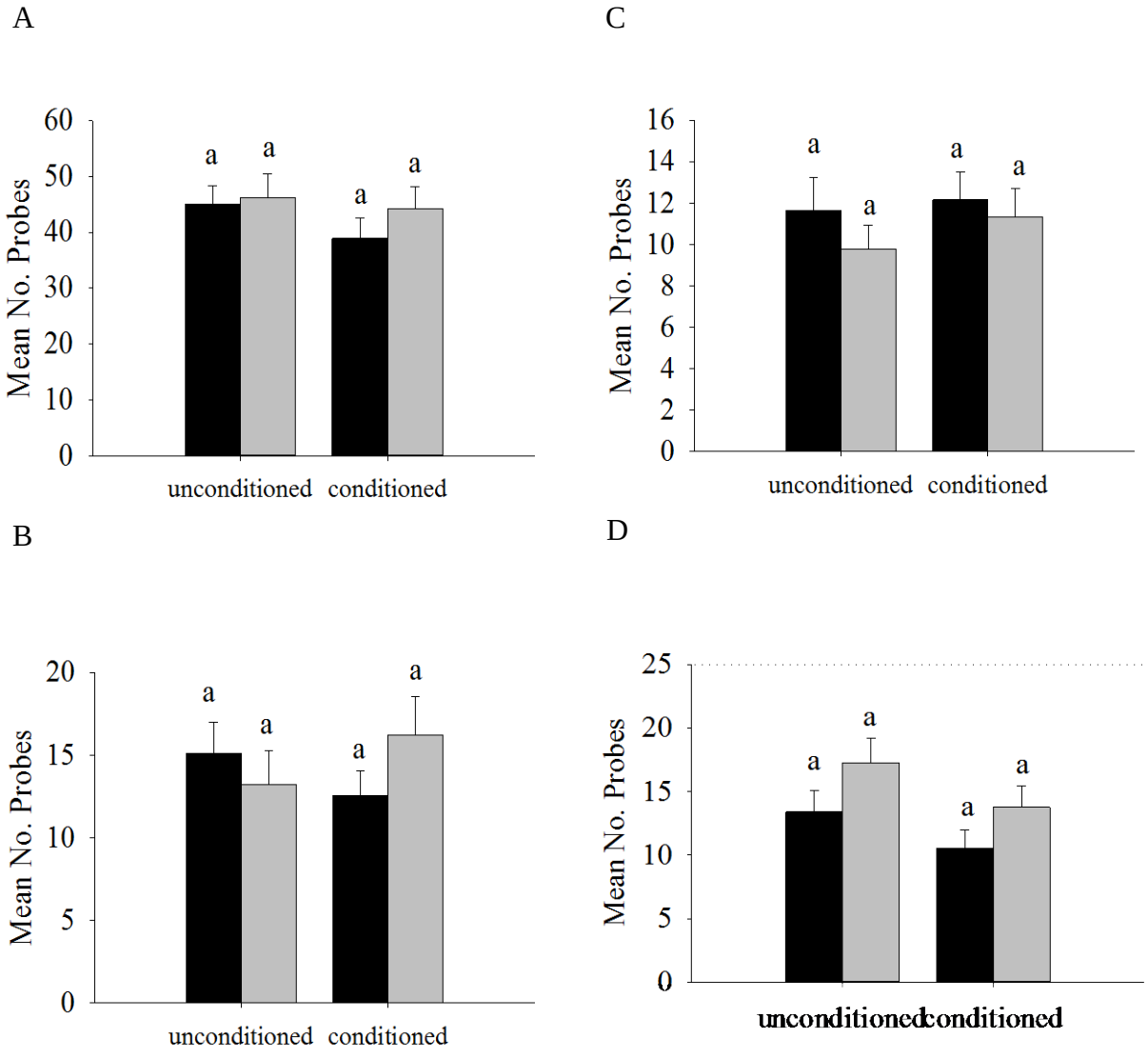


Figure 2.7 Psyllid probing response to SPLAT scented with banana extract (A), anisole (B), or anisaldehyde (C), limonene (D). Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT for each conditioning treatment. All ACP were exposed to the listed (x-axis). Different letters indicate statistically significance differences.

There was a difference among treatments after conditioning to vanilla extract ($p<0.0002$; α: 0.025) (figure 2.8). Vanilla scented SPLAT probed by psyllids exposed to the conditioning solution (treatment SS) had significantly more probes than the BB, BS, and SB treatments ($p=2.84E-06$; α: 0.0167). There was no statistical difference between BB, BS, and SB treatments ($p>0.6$).

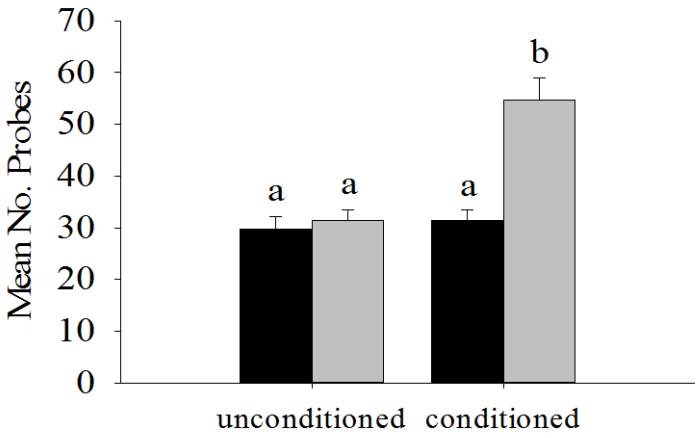


Figure 2.8 Psyllid probing response to SPLAT scented with vanilla extract. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT for each conditioning treatment. All ACP were exposed to the listed (x-axis) conditioning treatments for the previous 24 hours. The black bar represents unscented SPLAT. The gray bar represents scented SPLAT. Different letters indicate statistically significance differences.

