BEHAVIORAL ECOLOGY OF THE ASIAN CITRUS PSYLLID: HOW ASSOCIATIVE LEARNING INFLUENCES THE BEHAVIOR AND FITNESS OF AN INVASIVE CITRUS PEST, *DIAPHORINA CITRI* KUWAYAMA

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To Clair Louise Schiesser Stockton

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TABLE OF CONTENTS

page

CHAPTER

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

*C*Las	*Candidatus* Liberibacter asiaticus. The bacteria that causes citrus green disease.
CR	Conditioned Response. The response that is evoked upon presentation of the unconditioned stimulus. Later, after learning, the conditioned stimulus may also evoke the conditioned response.
CS	Conditioned Stimulus. The stimulus that becomes associated with the unconditioned stimulus. After repeated trials, the conditioned stimulus elicits the conditioned response.
EAG	Electroantennogram. A device used to measure the antennal response of insects to olfactory stimuli.
EPG	Electropenetrography. A device used to measure the probing patterns of insects within plant issue, or in some cases, within the tissue of an animal on which a hematophagous species is feeding.
HLB	Huanglongbing. Also known as citrus greening disease. A disease of citrus putatively caused by infection with the gram-negative alpha-proteobacteria, *Candidatus* Liberibacter asiaticus.
OJ	Orange Jasmine; *Murraya paniculata* (L.) Jack
PER	Proboscis Extension Reflex. A technique used to assess learning in some insects. The conditioned stimulus is paired with the unconditioned stimulus to elicit a response in the insect. The response is the extension of the proboscis.
US	Unconditioned Stimulus. The stimulus that naturally produces the conditioned response without training. In the Pavlovian example, dog salivation is the US.
VAL	‘‘Valencia’’ Orange; *Citrus* x *sinensis (*L.) Osbeck

Abstract of Dissertation Presented to the Graduate School
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Learning plays a role in modulating arthropod behavior. In the Asian citrus psyllid (*Diaphorina citri*), it is unclear how learning modifies behavior, although research in related taxa suggests that host finding in hemipteran vector species is likely plastic. In order to investigate how learning influences psyllid behavior, we studied associative learning in two contexts, feeding and reproduction. With regard to feeding, we studied the relative salience of olfactory and visual associations with the host plant. We found that *D. citri* is capable of visual and olfactory associative learning - both males and females learned to associate a novel stimulus with the host plant after simultaneous presentation. This suggests that psyllids associate environmental odors and appearance with the host, which is likely reinforced by successful feedings.

Next we examined whether host preference was influenced by early developmental experience. We found that host preference does appear linked to previous host plant experience, such that the natal host was preferred in lieu of alternative experience. Female psyllids also displayed learned oviposition site preference, preferring the natal host over novel species. Such host fidelity appears to

yield fitness benefits to offspring. We found that psyllid offspring were larger and developed more rapidly on the maternal host species, compared to an alternative host species.

In the last two Chapters we examined learning specifically in the context of mating and reproduction. First we investigated whether female mate choice was influenced by previous courtship experiences. We found that female psyllids modify mate choices such that orange males are more preferred than blue males after mating experience. Our data indicate that the difference between orange and blue males is likely behavioral rather than physiological. Next we investigated the associative strength of different forms of reinforcement in males. We found that males appear to differentially learn in the context of mating, where males learned a novel olfactory cue when paired with receptive females, but not in the context of food or general environmental exposure. This suggests that for males, mating and the search for females may drive host plant selection behaviors.

These results show that experience-dependent changes in behavior act as important mechanisms of adaptive plasticity in *D. citri*. Induced host preference and learned mate choice appear to directly affect fitness, benefiting reproductive efficiency and offspring survival. Furthermore, our results demonstrate the need for more regional specificity in psyllid traps and lures such that the experience of the insect is incorporated into the design.

CHAPTER 1
INTRODUCTION

A Review of Insect Learning

Most organisms possess intrinsic preferences. In the case of herbivorous insects, biologically hardwired olfactory and visual preferences guide resource detection and mate selection. However, innate responses are also readily shaped by the organism's experiences. That shaping is most commonly described as learning. Throughout this dissertation, we will refer to learning as the change in behavior for a period of time following experience. Some learning is short-term and is easily modified by subsequent experiences. In aphids, associations are made with the current host plant species are maintained for only 12 hrs (Tapia et al., 2015). In contrast, Watanabe et al. (2003) found that the cockroach, *Periplaneta americana*, demonstrated long-term retention up to four days when classically conditioned to peppermint odor. In other cases, learning has life-long effects, modifying insect behavior in the juvenile stages through the adult life span (Gandolfi et al., 2003; Barron, 2001; Matsumoto & Mizunami, 2002; Tully et al., 1994). In *Drosophila melanogaster*, for example, olfactory associations made as 3rd instar larvae, are maintained through pupation to guide adult host choice (Tully et al., 1994). There is even some evidence of epigenetic effects of learning in insects, where adult experiences are encoded via DNA methylation and histone modifications in the gametes to produce effects in the offspring (Maleszka, 2015; Yan et al., 2015; Ledón-Rettig et al., 2012). Although the field of insect learning is a relatively young science, it is increasingly apparent, based on decades of research, that insects are not only capable of learning, but can accomplish highly complicated tasks as a result.

Studies in insect learning have largely focused on four model organisms - the fruit fly *Drosophila melanogaster*, grasshoppers such as *Locusta migratoria*, parasitoid wasps like *Microplitis croceipes*, and the common honeybee *Apis mellifera*. Each taxon has provided its own unique contribution to learning theory. Fruit flies, for example, can associate olfactory stimuli 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). Larval *D. melanogaster* can do the same (Aceves-Pina & Quinn, 1979). Fruit flies are also capable of making visual associations and will avoid sources of illumination when paired with an aversive stimulus, as well as visual patterns paired with a harmful heat source (Folkers, 1982; Liu et al., 2006). Other studies have shown the importance of learning in reproductive viability. Male flies can draw associations between unsuccessful mating attempts and the female pheromones emitted during those failed experiences (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. 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). The honeybee exhibits the keenest abilities of all, 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).

For the topic of our work, we investigated the role of learning in the Asian citrus psyllid (*Diaphorina citri* Kuwayama) (Hemiptera: Psyllidae). This herbivorous insect feeds on phloem sap and transmits *Candidatus* Liberibacter asiaticus (CLas), the putative causal agent of citrus greening disease, or Huanglongbing (HLB), which threatens citrus production world-wide. As an oligophagous species, *D. citri* feeds and reproduces only on *Citrus* and closely related genera in the plant family Rutacae. The immatures feed only on young foliage, called flush, which is also to sole site of oviposition. This fact is important to the discussion of psyllid learning. Mathematical models have shown that learning is favored in species which encounter environmental unpredictability across generations (Stephens, 1993). Since flushing within its host plants may vary both spatially and temporally, learning to recognize stimuli associated with flush may be adaptive to psyllids.

Previous experiments have demonstrated that *D. citri* is capable of olfactory associative learning, where a non-host plant derived volatile was conditioned, through pairing with sucrose solution, to stimulate higher rates of feeding compared to novel presentation of the olfactory stimulus (Patt et al., 2014). Prior to that investigation, it was unknown whether *D. citri* would demonstrate the ability to learn and to what extent that ability would manifest. Due to the basic level of the initial study, and the fundamentality of the questions being addressed, it was designed to look at conditioned responses to novel odors, particularly those psyllids would rarely encounter in nature. It did not, however, investigate the natural function of learning in psyllids and uncover the sensory modalities subject to modification in the psyllid, explore the effects of learning on host choice, oviposition preference, or mate choice. The following dissertation aims to expand our knowledge about psyllid learning and ask questions regarding psyllid ecology, reproductive biology, and the mechanisms guiding associative learning in this species. It is plausible that the intrinsic preferences of *D. citri* for certain sensory stimuli are to an extent, plastic, and can be shaped depending on prior, reinforcing experiences. If so, that means that there is an area of flexibility within psyllid response to sensory stimuli that could have wide implications for how we study psyllid ecology and approach *D. citri* integrated pest management.

Mechanisms of Learning

Learning theory, which has largely been based on research with mammals, categorizes learning based on the mechanism through which a behavior is acquired. There are distinct differences in the mechanism for behavioral induction between classical and operant conditioning, both of which are forms of associative learning. Classical conditioning, also known as instrumental learning, occurs when a neutral stimulus becomes associated with a naturally evocative stimulus to produce a behavior. For example, a tone can be paired with meat to elicit salivation in a dog. The meat is the unconditioned stimulus (US), the tone is the conditioned stimulus (CS) and salivation is the conditioned response (CR). The meat (US) naturally elicits salivation in the dog (UR). Prior to learning, the tone (CS) does not elicit salivation. However, with repeated pairings, the tone (CS) elicits salivation (CR) upon presentation alone.

In contrast, operant conditioning depends on the consequence of a behavior, otherwise known as reward or punishment. B.F. Skinner’s research with pigeons showed how behaviors are readily acquired and maintained through reinforcement. Skinner devised an assay in which pigeons were required to peck an object, usually a key or a light, and as a result would receive a food-based reward. While at first the pecking behavior was random, the pigeon would eventually peck the correct object and a bit of food would mechanically drop in front of the pigeon. Very quickly the pigeon would restrict it’s pecking to the rewarding object. In this way, behavior can be modified via positive reinforcement.

Ultimately, depending on the behavior being manipulated and the organism being studied, certain mechanisms of learning are more or less applicable than others. Insect model species have demonstrated learning as defined by most of the known mechanisms. Watanabe and Mizunami (2005) replicated Pavlov's classic design using a cockroach, showing increases in salivation (CR) in response to odors (CS) after paired presentation with sucrose (US). Similarly, the desert locust, *Schistocerca gregaria*, has been shown to learn using Pavlovian mechanisms, where paired presentations of food (US) and a neutral odor (CS) led to increased maxillary movements (CR) when the odor was presented alone (Simões et al., 2011). Alternatively, operant conditioning, which is considered a more complex learning mechanism, has been repeatedly reported in honeybees (Kisch & Erber, 1999; Erber et al., 2000), the fruit fly *D. melanogaster* (Davis, 1996; Dill et al., 1993), locusts and cockroaches (Eisenstein & Carlson, 1994; Horridge, 1962).

Others have focused on non-associative learning such as habituation and sensitization. Building upon the classical habituation and sensitization work done on *Aplysia*, insect models have clarified the neurobiological mechanisms responsible for non-associative processes (Braun & Bicker, 1992; Scheiner, 2004). One study in honeybees found that low doses of imidacloprid, a neonicotinoid insecticide, were capable of inducing habituation (Lambin et al., 2001). This is particularly interesting considering the recent concern over imidacloprid as a possible cause for colony collapse disorder (Mao et al., 2013).

This brings up an important consideration when performing learning and memory research with insects. Some scientists have argued that it is often difficult to describe learning mechanisms in insects the same way we describe them in vertebrates (Papaj & Prokopy, 1989). Particularly in small insects where it is not always feasible to deliver the novel cue and the biologically significant cue separately, it can be unclear whether the biologically significant stimulus is acting as an unconditioned stimulus or a reward. As a result, some studies loosely describe the mechanism of learning as associative. This is not because entomologists are less diligent than psychologists, but because handling and performing behavioral experiments with minute insects often hinders the type of controlled associations that can be accomplished with mammals. However, it poses enough of a problem that some researchers do not attempt to describe the mechanism of learning at all, and as a result choose to use the phrase “induction of preference” to replace the word “learning.” Most often, induction of preference specifically refers to food.

We designed our experiments to test different aspects of learning throughout the following Chapters, including associative learning and induced preference. The small size and delicacy of *D. citri* makes it difficult to precisely manipulate the delivery of all stimuli in a way that can be clearly described as classical or operant conditioning. However, care will be taken to design all experiments in a way that makes certain that any shifts in behavior are due to the experimental stimulus rather than another environmental factor. Because psyllids are closely associated with the host plant, the novel stimuli will be presented simultaneously with the host plant for discrete periods of time. Afterwards, behavior will be measured and compared to naïve insects. Although this is not a traditional method of studying learning, where inter-trial intervals are typically used between repeated presentations of the conditioned and unconditioned stimulus, preliminary tests showed that a simultaneous presentation was sufficient to induce a learned response. Furthermore, this type of presentation may more accurately resemble the type of experiences psyllids encounter in nature.

Biological Constraints on Learning

Most organisms likely possess a degree of learning preparedness with regard to the innate ability to learn some cues more readily than others. Biological preparedness describes the nuance of learning phenomenon and takes into account the biological constraints of learning as it applies to each organism, which has evolved in its own unique ecological niche and inherent requirement for survival. Despite the extensive effort to study *D. citri* ecology and behavior, the extent to which psyllids are biologically prepared to respond to certain stimuli has yet to be evaluated and discussed in detail.

The original work on preparedness described how fear responses in a species were formed differentially to certain types of stimuli. Juvenile monkeys, for example, readily learn to avoid toy snakes through modeling of adult monkey behavior (Seligman, 1971). However, that same fear response is not as easily learned when the toy snake is replaced with an object such as a flower. The ability of young monkeys to quickly learn to fear a plastic snake and not a plastic flower is described as evidence that certain learned responses are evolutionarily hardwired. Traditionally, preparedness has been thought of as an evolutionary adaptation supporting survival mechanisms.

Similar work by Garcia and Koelling (1955) described the limits of organisms' ability to associate a neutral stimulus with a noxious unconditioned stimulus. Their work showed that rats with chemically induced radiation sickness are capable of associating gastric disturbance (the unconditioned stimulus) with gustatory but not visual or auditory stimuli (the conditioned stimulus). As such, rats quickly learned to avoid tastes that were paired with radiation sickness, but not sounds or objects that were similarly paired. This suggests that the ability to form an association between a conditioned stimulus and an unconditioned stimulus is not indiscriminate, but is dependent on related sensory modalities that have a common function in nature.