There was a difference among treatments after conditioning to almond ($p<0.01$; α:0.025), where scented SPLAT at the 3µl concentration had significantly more probes than 20µl SPLAT with and without prior exposure to almond scented sucrose solution (figure 2.9). Treatment BS3 yielded significantly more probes than BS20 ($p<0.01$; α: 0.025). Treatment BS3 yielded significantly more probes than SS20 ($p<0.007$; α: 0.025). There was no statistical difference between BS3 and SS3 ($p>0.9$; α: 0.0167), or BS20 and SS20 ($p>0.7$; α: 0.0167).

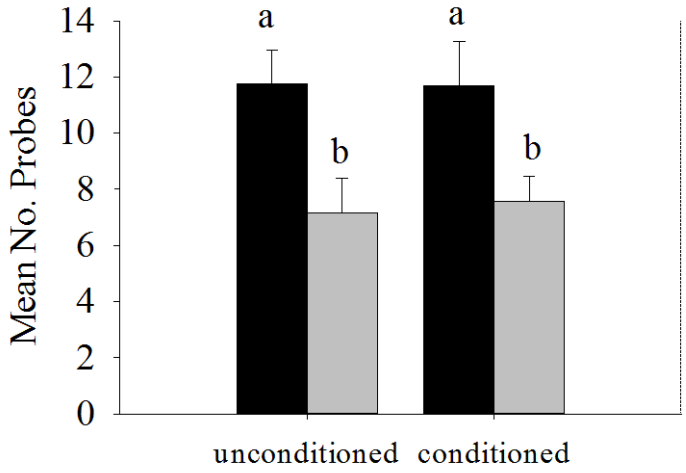


Figure 2.9 Psyllid probing response to SPLAT scented with almond extract. Bar graph data represents mean ± SEM number of probe spots on lines of SPLAT for each conditioning treatment. All ACP were exposed to the listed (x-axis) conditioning treatments for the previous 24 hours. The black bar represents unscented SPLAT. The gray bar represents scented SPLAT. Different letters indicate statistically significance differences.

4.3 Compound Stimulus Test

Vanilla and banana were investigated for interactive effects at different ratios. All psyllids were exposed to vanilla scented sucrose solution prior to testing. One way-ANOVA showed significant differences among treatments, $F(5,173) = 6.451$, $p<0.0001$ (figure 3.1). Pair-wise comparisons showed fewer probes on unscented SPLAT compared to banana scented SPLAT ($p<0.005$), vanilla scented SPLAT ($p<0.0001$), 1banana:1vanilla SPLAT ($p<0.0001$), or 1banana:3vanilla SPLAT ($p<0.03$). Banana scented SPLAT had significantly fewer probes than vanilla scented SPLAT ($p<0.01$). Vanilla scented SPLAT had significantly more probes than 1banana:1vanilla SPLAT ($p<0.03$), 3banana:1vanilla SPLAT ($p<0.0005$), or 1banana:3vanilla SPLAT ($p<0.04$). There were significantly more probes on 1banana:1vanilla SPLAT than

3banana:1vanilla SPLAT ($p<0.03$). There were no differences between 1banana:3vanilla SPLAT and 3banana:1vanilla SPLAT ($p>0.1$) or 1banana:1vanilla SPLAT ($p>0.7$).

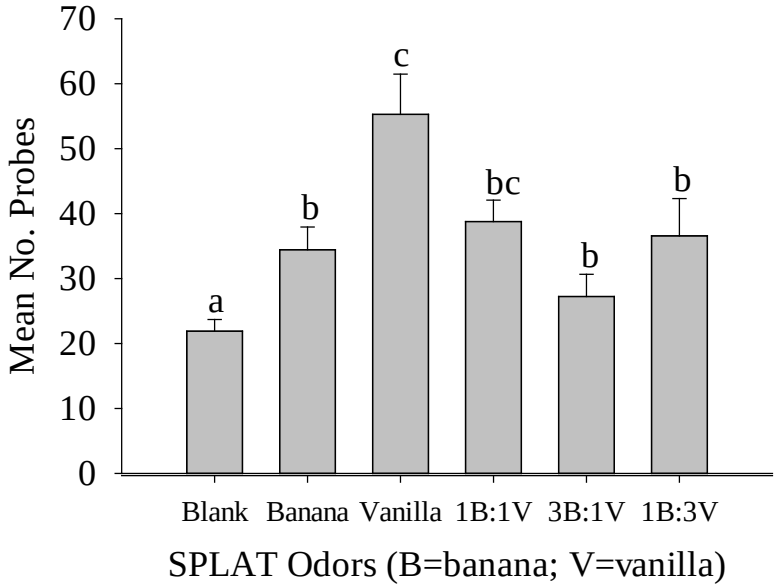


Figure 3.1 Relative rates of probing for six SPLAT treatments – different ratios of vanilla and banana extract - (n=30) following conditioning to vanilla-scented sucrose solution. Data represents mean ± SEM number of probe spots on lines of SPLAT. Sample size refers to each treatment for every odor tested. Different letters indicate statistically significance differences.