While most research on biological preparedness has focused on mammals, and in the context of fear responses, there is evidence of preparedness in insects, which consequently extended the originally defined notions of the role of preparedness in learning. Honey bees, for example, appear to acquire learned associations with the color violet faster than associations to blue or green (Menzel, 1967). Additionally, floral odors such as geraniol are more easily learned by bees than non-floral odors such as propanol (Mashur & Menzel, 1972).

As such, it is clear that biological preparedness can be used to describe constraints on learning in contexts other than avoidance learning Most simply, it describes the phenomenon by which the nervous system “weights” different stimuli (Menzel, 1985). If Skinner had attempted to train his pigeons, for example, to sit on a key rather than peck it, he might not have been able to show that reinforcement encourages a behavior to be repeated. Skinner exploited a naturally abundant behavior of pigeons to observe how pigeons learn. Likewise, learning research in insects requires that behaviors that are biologically relevant are taken into consideration and used appropriately. The diversity of insects can make this a particular challenge. Assays most often must be developed to suit each individual species being studied.

Most of the research on learning in insects has been performed on a small set of model species - bees (*Apis mellifera*), flies (*Drosophila melanogaster*), and parasitic wasps. As a result, very few measures of behavior have been established to study learning in insects. Studies in olfactory learning in bees and flies have used a combination of orientation behavior measured with an apparatus known as a Y-maze, and a physiological measure of olfaction, known as the proboscis extension reflex, to quantify shifts in response. Alternatively, learning in parasitic wasps has largely described learning in terms of oviposition, where previous experience has been shown to influence future host selection.

Occasionally studies on untraditional models are published which describe very detailed displays of learning and apparatus used to test such behavior. One unique approach was described in a study on antlion larvae (Neuroptera: Myrmeleontidae), which showed that antlions learn to associate certain vibrational cues with potential prey (Guillette, et al., 2009). Importantly, the antlions were studied using species-specific parameters. If the scientists had attempted to measure antlion learning using the Y-maze olfactometry, it would not have been appropriate being that antlion larvae are sedentary and do not detect prey using olfactory cues. Antlion larvae could be described as being biologically prepared to associate vibrational cues with a food source more readily than visual or olfactory cues.

Sensory Modalities and Behaviors of Interest

For these reasons, it is important while attempting to investigate learning in *D. citri* that care is taken to use measures that are biologically relevant to the ecology of psyllids. Two modalities of learning are currently of interest –visual learning and chemosensory learning. While there will be overlap between the sensory modalities, particularly in cases of oviposition learning, each will be discussed separately as independent objectives.

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). As such yellow sticky-traps are the most effective method of psyllid population monitoring (Hall & Albrigo, 2007). In experimental conditions, *D. citri* appears most attracted to colors with reflectance values approximately 500nm in wavelength, on the border between blue and green, which is similar to the color of flushing citrus leaves, the exclusive site of psyllid oviposition (Sanchez, 2008; Wenninger et al., 2009). As a result, lime green sticky traps are now used to monitor *D. citri* populations by the Texas Department of Agriculture. It is possible that *D. citri* 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 Hemipterans, such as aphids, are documented to have keen visual abilities (Doring & Chitka, 2007). The whitefly, *Trialeurodes vaporariorum,* locates its host plant entirely using visual cues (Vaishampayan et al., 1975). 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). However, Wenninger (2009) found that while *D. citri* 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 may not be surprising considering that not only are the primary volatiles emitted by citrus (terpenes) 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 (Thorsteinson, 1960; 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). It is well established that plant volatiles can both attract and repel herbivores (Visser & Thiery, 1986; Foster & Harris, 1997). For this reason, it is presumed that *D. citri*, to some extent, employ olfactory cues to aid in the detection and selection of host plants for feeding and oviposition.

Most likely, however, *D. citri* host-plant discrimination is based on complex multimodal cues including combinational visual, olfactory, and even gustatory stimuli. There is evidence that compound cues in nature 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 (Borden, 1982). A recent study on *D. citri* attraction found that visual, olfactory, and gustatory cues, when combined produced a strong increase in probing behavior (Patt et al., 2011).

One way to study the effects of complex stimuli is compound conditioning, which involves the acquisition of a learned response to multiple stimuli presented concurrently. After acquisition, each part of the compound stimulus can be studied individually to demonstrate the salience of each component. In nature, *D. citri* interact with countless chemosensory cues. Certainly, their own host plants emit highly varied and enormously complex volatile profiles. Chapter 2 specifically addresses preparedness by comparing the learned response to visual and olfactory stimuli. First we determined whether learning readily occurs in the context of visual and olfactory stimuli. Then we used compound conditioning to compare the associative strength of visual and olfactory stimuli. The Chapters thereafter take those data into account in the experimental design. In Chapter 3, olfactory associations were used as a primary method of determining host species preference. This was because we determined that olfactory cues were biologically relevant to psyllid learning. In Chapter 4, we revisit visual learning in the context of mate choice.

Within each sensory modality certain biologically relevant behaviors can be observed that may independently demonstrate the ability of *D. citri* to learn. In order to understand the extent to which learning guides *D. citri* host plant selection, each behavior must be given special attention. The first two behaviors of interest are feeding and orientation, and were described in detail previously. Both feeding and orientation are the most generalized among learning literature and are the focus of attention in almost all invertebrate models.

Feeding studies in insect models commonly employ the proboscis extension reflex (PER) to measure feeding behavior. PER involves measuring a learned response as an extension of the proboscis, or the siphoning tube of piercing-sucking insects. To do this, an odor is often paired with sucrose to stimulate behavior. However, the size of *D. citri* limits the use of PER. A novel method of studying psyllid feeding behavior was developed by Patt et al. (2011). Using this assay, referred to as the 'leaf assay' because it mimics the feeding of *D. citri* on a leaf, feeding behavior can be quantified as probe spots left on a line of emulsified wax known as SPLAT (Specialized Pheromone Lure Application Technology; ISCA Technologies™). After feeding on the SPLAT for a pre-designated amount of time, the SPLAT is stained and the probe spots are counted under magnification. More recently, much work has been done on the feeding timing and location of feeding within the leaf epithelium using electropenetragraphy (EPG).

To study olfactory orientation responses in insects, an apparatus such as a Y-maze is commonly used to evaluate changes in orientation behavior in response to experience. Throughout our experiments, the Y-maze was used to study psyllid preference. This assay consists of a glass tube with two arms that provide an odor choice. We also occasionally employed a variation on the Y-maze, known as the T-maze (Mann et al., 2012). Orientation assays involve presentation of a stimulus, usually olfactory, on one side of a two-choice glass tube. The insect is given the option of entering the control arm (unscented) or the treatment arm (scented). This method can be modified to look at orientation response to visual cues as well, where lights are used as the motivating stimulus at the distal ends of the t-maze chambers. In both olfactory and visual modalities, this assay is aided by the positive phototaxis and negative geotaxis demonstrated by *D. citri*.