4.4 Visual Learning

Psyllids were evaluated for changes in behavior following twenty-four hour exposure to a non-stimulatory color, blue. One way-ANOVA showed a significant difference between treatments, $F(3, 36) = 4.694$, $p=0.0072$. Paired samples t-tests showed the scented blue solution produced significantly more probes than blue sucrose solution ($p<0.05$), but that scented blue sucrose solution produced significantly more probes than plain blue ($p<0.05$) or blue sucrose solution ($p<0.005$) (figure 3.2). There were no significant differences among the control dishes (neon green SPLAT) prepared with ACP from each experimental treatment (figure 3.2).

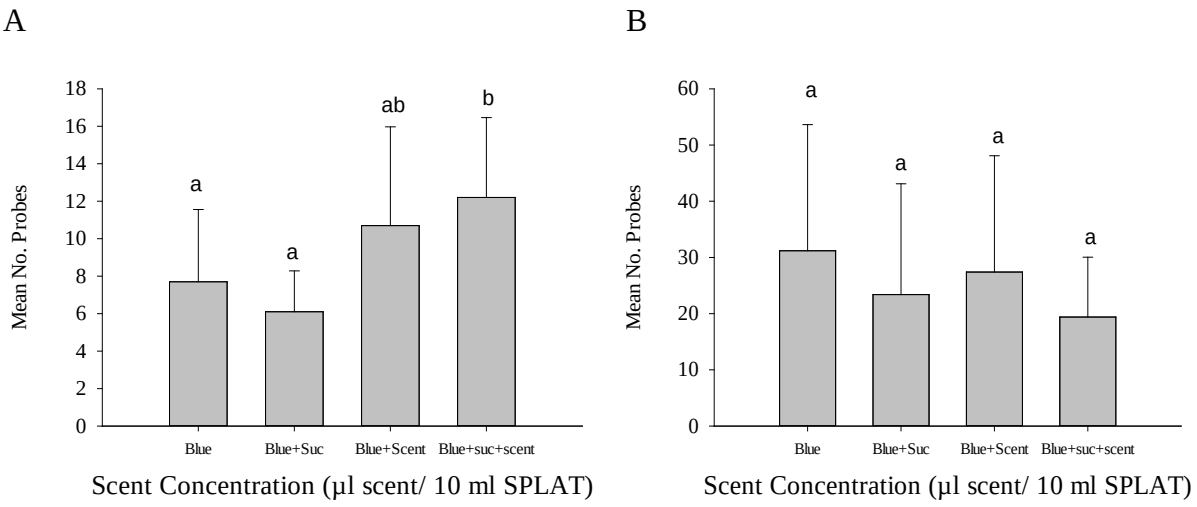


Figure 3.2 Mean ± SEM number of probe spots on lines of blue SPLAT, (A) (n=10) and green SPLAT, (B) (n=5) for each conditioning treatment. The ACPs were exposed to the treatment listed (x-axis) for the previous 24 hours. Different letters indicate statistically significance differences.

Overnight mortality increased significantly due to treatment. ACP exposed to unscented blue water had fewer survivors than ACP exposed to blue sucrose, blue scented, or blue scented sucrose solution $F(3,36)=11.984$, $p<0.0001$ (figure 3.3).

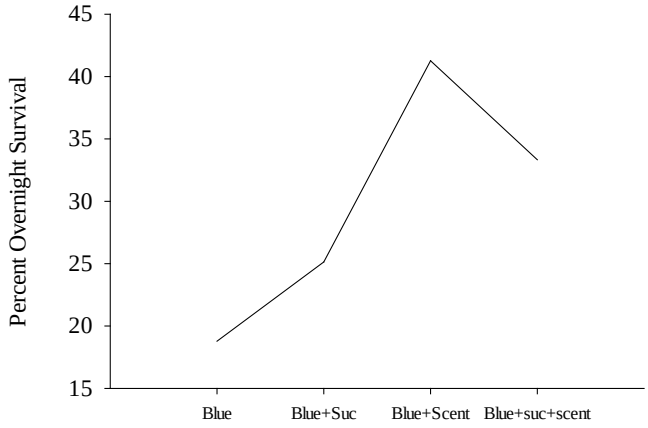


Figure 3.3 Line graph showing percent overnight survival rates for ACP exposed to different conditioning treatments.

CHAPTER V

DISCUSSION

5.1 Innate Response

Dose response curves for the eight compounds under investigation showed differential response to odor and concentration. Vanilla extract did not elicit an innate response from *D. citri.* Neither did anisole (methoxybenzaldehyde) nor anisaldehyde (4-methoxybenzaldehyde), two odors with molecular structures closely resembling vanilla extract's primary constituent, vanillin (4-hydroxy-3-methoxybenzaldehyde). These three compounds may be considered true neutral stimuli.

Banana extract and eugenol, two structurally unrelated compounds, yielded a decrease in response. While eugenol shares a benzene ring, an alcohol group, and a methoxy group with vanillin; it also has a three carbon chain instead of an aldehyde group. Isoamyl acetate, the primary component in banana extract however, is a poly-carbon chain fruit ester, structurally unrelated to the other chemicals tested. In the case of both odors, the response level decreased at 9µl, stayed in decline at 30µl, but increased again at 90µl. One possible explanation is that the receptors for these two chemicals became "washed out" at such a high concentration, meaning that the ACP went into sensory overload above a certain concentration

The last three odors, almond extract (benzaldehyde), limonene, and menthol were all positive stimuli, increasing ACP response. While all three compounds share an aromatic ring, their structures differ significantly. Almond extract elicited a response only at the lowest

concentration (3µl). This was the only odor that elicited a response at this concentration. Menthol and limonene show an inverted pattern of dose dependent response relative to banana extract and eugenol. Response level increased at 9µl and declined again at 90µl. This suggests that the optimal concentration is approximately 30µl volatile odor per 10ml SPLAT. Qualitatively, this is a fairly strong odor, depending on the compound, however perception of odor intensity at a given concentration varies from odor to odor.