We also studied the effects of learning on oviposition, the complex egg laying behavior displayed by insects. Oviposition is likely dependent on multiple types of sensory stimulation. In *D. citri*, oviposition is exclusive to the new flush shoots of citrus and related Rutacea such as *Murraya paniculata* and *Murraya koenigii* (Halbert, 2004). Surveyors have noticed rapid decline in the number of eggs laid on flush after stage 3, when the leaves begin to enlarge and elongate (Setamou et al. 2014). Odor profile and color shifts during leaf maturation are likely responsible for the decrease in selection. It is possible that beyond the innate cues that stimulate female *D. citri* to select an oviposition site, learning may encourage selection where previous odor profiles are associated with the appropriate stimulation produced by the visual appearance of young flush.

Learning in an Applied Context

Pest management is most effectively and responsibly accomplished using integrated pest management (IPM), which aims to take a thorough ecological approach to bringing pest populations below the economic threshold without causing damaging extremes in non-target and beneficial insect populations. To do this, a variety of measures are taken including biological control, which controls pest populations with natural enemies; genetic modification, which may involve increased plant resistance or pest sterilization; and environmental management (Prokopy & Lewis, 1993). These tools are key to sustaining healthy crops and increasing profitability for growers. However, another measure that has gained increased attention is the application of behavioral manipulation, specifically, learning in insects. While very few studies have looked directly at the impact of learning on pest management, the growing body of knowledge about the importance of learning ability in insects suggests that it may deserve more attention than it has previously received. In this next section, we will review the body of work on learning in pests and beneficial species affected by pest management. Then, we will discuss how our research approaches these topics with regard to psyllids and citrus in agroecosystems.

As previously discussed, learning allows insects to efficiently forage (Menzel, 1985; Carter 2004), communicate with other insects (Dyer, 2002), find mates (Ejima et al., 2005; Dukas, 2005; Yurkovic et al., 2006) and avoid potentially harmful stimuli after experience (Temple et al., 1983; Quinn et al., 1974; Aceves-Pina & Quinn, 1979; Folkers, 1982; Lui et al., 2006). In insects, the information stored during the learning process is entirely ecologically relevant to their success as a species. Without the neural plasticity of learning, insects, like all animals, would not be able to survive a dynamic environment with unpredicTable resources and threats. Such work has highlighted the important influences learning may have on many aspects of IPM. Critical components of pest management programs, such as monitoring and the application of feeding deterrents or insecticides, can be affected by learning phenomenon in both pest and beneficial populations.

One critical component of IPM, population monitoring, involves the frequent sampling of target insects and is required to determine the economic thresholds that necessitate the application of pest control measures (Whitfield & Purcell, 2013). Without accurate sampling estimates, effective pest control is not possible. Southwood (1978) suggested that sampling can be affected by many dynamic environmental factors and that sampling accuracy could be influenced by changes in insect behavior as a result of such environmental shifts. True to the authors prediction, it appears that certain sampling methods such as capture-recapture may result in learned response. Incidentally, some of the clearest examples of the impact of insect learning are seen in such situations.

Capture-recapture sampling involves is used to monitor populations by releasing a known number of marked individuals and allowing those individuals to mingle with the natural unmarked population (Begon, 1978). As a result, re-sampling should collect a number of marked and unmarked individuals at a certain ratio, which can be used to estimate the level of the natural population in the field. However, due to laboratory or forced human exposure, marked individuals are prone to behavior modifications as a result of experience with the trapping exercise.

Mallet et al. (1987) describes work with *Heliconius* (Lepidoptera: Nymphalidae) butterflies that reflects such a scenario. For some time, it was unknown why some butterflies disappeared after being captured. Previous studies had suggested that the disappearance was due to factors other than learning including increased dispersal and mortality (Singer & Wedlake 1981). However, others thought the disappearance might have been due to learned avoidance. To test those hypotheses, butterflies were captured in a net and were labeled with a serial number and rereleased. The effects of such handling were recorded as latency to return to the site of capture over a series of days. Mortality and dispersal were recorded as well. The authors concluded that the disappearance was in fact the product of learned avoidance. *Heliconius* butterflies are less likely to return to the flower on which they were foraging or their preferred roost site, up to one day after handling.

Another study looked at the effect of previous experience on the efficacy of traps used to collect *Rhagoletis pomonella* (Diptera: Tephritidae) apple maggot flies (Prokopy et al., 1990). To capture the flies, growers used red sticky spheres resembling apples with a volatile lure to bait them away from the nest. However, the traps were only effective if they closely resemble apples from orchards in nearby areas. It appears that *R. pomonella* readily acquires information about its experience with previous apples' size, color and shape and significantly prefers traps resembling such cultivars.

These two examples demonstrate how associative learning, when left unaccounted for in IPM programs, can decrease the effectiveness of pest management. Processes other than associative learning can affect IPM as well. Non-associative learning, a form of neural reflex that is acquired by experience with stimuli, can cause reductions in the efficacy chemical products in an agricultural setting such as anti-feedants. Feeding deterrents are used in agriculture to decrease the likelihood of damage to a particular crop by introducing a volatile or other olfactory/gustatory stimulus that triggers innate repellency. Recently, it has been noted that a variety of insects have demonstrated habituation to applied feeding deterrents. Habituation is a form of non-associative learning in which an organism shows a decrease in avoidance (reflexively) with repeated exposure to an aversive stimulus.

The Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabeidae), a polyphagous herbivore that can feed on more than 300 host plants, demonstrated habituation to a feeding deterrent, azadirachtin, after repeating presentation (Held et al., 2001). Azadirachtin was applied to the foliage of a preferred host plant in both choice and no-choice test situations. The results of the study showed that not only did *P.*

japonica proportionally increase feeding on the treated plants with time in choice tests, in no-choice tests, feeding on treated plants was equivalent to control plants after three days of exposure. While the authors comment on the amplified response in laboratory studies compared to field settings, it is clear that phenomenon such as habituation are important to consider and regularly monitor when anti-feedants are used in agriculture, due to the rapidly decline in efficacy as insects adapt to exposure.

Similar results have been demonstrated with other insect orders including Lepidopterans. Plant latex acts as an oviposition deterrent on naïve cabbage looper moths, *Trichoplusia ni* (Lepidoptera: Noctuidae). However, larval moths fed on an artificial diet including plant latex in the final stages before pupation show no oviposition deterrence as adults (Shikano & Isman, 2009). This indicates that the repellant effects of latex are not only habituated in larval insects, but can carry over through metamorphosis, despite dramatic neurological reorganization.

This work has forced agricultural scientists to reconsider the approach to pest management, even with the use of pesticides. Unlike feeding deterrents, pesticides are designed to kill insect that come into contact, and at initial consideration, might be considered exempt from the effects of learning. After all, if the insect dies, how can it learn anything? However, recent studies have shown that learned avoidance, in combination with genetic resistance, can dramatically reduce the effectiveness of chemical control methods due to the gradual uptake of some pesticides such as systemics, as seen at alarmingly high proportions of insecticide controlled agricultural pests, (Reierson & Rust, 2012). This is rooted in the evolutionary mechanisms that protect phytophagous insects by reducing the likelihood of feeding on toxic plants (Hagen, 2009).