5.2 Classical Conditioning

Based on the innate response results five odors were chosen for the conditioning trials. Vanilla extract, anisole, and anisaldehyde were chosen for conditioning because they performed as neutral stimuli in the innate response tests. While there was no evidence of conditioning to anisole and anisaldehyde, vanilla extract showed a strong increase in response after conditioning.

Limonene, an odor present in ACPs natural environment, was chosen for conditioning in an attempt to investigate whether high innate response to an odor can be further augmented by conditioning. There was no evidence of conditioning to limonene, suggesting that in cases with high baseline rates of response, there is a maximum threshold of response that cannot be exceeded by conditioning.

Banana extract was chosen for a similar reason to limonene. Since banana extract caused a decrease in response, conditioning trials were run in an attempt to show whether conditioning can improve the response to odors ACP naturally find aversive or perhaps just non-appetitive. There was no evidence of conditioning to banana extract. The primary component in banana extract is a fruit ester called isoamyl acetate and is fairly ubiquitous in nature. A low level of probing behavior in the presence of isoamyl acetate, despite pairing with an unconditioned

appetitive stimulus (sucrose), may reflect a mechanism by which selective feeders refuse food sources. Further research is needed to investigate the neurobiological mechanisms responsible for the decrease in probing response, akin to repellency, in ACPs. There are two immediate possibilities: either reward neurotransmitters are blocked upon exposure to non-citrus odors profiles, or contact with those odors may initiate an inhibitory neurotransmitter cascade limiting both behavior and reward to behavior.

The low-dose-dependent response to almond extract was investigated further through conditioning as well. Trials with almond extract attempted to show that baseline levels of dose specificity can be expanded by conditioning, meaning that doses that did not elicit innate probing may do so after conditioning. However, the results showed that almond extract only elicited responses at low concentrations regardless of the conditioning treatment. Almond extract does not appear to be involved in the mechanisms involved in ACP olfactory learning.

This information leaves many questions. Why can ACP learn to associate vanilla with a food source but not anisole or anisaldehyde? The molecular structure of vanillin, which includes three functional groups (an aldehyde, a methoxy, and an alcohol), may coordinate to stimulate the neural mechanisms necessary for learning. Further research is needed to clarify the exact reasons for this phenomenon.

5.3 Compound Stimulus Test

A gradient test was designed to demonstrate the effect of competing stimuli on learning. A compound stimulus was created through the introduction of banana extract, a negative stimulus, into SPLAT after conditioning the psyllids to vanilla extract. The amount of banana extract was varied to create several scented treatments. There was decreased probing with

increased amounts of banana extract. This may indicate that olfactory "noise" disrupts the conditioned response after exposure to the conditioning protocol. In psychology, a similar phenomenon called external inhibition is well documented. External inhibition is the decrease in conditioned response to the conditioned stimulus observed when a second, often neutral, stimulus is introduced. It is possible that the decrease in conditioned response to vanilla extract is an example of this phenomenon, whereby banana extract is acting as the inhibitory stimulus.

It must be noted that this portion of the study revealed some results that cannot currently be explained. The results showed a statistically significant increase in probing on banana scented SPLAT relative to the control. Being that the ACP were not conditioned to banana, and since it is known from previous innate response data that ACP respond neutrally to banana extract, we would have expected a similar neutral response to banana even after conditioning to vanilla. However, there was an increase probing, as if the vanilla in some way primed the ACP to respond to novel, non-innate stimuli. This is particularly curious because the molecular structures of the fruit esters, which comprise banana odor, compared to vanillin, which is an aromatic, are unrelated structurally and would most certainly bind to different sensory receptors types.

The other counterintuitive observation was the dissimilarity of the 1banana: 3vanilla treatment to the pure vanilla treatment. While the 1banana:1vanilla treatment showed statistical similarity to vanilla, the alternative treatment with a higher concentration of vanilla did not. This potentially contradicts the external inhibition idea. If external inhibition were occurring, we would expect a consistent decrease in response relative to concentration of vanilla. Both this quirk and the elevation in response to pure banana extract following conditioning to vanilla deserve attention in future studies.

5.4 Visual Learning

In trials in which ACPs were conditioned to the color blue, there is sufficient evidence that ACPs can learn to associate a non-innate visual stimulus with biologically significant stimuli. This experiment attempted to condition ACPs to several different blue solutions since there was no pre-existing knowledge about what type of stimulus would most effectively serve as an unconditioned stimulus. We attempted conditioning with both sucrose and a stimulatory scent derived from citrus volatiles. The data suggests that scent was a more powerful unconditioned stimulus than sucrose, but that the combination of the two was even more effective at inducing a conditioned response. Further work is needed to understand the limits of visual learning in the Asian citrus psyllid. Previous data suggests that magenta might also be a color suitable to visual learning in ACPs.

5.5 Conclusions

The present study is the first to demonstrate visual learning and the second to demonstrate olfactory learning in the family *hemiptera*. Psyllids can not only learn to associate a nutritive, biologically, significant stimuli (sucrose) with a non-innate chemosensory cue (vanilla extract); they can discriminate between molecularly similar compounds (anisole and anisaldehyde). There is, in addition, preliminary evidence of visual learning in ACPs, where multimodal stimulation produces the most significant response.