Laboratory evidence of such phenomena can be recreated with the bacterial endotoxin *Bacillus thuringiensis*. While naïve insects such as the light brown apple moth, *Epiphyas postvittana* (Lepidoptera: Tortricidae) show no preference for treated and control diets, after one day moths exclusively feed on diets without *B. thuringiensis* (Foster & Harris, 1997). Similar results have been found in several other unrelated species. It appears however, that such effects are most common in insects with well-developed chemosensory systems such as Lepidoptera and Diptera (Hoy et al., 1998). It is also important to point out that such phenomena are only possible when the rate of exposure is below the threshold for other physiological effects like mortality or reduced fecundity.

Despite the situations in which insect learning can deleteriously affect pest control measures, it is important to consider the potential benefits of incorporating the effects of learning, particularly in beneficial species, into IPM strategies. Of particular interest is the potential for specialized commercially-conditioned predators. Food imprinting is a form of pre-imaginal conditioning in which information acquired during the immature stages of development shapes preferences as an adult. One recent study reported the profound effects of food imprinting in the pre-imaginal predatory mite *Neoselulus californicus* (Schausberger et al., 2010). While naïve adult mites prefer to feed on spider mites, one 24-hour forced exposure to a diet of thrips during the late immature stage resulted in decreased attack latencies and increased predation on thrips throughout adulthood. Using such techniques, it may be possible to release preprogrammed predators that target the individual pest species of concern during a particular season.

For beneficial insects, learning is one of the most important processes required for the acquisition of resources. Parasitoids, one of the most commonly imported biological control agents of invasive exotic pests, rely heavily on learned associations between the hosts plants' volatile profile and appearance to locate hosts for reproduction. As a result, parasitoid 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 *Microplitis croceipes* (Hymenoptera: Braconidae) wasps were provided with hosts in combination with different odors such as chocolate, a non-innate volatile. After pairing, the wasps were significantly more likely to seek out hosts in chocolate scented areas (Lewis & Takasu, 1990).

The abilities of *M. croceipes* have gained attention since the proposal that parasitic wasps may be used a sort of sniffing dog. Although it was first suggested that wasps may be used as an alternative to dogs as detectors for illegal products such as bombs or drugs, many studies have shown the potential benefits parasitoid wasps may provide to the agricultural community (Olsen et al., 2003). Using a device called a "wasp hound," samples of air can be rapidly evaluated by the aggregation patterns of wasps near the odor source. Most recently this technique was used to successfully identify noxious odors on hogs that make the meat unpalaTable but are usually only detected after the expensive and labor intensive de-boning process is complete (Wäckers et al., 2010).

Unfortunately, widespread use of pesticides has been shown to disrupt learning in many beneficial species, including parasitoids. Of particular concern is the disrupted navigation seen in parasitoids after pesticide exposure. In *M. croceipes*, nutritive exposure to sublethal doses of the systemic neonicotinoid insecticide, imidacloprid, causes more than a 70% reduction in successful orientation towards host odors (Stapel et al., 2000). Additionally, female *Trissolcus basalis* (Hymenoptera: Scelionidae) parasitoids spend less time on host eggs during tasting and oviposition after exposure to the pyrethroid insecticide, deltamethrin (Salerno et al., 2002). These effects may significantly impact the use of biological control as an IPM strategy. Unfortunately, the sublethal effects are rarely studied because USDA guidelines for pesticide approval currently do not require such data be evaluated (Desneux et al., 2007).

Besides parasitoids, beneficial pollinators are also deeply impacted by sublethal pesticide exposure. Due to increased concern over the causes of colony collapse disorder in domestic honeybees, studies have begun to look at the possible role of pesticides like imidacloprid on behaviors such as bee foraging. In laboratory studies using the proboscis extension reflex (PER) conditioning paradigm, honey bees consistently show reduced learning following chronic and acute imidacloprid exposure (Decourtye et al, 2003). Similar results are seen with honey bee exposure to fipronil, deltamethrin, endosulfan, and prochloraz (Decourtye et al., 2005). It appears that neonicotinoids, which are neurotoxic in insects, may reduce the ability of honeybees to make associative connections (Desneux et al., 2007). As a result, there is a significant impact on foraging efficiency that can lead to hive decline, reduced honey production, and reduced cross-pollination required by growers to produce crops.

Studies on the effect of imidacloprid on nonassociative learning in honeybees have shown that at low doses, bees not only habituate to the toxin, but that neuromodulatory insectides may inherently facilitate habituation due to their mode of action (Lambin et al, 2001). There also appear to be differential rates of habituation in bees depending on age, where older bees habituated more rapidly than younger bees (Guez et al., 2001). This is particularly important since older bees are responsible for the foraging activities of the colony.

While this demonstrates the potent harm of some pesticides on overall ecology, it has also increased the awareness of scientists, growers, and the general community of the importance of taking a holistic view of the entire ecological system when preparing pest management programs. As a result, slow but steady change is taking place. Neonicotinoids are now recommended for restriction to application windows that attempt to limit the exposure of beneficial insects by avoiding application during high foraging periods such as spring blooms (Riedl et al., 2006). Future studies may increasingly include references to the application of insect learning in pest management as it is an ecologically relevant behavior for both pest and beneficial insects in areas addressing monitoring strategies and pesticide application.

In our work with *D. citri*, we looked at two aspects of behavior relevant to learning and pest management. First, we studied the effects of learning on host plant preference and determined whether the natal host species significantly affected host preference for feeding and oviposition as adults. If psyllids predictably prefer to the host species on which they were reared, this has profound implications for current best management practices in citrus. Currently, only general citrus lures are being investigated. These are often conceived in one state and employed in another or several other states with no change in formulation depending on the place of use or grower specialty. The results have been modest at best. It is possible that lures designed to mimic the odors of plants grown regionally, such a navel orange lure for orange groves in Florida, grapefruit lures in South Texas, etc., would improve the rates of capture by capitalizing on the induced preferences of psyllids in different regions of the country.

Second, studied learned mate choice in female and male *D. citri*. We examined the physiological and behavioral differences between blue and orange phenotype males. Then we determined whether female *D. citri* show an innate or learned preference for the different male phenotypes. We also studied male response to female odor and determined whether male psyllid attraction to female odor is innate or learned. Due to increased demand for non-chemical control measures, mating disruption technology is increasingly important in IPM. There are currently efforts to develop a synthetic female for use in the field, which exploits the vibrational communication psyllids use along the plant surface. If coupled with olfactory and visual stimuli, mating disruption could be enhanced. However, it is necessary to first understand how psyllid mate choice is determined.