The current study also demonstrated what may be an example of external inhibition. When a learned odor was presented with a learning resistant odor, the conditioned response weakened. This is particularly interesting because of the implications to phenomenon in nature. External inhibition usually occurs when there is introduction of a distraction stimulus. The

results suggest that banana extract may have performed as a distraction stimulus even at low concentrations. This raises the question, if ACPs are distracted by one additional stimulus, what would be the effect in nature of ACP learning to associate chemosensory cues with a nutritive-oviposition site?

One cautious interpretation is that ACPs possess easily disrupted learning systems. If that is the case, limited learning abilities can currently be explained one of two ways: either learning was a maladaptive trait, or it may not have been necessary for survival, meaning ACPs never felt the evolutionary pressure to learn. If learning was maladaptive to ACP feeding, Hollis's proposal of the evolution of inflexibility is supported. If learning failed to develop because ACPs never needed to learn, then Dunlap's learning hypothesis of necessary environmental conditions is supported. ACPs did demonstrate the ability to learn vanilla extract, therefore the results might support Hollis's claim. If so, it is a reasonable hypothesis that while ACPs can learn, that ability is largely unused in nature, where there is an excess of olfactory noise.

Recent data on alternative ACP host-plants may support this hypothesis. There are several plants including *Amyris madrensis, Ptelea trifoliata,* and *Zanthoxylum fagara,* on which ACPs have been found to feed and oviposit, however both the health of the ACP and the successful maturation of eggs and nymphs is compromised on these plants (Sandoval, 2010). There is currently no explanation regarding ACP orientation towards unsuitable host plants. The delicacy of learning and memory in ACPs may help explain this phenomenon. If ACPs have limited or even suppressed learning systems, ACPs are less likely to associate olfactory cues with a host plant and thus revisit that host plant in the future based on those olfactory cues. Given that ACPs occasionally select a plant such as *Z. fagara* on which to feed and reproduce, a

selection that is evolutionarily maladaptive, mechanisms to reduce the likelihood of reoccurrence would be beneficial. In an insect that makes reproductively detrimental decisions regarding host plant species, there might have been an evolutionary advantage to develop mechanisms that suppress learning. These possibilities should be explored. However, so little known about ACP learning in general it might be some time before there is enough data to confidently move forward to larger studies in non-learning. A great deal of work is needed to test this early and perhaps brash claim.

One counter-point to the idea of non-learning in ACP comes from another well documented concept in psychology, biological preparedness, which is the idea that organisms are hardwired to learn some stimulus-stimulus associations more readily than others. A classic example of biological preparedness comes from work with monkeys (Seligman, 1971). Juvenile monkeys are more apt to learn to associate, via modeling by wild monkeys on video tape, a fear response to snakes compared to an inert object like a flower. Further work with rats showed that animals can be prepared to learn more than just phobic-like behaviors - they can more rapidly associate a food source with radiation sickness than a food source with electric shock (Garcia & Koelling, 1966). This demonstrates that awareness of the stimulus is unnecessary for learning in the context of biological preparedness to occur. The unconscious level of behavior is important to the relevance and potential application of such psychological concepts, which have been studied almost exclusively in relatively intelligent animals, to neurobiologically simplified animals such as insects. However, there is a potential problem with the fundamental definition of biological preparedness, which has traditionally referred to fear associations exclusively. If there is more flexibility within the concept of biological preparedness than Seligman originally conceived, to encompass a wider range of learning circumstances, it may also apply to the

nuanced behavior reported in this study. As such, ACP might be biologically prepared to learn some stimuli, such as those found in vanilla extract, rather than others, such as those found in banana extract. The possibilities described are all worthy of further investigation.

Applications of this research may be applied to multiple areas of science. In the field of psychology there are limited examples of research that takes such an interdisciplinary approach to explain phenomena. The current research may help the psychology community study the complex processes of learning and memory as well as recognize the benefits of studying the simplified sensory systems such as that found in the Asian citrus psyllid. Evolutionary theory is similarly deprived of enough interdisciplinary approaches to evidencing hypothesized models of how traits evolved in species across time. By combining multiple areas of science, psychology, entomology, and chemical ecology, valuable inferences can be made about the origins of animal behavior and how that behavior can possibly relate to human development. However, applied agricultural science may benefit from this work most of all. Understanding the limits of ACP sensory systems and subsequent manipulation is pivotal to local and national citrus grower's efforts to eliminate *D. citri* and slow the transmission of Huanglongbing to new groves.

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APPENDIX A

APPENDIX A

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO VANILLA EXTRACT

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
blank	30	115	3.833	7.247	2.69	0.49
Vanilla 3	30	125	4.167	15.454	3.93	0.72
Vanilla 9	30	124	4.133	11.499	3.39	0.62
Vanilla 30	30	126	4.200	14.855	3.85	0.70
Vanilla 90	30	123	4.100	15.403	3.92	0.72

**Descriptive statistics for innate response data showing vanilla odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.573	4.000	0.643	0.050	0.995	2.434
Within Groups	1869.300	145.000	12.892			
Total	1871.873	149.000				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*p-value*	*F2: α is 0.008*	*p-value*		*p-value*		*p-value*
blk x v3	0.703	v3x v9	0.972	v9 x v30	0.944	v30 x v90	0.921
blk x v9	0.706	v3x v30	0.974	v9 x v90	0.972		
blk x v30	0.671	v3 x v90	0.948				
blk x v90	0.760						
F3: α is 0.05							
blank x vanilla	0.663						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX B

APPENDIX B

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO ALMOND EXTRACT

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
Blank	30	499	16.633	78.654	8.87	1.62
Almond 3	30	784	26.133	187.085	13.68	2.50
Almond 9	30	582	19.400	146.524	12.10	2.21
Almond 30	30	667	22.233	205.909	14.35	2.62
Almond 90	30	737	24.567	313.495	17.71	3.23

**Descriptive statistics for innate response data showing almond odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3356.267	5.000	671.253	4.045	0.002	2.266
Within Groups	28872.5	174.000	165.934			
Total	32228.8	179.000				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*p-value*	*F2: α is 0.008*	*p-value*		*p-value*		*p-value*
blk x a3	0.002	a3 x a9	0.048	a9 x a30	0.403	a30 x a90	0.555
blk x a 9	0.317	a3 x a30	0.286	a9 x a90	0.192		
blk x a30	0.074	a3 x a90	0.703				
blk x a90	0.032						
F3: α is 0.05							
blank x Almond	0.023						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX C

APPENDIX C

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO BANANA EXTRACT

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
Blank	30	499	16.633	78.654	8.87	1.62
Banana 3	29	416	14.345	137.734	11.74	2.14
Banana 9	30	287	9.567	36.047	6.00	1.10
Banana 30	30	280	9.333	57.954	7.61	1.39
Banana 90	29	341	11.759	78.190	8.84	1.61

**Descriptive statistics for innate response data showing banana odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1181.212	4	295.303	3.821	0.006	2.435
Within Groups	11052.9	143	77.293			
Total	12234.1	147				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*p-value*	*F2: α is 0.008*	*p-value*		*p-value*		
blk x b3	0.401	b3 x b9	0.053	b9 x b30	0.896	b30 x b90	0.263
blk x b 9	0.001	b3 x b30	0.056	b9 x b90	0.269		
blk x b30	0.001	b3 x b90	0.347				
blk x b90	0.039						
F3: α is 0.05							
blk x banana	0.007						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX D

APPENDIX D

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO LIMONENE

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
blank	15	207	13.80	35.46	5.955	1.537
Limonene 3	15	216	14.40	81.97	9.054	2.338
Limonene 9	15	357	23.80	58.31	7.636	1.972
Limonene 30	15	420	28.00	341.57	18.482	4.772
Limonene 90	15	377	25.13	123.84	11.128	2.873

**Descriptive statistics for innate response data showing limonene odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2539.813	4.000	634.953	4.952	0.001	2.503
Within Groups	8976.133	70.000	128.230			
Total	11515.9	74.000				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*P-value*	*F2: α is 0.008*	*P-value*		*P-value*		*P-value*
blk x a3	0.832	a3 x a9	0.005	a9 x a30	0.423	a30 x a90	0.611
blk x a9	0.0004	a3 x a30	0.016	a9 x a90	0.705		
blk x a30	0.008	a3 x a90	0.007				
blk x a90	0.002						
F3: α: 0.025							
blank x Limonene	0.011						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX E

APPENDIX E

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO MENTHOL

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
blank	30	354	11.800	95.338	9.76	1.78
Menthol 3	30	242	8.067	45.306	6.73	1.23
Menthol 9	30	634	21.133	133.637	11.56	2.11
Menthol 30	30	573	19.100	99.955	10.00	1.83
Menthol 90	30	355	11.833	66.075	8.13	1.48

**Descriptive statistics for innate response data showing menthol odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3626.573	4.000	906.643	10.296	0.000	2.434
Within Groups	12769	145.000	88.062			
Total	16395.6	149.000				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1:α:0.0125	*p-value*	*F2:α:0.008*	*p-value*	*p-value*		*p-value*	
blk x m3	0.090	m3 x m9	0.000	m9 x m30	0.469	m30 x m90	0.003
blk x m9	0.001	m3 x m30	0.000	m9 x m90	0.001		
blk x m30	0.006	m3 x m90	0.055				
blk x m90	0.940						
F3: α is 0.05							
blank x menthol	0.131						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX F

APPENDIX F

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO EUGENOL

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
blank	15	207	13.8	57.6	7.6	2.0
Eugenol 3	15	168	11.2	40.314	6.3	1.6
Eugenol 9	15	91	6.067	13.067	3.6	0.9
Eugenol 30	15	55	3.667	4.667	2.2	0.6
Eugenol 90	15	171	11.4	53.542	7.3	1.9

**Descriptive statistics for innate response data showing eugenol odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1056.48	4	264.12	7.8054	2.9E-05	2.50266
Within Groups	2368.67	70	33.8381			
Total	3425.15	74				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*p-value*	*F2: α is 0.008*	*p-value*		*p-value*		*p-value*
blk x e3	0.32	e3 x e9	0.01	e9 x e30	0.04	e30 x e90	0.001
blk x e9	0.001	e3 x e30	0.0002	e9 x e90	0.02		
blk x e30	0.00003	e3 x e90	0.94				
blk x e90	0.385						
F3: α is 0.05							
blank x eugenol	0.003						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX G

APPENDIX G

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO ANISOLE

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
blank	45	230	5.111	27.328	5.2	1.0
Anisole 3	45	297	6.600	18.200	4.3	0.8
Anisole 9	45	203	4.511	22.165	4.7	0.9
Anisole 30	45	203	4.511	9.392	3.1	0.6
Anisole 90	45	237	5.267	17.973	4.2	0.8