CHAPTER 2
ASSOCIATIVE LEARNING AND HOST PREFERENCE

Chapter Abstract

Although specialist herbivorous insects are guided by innate responses to host plant cues, host plant preference may be influenced by experience and is not dictated by instinct alone. The effect of learning on host plant preference was examined in the Asian citrus psyllid, *Diaphorina citri*; vector of the causal agent of citrus greening disease or huanglongbing. We investigated: a) whether development on specific host plant species influenced host plant preference in mature *D. citri*; and b) the extent of associative learning in *D. citri* in the form of simple and compound conditioning. Learning was measured by cue selection in a 2-choice behavioral assay and compared to naïve controls. Our results showed that learned responses in *D. citri* are complex and diverse. The developmental host plant species influenced adult host plant preference, with female psyllids preferring the species on which they were reared. However, such preferences were subject to change with the introduction of an alternative host plant within 24-48 hrs, indicating a large degree of experience-dependent response plasticity. Additionally, learning occurred for multiple sensory modalities where novel olfactory and visual environmental cues were associated with the host plant. However, males and females displayed differing discriminatory abilities. In compound conditioning tasks, males exhibited recognition of a compound stimulus alone while females were capable

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of learning the individual components. These findings suggest *D. citri* are dynamic animals that demonstrate host plant preference based on developmental and adult experience and can learn to recognize olfactory and visual host plant stimuli in ways that may be sex specific. These experience-based associations are likely used by adults to locate and select suiTable host plants for feeding and reproduction and may suggest the need for more tailored lures and traps, which reflect region-specific cultivars or predominate Rutaceae in the area being monitored.

Background Information

Although herbivorous insects rely heavily on innate olfactory and/or visual preferences to locate and select host plants (Mori, 1982; Bernays et al., 1985), innate responses may be shaped by the organism's experiences in a process known as learning (Weiss, 1997; Kelber, 2002; Riffell et al., 2008). Insects are not only capable of learning, but can accomplish highly complicated tasks as a result. Fruit flies can associate odor, color, and visual patterns with both appetitive and aversive conditions (Temple et al., 1983; Quinn et al., 1974); and grasshoppers have the sophisticated ability to learn visual stimuli associated with nutritionally valuable food (Simpson & Raubenheimer, 2000; Dukas & Bernays, 2000). Hymenopterans display some of the most sophisticated learning abilities investigated thus far. The honeybee is capable of learning and interpreting complex waggle dances to encode information about flower location (von Frisch, 1967; Menzel & Giurfa, 2001; Menzel & Muller, 1996; Dyer, 2002). Meanwhile the bumble bee, *Bombus terrestris,* is known to use spatial learning to efficiently locate and navigate flowers (Carter, 2004; Laverty, 1994) and recent work has identified observational learning and even false memories in this species (Leadbeater &

Chittka, 2007; Dawson et al., 2013; Hunt & Chittka, 2015). However, beyond the models described above, much less is known about learning in other insect taxa, particularly phytophagous Hemipterans. This is important because Hemipterans represent a diverse and threatening group of agricultural pests, many of which transmit plant disease pathogens, particularly those in the monophyletic suborder Sternorrhyncha, which includes aphids, whiteflies, psyllids and scale insects. Current models predict that within a century, the occurrence and abundance of agricultural disease vectors will spread latitudinally as climate change expands the range of migration, and across geographically isolated regions as human movement continues to perpetuate invasions by non-native species (Githeko et al., 2000; Parmesan, 2006; Watson et al., 1996).

To our knowledge, only a few species in the order Hemiptera have been investigated in the context of learning. Patt & Setamou (2010) found evidence of olfactory learning in nymphs of the glassy winged sharpshooter (*Homalodisca vitripennis*). Orientation towards a visual target was reportedly influenced by experience in a species of the minute pirate bug, *Orius majusculus* (Henaut et al., 1999). Finally, visual and olfactory learning in the context of host preference have been documented in the predatory bug, *Rhodnius prolixus* (Aldana et al., 2008; Abramson et al., 2005; Vinauger et al., 2011a; Vinauger et al., 2011b; Vinauger et al., 2012). While there is a general lack of knowledge about learning in Hemipterans, even less is known about how learning may affect pest management (Prokopy & Lewis, 1993). Furthermore, the increased demand for pest management strategies, alternative to traditional synthetic pesticides, necessitates investigation of the behavioral and cognitive ecology of agricultural pests.

Of current concern is the introduction and spread of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), a phloem-feeding citrus pest that transmits *Candidatus* Liberibacter asiaticus (*C*Las), the putative causal agent of citrus greening disease or huanglongbing (HLB) (Halbert & Manjunath, 2004; Bove, 2006). Management of HLB relies on the intensive use of insecticides to reduce *D. citri* populations (Bove, 2006; Grafton-Cardwell, 2013; Hodges & Spreen, 2012); however, insecticide resistance has recently been detected in *D. citri* (Tiwari et al., 2011). The dependence upon chemical controls in the face of emerging insecticide resistance, along with the recent concerns about neonicotinoids, necessitates the development of alternative control techniques for managing *D. citri* populations (Martini et al., 2015; Croxton & Stanley, 2014). As a result, better tools are needed to detect and monitor this pathogen vector. Development of successful alternative control techniques, such as trap cropping and 'attract and kill' strategies will require a thorough understanding of the insect's ecology and cognitive abilities with respect to finding host plants and conspecifics (Cook et al., 2006).

Currently, we know that *D. citri* employ several sensory modalities when selecting hosts including vision (Hall et al., 2007; Wenninger et al., 2009; Paris et al., 2015; Setamou et al., 2014; Patt et al., 2011), gustation (Patt et al., 2011), audition (substrate-borne) (Mankin et al., 2013; Rhonde et al., 2013), and olfaction (Patt et al., 2011, Mann et al., 2012; Martini et al., 2014; Patt & Setamou, 2010). Furthermore, the response of the insect to olfactory cues is affected by bacterial infection of plants, which changes the bouquet of volatiles released from infected plants (Aksenov et al., 2014; Mann et al., 2013). To some extent, the behaviors of *D. citri* have already been exploited for pest management. Traps for monitoring *D. citri* populations have been optimized to exploit color preferences (Setamou et al., 2014) and olfactory lures based on preferred host plant volatile profiles are under development (Godfrey et al., 2013; Coutinho-Abreu et al., 2014). However, the success of such applications has been limited, and it is unclear whether the preferences displayed by *D. citri* are learned or innate.

Previous research on feeding behavior in adult *D. citri* indicates a possible role for experience in host selection and acceptance (Patt et al., 2014), despite strong innate responses to certain host plant volatiles (Wenninger et al., 2009; Patt et al., 2011; Mann et al., 2012; Patt & Setamou, 2010). It remains unclear whether information about stimuli, perceived by developing psyllids, is retained and used in host plant selection as reproductively mature adults. This is an important pest management consideration because if *D. citri* host preferences are experience-dependent, then differences in local citrus cultivar abundance may influence monitoring (i.e., orientation to a target) and the development of alternative control measures (i.e., selection of trap crop cultivar) (Patt et al., 2014).