**Descriptive statistics for innate response data showing anisole odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	131.467	4	32.866667	1.7287768	0.14462	2.412682
Within Groups	4182.53	220	19.011515			
Total	4314	224				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*P-value*	*F2: α is 0.008*	*P-value*		*P-value*		*P-value*
blk x a3	0.14	a3 x a9	0.03	a9 x a30	1.00	a30 x a90	0.34
blk x a9	0.57	a3 x a30	0.01	a9 x a90	0.43		
blk x a30	0.51	a3 x a90	0.14				
blk x a90	0.88						
F3: α is 0.05							
blank x Anisole	0.88						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX H

APPENDIX H

INNATE RESPONSE TABLES FOR ACP PROBING RESPONSE TO ANISALDEHYDE

Groups	*Count*	*Sum*	*Average*	*Variance*	*SD*	*SEM*
blank	30	205	6.8333333	22.074713	4.7	0.9
Anisaldehyde 3	30	248	8.2666667	59.857471	7.7	1.4
Anisaldehyde 9	30	348	11.6	125.83448	11.2	2.0
Anisaldehyde 30	30	240	8	64.275862	8.0	1.5
Anisaldehyde 90	30	383	12.766667	158.25402	12.6	2.3

**Descriptive statistics for innate response data showing anisaldehyde odor concentrations, the sample size for each concentration, and the corresponding statistics showing probing response.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	778.893	4	194.72333	2.2626643	0.06524	2.4340651
Within Groups	12478.6	145	86.05931			
Total	13257.5	149				

** One-way analysis of variance (α: 0.05) of innate response data looking at overall treatment effect.*

F1: α is 0.0125	*P-value*	*F2: α is 0.008*	*P-value*	*P-value*			*P-value*
blk x a3	0.39	a3 x a9	0.19	a9 x a30	0.16	a30 x a90	0.085
blk x a 9	0.04	a3 x a30	0.90	a9 x a90	0.71		
blk x a30	0.49	a3 x a90	0.10				
blk x a90	0.02						
F3: α is 0.05							
blk x Anisaldehyde	0.084						

** Paired samples t-test results systematically compared at each odor concentration.*

APPENDIX I

APPENDIX I

CONDITIONING TABLES FOR ACP RESPONSE TO VANILLA EXTRACT

Groups	*Mean*	*StDev*	*SEM*	*n*
BB	29.73	15.22	2.41	40
BS	31.35	17.18	2.72	40
SB	31.35	13.51	2.14	40
SS	54.70	27.33	4.32	40

** Elementary statistics for conditioning data showing each of the treatment groups. The first letter represents the conditioning treatment, unscented sucrose solution (B) or scented sucrose solution (S). The second letter represents the SPLAT treatment, unscented SPLAT (B) or scented SPLAT (S). Data reflects ACP probing response to SPLAT.*

F1: α is 0.0167	*p-value*	*F2: α is 0.025*	*p-value*
BB x BS	0.656	blk X vanilla	0.000
BB v SB	0.615		
BB v SS	0.000		

** Table shows t-test results systematically compared at each odor concentration.*

APPENDIX J

APPENDIX J

CONDITIONING TABLES FOR ACP RESPONSE TO ANISOLE

Groups	*Mean*	*StDev*	*SEM*	*n*
BB	15.07	10.29	1.91	29
BS	13.21	11.22	2.08	29
SB	12.55	7.97	1.48	29
SS	16.21	12.49	2.32	29

** Elementary statistics for conditioning data showing each of the treatment groups. The first letter represents the conditioning treatment, unscented sucrose solution (B) or scented sucrose solution (S). The second letter represents the SPLAT treatment, unscented SPLAT (B) or scented SPLAT (S). Data reflects ACP probing response to SPLAT.*

F1: α is 0.0167	*p-value*	*F2: α is 0.025*	*p-value*
BB x BS	0.513	blk X anisole	0.189
BB x SB	0.302		
BB x SS	0.706		

** Table shows t-test results systematically compared at each odor concentration.*

APPENDIX K

APPENDIX K

CONDITIONING TABLES FOR ACP RESPONSE ANISALDEHYDE

Groups	*Mean*	*StDev*	*SEM*	*n*
BB	11.62	10.67	1.59	45
BS	9.76	7.86	1.17	45
SB	12.16	9.10	1.36	45
SS	11.33	9.21	1.37	45

** Elementary statistics for conditioning data showing each of the treatment groups. The first letter represents the conditioning treatment, unscented sucrose solution (B) or scented sucrose solution (S). The second letter represents the SPLAT treatment, unscented SPLAT (B) or scented SPLAT (S). Data reflects ACP probing response to SPLAT.*

F1: α is 0.0167	*p-value*	*F2: α is 0.025*	*p-value*
BB x BS	0.347	blk X anisaldehyde	0.872
BB x SB	0.799		
BB x SS	0.891		

** Table shows t-test results systematically compared at each odor concentration.*

APPENDIX L

APPENDIX L

CONDITIONING TABLES FOR ACP RESPONSE TO ALMOND EXTRACT

Groups	*Mean*	*StDev*	*SEM*	*n*
BB	11.77	6.59	1.20	30
BS	7.17	6.75	1.23	30
SB	11.70	8.57	1.56	30
SS	7.57	4.94	0.90	30