The goals of the present study were to investigate the extent of associative learning in *D. citri* in the form of simple and compound conditioning tasks while exploring memory duration, and the relative salience of visual versus olfactory information. Due to the economic importance of this pest species, an ancillary goal was to determine the factors that may contribute to effective application of behavioral modification as a future management tool for *D. citri* and, by extension, other phytopathogen vectors. For this reason, we sought to study learning in the context of host preference. If learning plays an important role in *D. citri* host plant preference, traps and lures tailored to regional *Citrus* or ornamental *Murraya* diversity and abundance may enhance the efficacy of current monitoring and disruption techniques.

Methods and Materials

Insect Colony and Host Plant Maintenance

D. citri were obtained from a *C*Las-free colony maintained at the University of Florida Citrus Research and Education Center in Lake Alfred, Florida. The psyllids were originally obtained from 'Valencia' orange (*Citrus* x *sinensis* (L.) Osbeck) trees and were subsequently maintained on 'Valencia' orange and orange jasmine (*Murraya paniculata* (L.) Jack). The trees were fertilized with a granular fertilizer monthly, and weekly with a soil drench fertilizer (MaxiGro™). To establish colonies reared on a specific host plant species, the psyllids from the general colony were moved into cages with either potted orange jasmine or sour orange (*Citrus* x *aurantium* L.) plants. After initial oviposition, general colony adults were removed to ensure that all developing insects were only experienced with the developmental host plant species; either orange jasmine or sour orange. All colonies were maintained at 28°C under a L14:D10 light cycle. To maintain *D. citri* reproduction and ensure maximum plant health, host plants were rotated out of colony cages once per month.

Y-maze Behavioral Assay Set-Up

Olfactory and visual preference tests were performed with a Y-maze apparatus with a 1 cm inner diameter. The length of the Y-maze measured 13 cm from the crux to the release end. The two arms of the Y-maze measured 8 cm from the crux to the odor source ends. The arms of the Y-maze received charcoal filtered and humidified air pumped at 0.2 liters per minute (LPM) from a flowmeter (ARS Inc., Gainesville, FL). The Y-maze was mounted vertically with two white compact fluorescent lights (Sylvania; 13W, 800 lumens) suspended at equal heights from the distal ends of the Y-maze arms. A vertical Y-maze mount was used due to positive phototaxis and negative geotaxis displayed by *D. citri* (Stelinski et al., 2013; Mann et al., 2011). White bulbs were used unless otherwise stated. In visual conditioning experiments involving different light colors, the white bulbs were replaced with colored compact florescent bulbs of the same intensity (Mood-lites®; 13W, 800 lumens).

To remove the effects of possible differences in heat and light intensity between the different bulbs, the bulbs were presented within a ‘shade’ container that was constructed from white cardstock (white bulb: 81°C unshaded, 39°C shaded; blue bulb: 80°C unshaded, 38°C shaded). This diffused the light and created a consistent 5 cm buffer between the bulbs and the arms of the Y-maze. Using a shade, the heat measured on the arms of the Y-maze was approximately 26°C regardless of bulb color. Ambient room temperature was approximately 23°C. Temperature was measured with a Fluke 62 Max+ Infrared (IR) thermometer. To control for positional bias, the Y-maze was rotated 180° every 10 trials. To control for potential chemical deposition, which could influence *D. citri* behavior (Martini et al., 2014), the Y-maze was cleaned and replaced every 5 trials. All glassware was cleaned with Sparkleen detergent (Fischerbrand), rinsed with distilled water, rinsed again with acetone, and dried in an oven at 80°C for at least 15 min. Each trial lasted 300s, or until an individual made a selection, which was designated as a minimum 1 cm entry into a particular arm. When a selection was made, the insect was removed from the Y-maze and the trial was ended. Individuals that failed to make a selection within 300 s were designated “nonresponsive.” Data were collected only on days when the response rate was above 80 %. The usual response rate was about 90-95 % but varied based on barometric pressure. The latency to selection and orientation of the selected arm data (left or right) were recorded in addition to odor selection data. Time to selection data were used to compare response time between male and female *D. citri*, as well as, naïve and experienced *D. citri* in experiment 3.

Host Plant Preference

To determine the effect of host plant experience on adult female host plant preference, *D. citri* were reared as described above on either sour orange (SO) or orange jasmine (OJ) for two generations. Female *D. citri* collected from each host plant colony were then assayed using a Y-maze olfactometer for host plant odor preference. Each arm was baited with odor from either 0.25 g sour orange leaves or 0.25 g orange jasmine leaves. Foliage used in testing was obtained from caged, undamaged plants that had never been fed upon. The age of the plants used in the olfactometer assays was the same as the plants used in the colonies; approximately 2 years. Both sets of plants were treated similarly (e.g., watering, pruning and fertilizer schedules) with the exception of *D. citri* herbivory. Only young flushing shoots were used and all leaves used in this experiment were visually approximated to maintain consistent leaf age. Adult psyllids used in these tests ranged in age from 4-7 days post-emergence. Only females were used in experiment 1 due low male response level in preliminary tests.

To determine the plasticity of such preferences, shifts in host plant preference after short-term experience feeding on an alternative host plant species was measured in adults. In this test, four-day old adult females reared on orange jasmine were moved to sour orange and assayed for orange jasmine or sour orange preference at 0-, 24-, 48-, and 72 hrs post host plant transfer. To control for handling and transfer of *D. citri*, a separate group was moved from the original host plant (orange jasmine) and placed on new orange jasmine plants in a different cage. These psyllids were assayed at the same time points as the experimental group. This experiment was repeated using sour orange as the natal host plant and orange jasmine as the novel adult host plant.

Single Stimulus Conditioning

Two experiments were conducted to investigate differences in stimulus acquisition by adult psyllids across sensory modalities. The first experiment evaluated single stimulus conditioning toward a novel, non-host plant associated volatile, vanillin, while the second experiment evaluated single stimulus conditioning to another novel, non-host plant associated stimulus, blue light. In the first experiment, adult *D. citri* were released onto caged sour orange trees baited with vanillin (Sigma-Aldrich; CAS 121-33-5). Baits were created by adding 5 ml of a 2.5% ethanolic vanillin solution to a cotton wick. Vanillin was dissolved into solution with 100% ethanol. To prevent direct contact by *D. citri* with vanillin, the wicks were enclosed in perforated plastic cups with lids. One bait cup was placed inside the pot of each sour orange plant. *D. citri* were allowed to feed freely on the vanillin baited plants for 72 hrs (Figure 2-1). After 72 hrs, male and female *D. citri* were assayed for orientation response to vanillin using the Y-maze olfactometry described above. One arm of the Y-maze was baited with 1ml 2.5% vanillin solution on a cotton wick. The other arm was used as a control and was baited with 1 ml of ethanol on a cotton wick. The wicks were air dried for 30 min prior to use to allow the ethanol to fully evaporate. The results of the vanillin experienced *D. citri* were compared with naïve *D. citri*.