** Elementary statistics for conditioning data showing each of the treatment groups. The first letter represents the conditioning treatment, unscented sucrose solution (B) or scented sucrose solution (S). The second letter represents the SPLAT treatment, unscented SPLAT (B) or scented SPLAT (S). Data reflects ACP probing response to SPLAT.*

F1: α is 0.0125	*p-value*	*F2: α is 0.025*	*p-value*
B3 x B20	0.010	3µl X 20µl	0.010
B3 x S3	0.973		
S3 x S20	0.007		
B20 x S20	0.794		

** Table shows t-test results systematically compared at each odor concentration.*

APPENDIX M

APPENDIX M

CONDITIONING TABLES FOR ACP RESPONSE TO BANANA EXTRACT

Groups	*Mean*	*StDev*	*SEM*	*n*
BB	45.05	20.97	3.32	40
BS	46.18	27.16	4.29	40
SB	38.93	23.33	3.69	40
SS	44.25	24.69	3.90	40

** Elementary statistics for conditioning data showing each of the treatment groups. The first letter represents the conditioning treatment, unscented sucrose solution (B) or scented sucrose solution (S). The second letter represents the SPLAT treatment, unscented SPLAT (B) or scented SPLAT (S). Data reflects ACP probing response to SPLAT.*

F1: α is 0.0167	*p-value*	*F2: α is 0.025*	*p-value*
BB x BS	0.836	blk X banana	0.399
BB x SB	0.220		
BB x SS	0.876		

** Table shows t-test results systematically compared at each odor concentration.*

APPENDIX N

APPENDIX N

TABLES WITH ADDITIONAL STATISTICS ASSOCIATED WITH EXPERIMENT III – ENVIRONMENTAL NOISE

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
Blank	29	635	21.90	106.31	10.31	1.79
Banana	30	1033	34.43	405.56	20.14	3.51
Vanilla	30	1658	55.27	1267.72	35.61	6.20
1B:1V	30	1163	38.77	359.29	18.95	3.30
3B:1V	30	817	27.23	384.25	19.60	3.41
1B:3V	30	1097	36.57	1089.63	33.01	5.75

** Elementary statistics showing treatment groups, sample size, and relevant statistics for the environmental noise test. Data reflects ACP probing response to SPLAT.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	19513.59	5	3902.72	6.451	1.595E-05	2.266
Within Groups	104664	173	604.994			

** Analysis of variance of environmental noise data looking at overall treatment effect.*

F1: α is 0.05	*p-value*		*p-value*		*p-value*
blk X Scented	0.002	ban x van	0.007	1B:1V x 3B:1V	0.024
blk x banana	0.004	ban x 1B:1V	0.394	1B:1V x 1B:3V	0.753
blk x vanilla	0.00001	ban x 3B:1V	0.166	3B:1V x 1B:3V	0.188
blk x 1B:1V	0.0001	ban x 1B:3V	0.764		
blk x 3B1V	0.198	van x 1B:1V	0.029		
blk x 1B3V	0.026	van x 3B:1V	0.0004		
		van x 1B:3V	0.0392		

** Table showing t-test results systematically compared at each odor ratio.*

APPENDIX O

APPENDIX O

TABLES WITH ADDITIONAL STATISTICS ASSOCIATED WITH VISUAL LEARNING

Groups	*Count*	*Sum*	*Average*	*Variance*	*StDev*	*SEM*
Blue	10	77	7.700	14.900	3.86	1.22
Blue + Sucrose	10	61	6.100	4.767	2.18	0.69
Blue + Scent	10	107	10.700	27.789	5.27	1.67
Blue + Sucrose + scent	10	122	12.200	18.178	4.26	1.35

** Elementary statistics showing treatment groups, sample size, and relevant statistics for the environmental noise test. Data reflects ACP probing response to SPLAT.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	231.075	3.000	77.025	4.694	0.007	2.866
Within Groups	590.7	36.000	16.408			
Total	821.775	39.000				

** Analysis of variance of number of probes on blue SPLAT. Data reflects ACP probing response to SPLAT.*

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	231.075	3.000	77.025	4.694	0.007	2.866
Within Groups	590.7	36.000	16.408			
Total	821.775	39.000				

** Analysis of variance of number of probes on green SPLAT. Data reflects ACP probing response to SPLAT.*

F1: α is 0.05	*p-value*		*p-value*		*p-value*
1 v 2	0.269	2 v 3	0.020	blue x green	1.30409E-06
1 v 3	0.164	2 v 4	0.001		
1 v 4	0.024	3 v 4	0.493		

** Table showing t-test results systematically compared at each odor ratio,*

BIOGRAPHICAL SKETCH

Dara G. Stockton is a native of the Rio Grande Valley and was raised in McAllen, Texas. After graduating from McAllen Memorial High School in 2004, with an adjunct diploma from the International Baccalaureate program at Lamar Academy, she attended Tulane University, in New Orleans, Louisiana.

In 2007 she received a Bachelor's of Science degree in neuroscience. Since that time she has worked at research institutions including Baylor College of Medicine, the United States Department of Agriculture – Agricultural Research Services, and Texas A&M Kingsville – Citrus Center.

Following the completion of her Masters of Arts degree in Experimental Psychology from the University of Texas – Pan American, she is relocating to Florida, where she will begin a Ph.D. program in Entomology through the University of Florida – Citrus Research and Education Center.

While completing her doctorate she intends to study the neurobiological mechanisms underlying insect olfactory learning as well as the evolutionary implications of non-learning. She will also be working on integrated pest management strategies in relation to the Asian citrus psyllid.

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