In the second experiment, adult *D. citri* (age non-specific) were released onto caged sour orange trees illuminated with blue light (Mood-lites®; compact florescent, 13W) (Figure 2-1). *D. citri* were allowed to feed freely on the blue illuminated plants for 72 hrs. After 72 hrs, male and female *D. citri* were assayed for orientation response to blue light using modified Y-maze olfactometry. The test treatment arm of the Y-maze was illuminated with blue light as described above, while the control arm was illuminated with white light. The illumination from each light source was isolated by using a solid white divider that was placed between the two arms of the Y-maze, resting upon the crux.

The results of the blue-light experienced *D. citri* were compared with naïve *D. citri*. All adult *D. citri* were at least 4 days old (fully sclerotized and reproductively mature) before use in any part of experiment 2.

Compound Conditioning

To evaluate the relative salience of olfactory versus visual stimuli, as well as, the ability to acquire complex multi-modal information, compound conditioning was performed on male and female *D. citri* using the olfactory stimulus, vanillin, and the visual stimulus, blue light. *D. citri* (age non-specific) were released onto caged sour orange trees illuminated with blue light and baited with 5 ml of 2.5% vanillin solution. *D. citri* were allowed to feed freely on the plants for 72 hrs. After 72 hrs, male and female *D. citri* were placed in one of six test groups and assayed using Y-maze olfactometry (Table 2-1). The test groups represented Y-maze choice-test permutations, which quantified responses to vanillin and blue light independently and as a compound stimulus. The behavioral results of the experienced *D. citri* were compared with naïve *D.*

citri. Similar to experiment 2, all adult *D. citri* were at least 4 days old before use in any part of this study.

Statistical Analysis

Y-maze data from the host preference tests and the simple and compound conditioning tests were analyzed with Chi-square tests for within-group comparisons and Chi-square 2 x 2 contingency Tables for between-group comparisons, alpha ≤ 0.05. Within-group comparisons were performed for selection differences towards either arm of the Y-maze (i.e. orange jasmine odor versus sour orange odor; vanillin odor versus blank; white light versus blue light). Between-group comparisons were performed for overall selection differences between groups (i.e. orange jasmine psyllids versus sour orange psyllids; naïve psyllids versus experienced psyllids). Differences between groups at each time point in Experiment 1 were calculated with chi-square contingency Tables. Standard error was calculated as $SE = \sqrt{(pq/n)}$ for binomial data where p is the proportion selecting the target, q is the proportion selecting the alternative target, and n is the number of Bernoulli trials.

Latency data from the compound conditioning experiment were compared using a generalized linear model with a Gaussian distribution. The model was simplified following a stepwise deletion to remove insignificant interactions ($\alpha > 0.10$). Only latency data from single stimulus versus control tests were included in those analyses. Latency data were not reported for single stimulus conditioning experiments because the sample size was too small to provide adequate statistical power.

At least two replicates, and as many as eight, were performed for each experiment. The number of individuals tested per replicate was dependent on the variation in response and the number of individuals required for statistical power for that particular experiment. For the analyses described above, the data from all replicates within a given experiment were pooled. All analyses were run in R (Version 3.1.3; the R Foundation for statistical software R; Vienna, Austria).

Results

Host Plant Preference

Between group comparisons of the overall response of sour orange-experienced psyllids and orange jasmine-experienced psyllids suggests that host plant preference was significantly affected by natal host plant type (χ^2=7.89, *df*=1, *p*=0.005) (Figure 2-2). Within group comparisons showed that female *D. citri* reared on sour orange plants preferred sour orange to orange jasmine leaf volatiles (χ^2=4, *df*=1, *p*=0.045). Conversely, female psyllids reared on orange jasmine significantly preferred orange jasmine odor as compared with sour orange (χ^2=3.9, *df*=1, *p*=0.048).

However, when insects reared on sour orange were transferred to orange jasmine plants as adults, host plant preference shifted towards orange jasmine 48 hrs post-transfer (Figure 2-3a) (χ^2=5.76, *df*=1, *p*=0.016). Similarly, a transfer of adult female *D. citri* from the developmental host plant species, orange jasmine, to the alternative host plant species, sour orange, changed those preferences in favor of the most recent host plant that was experienced (Figure 2-3b). By 48 hrs post host plant transfer, *D. citri* significantly preferred sour orange compared with *D. citri* that had constant exposure to the developmental host plant, which maintained preference for orange jasmine (χ^2=5.32, *df*=1, *p*=0.021).

Single Stimulus Conditioning

There was a significant effect of treatment in the single stimulus learning experiments, with experienced insects showing greater response to the test stimulus than naïve insects. Feeding experience on vanillin-baited plants for 72 hrs was sufficient to produce a significant change in response to that volatile in adult *D. citri* (Figure 2-4a). Compared to naïve insects, experienced females (χ^2=7.57, *df*=1, *p*=0.006) and males (χ^2=3.99, *df*=1, *p*=0.045) showed significantly greater selection of vanillin. Similarly, feeding experience on plants illuminated with blue light induced a significant change in response compared with naïve insects in both females (χ^2=9.99, *df*=1, *p*=0.002) and males (χ^2=4.84, *df*=1, *p*=0.027) (Figure 2-4b).

Compound Conditioning

Experience with a bimodal compound stimulus (vanilla odor + blue light) resulted in significantly different responses in female *D. citri* to each stimulus when presented individually (Figure 2-5a-b). In a naïve state, female *D. citri* oriented towards vanillin at the same rate as the unscented control arm, meaning that response to vanillin was neutral. However, after experience, this percentage increased by approximately 19%. Between group comparisons showed that this difference was statistically significant (χ^2=3.81, *df*=1, $p \leq 0.05$). Naïve female response to blue light increased similarly after experience, from 19 to 45% (χ^2=8.96, *df*=1, *p*=0.003). Males did not show increases in response to each stimulus when presented individually.

When the two stimuli were presented on either end of the Y-maze simultaneously, preference shifted as a result of experience (Figure 2-5c). Naïve males (χ^2=5.67, *df*=1, *p*=0.02) and females (χ^2=9.28, *df*=1, *p*=0.002) preferred the arm emitting the olfactory stimulus, while insects that had experienced a combination of vanilla odor + blue light selected the colored and scented arms equally.

When the compound stimulus was presented opposite of the blank control, there were no differences between groups (Figure 2-5d). However, there was a significant difference in naïve female selection, with preference shifted towards the control arm (χ^2=11.27, df=1, p<0.0008). This difference was abolished after experience - experienced females showed no preference for either the blank arm or the arm with the compound stimulus. The opposite trend occurred for males; naïve males showed no preference between the compound stimulus and the control; whereas experienced males preferred the control (χ^2=4.59, df=1, p=0.03).

The final two tests involved presenting the compound stimulus opposite each of the stimuli individually (Figure 2-5ef). When the compound stimulus was presented opposite of the olfactory stimulus alone, experienced males showed an increase in response to the compound-stimulus (χ^2=4.85, df=1, p=0.03) (Figure 2-5e). There was no difference in female response, (χ^2=2.88, df=1, p=0.09). When the compound stimulus was presented opposite of the visual stimulus alone (Figure 2-5f), there was no statistically significant difference between naïve and experienced insects, although experienced females showed an 18% increase in response to the compound stimulus (χ^2=3.55, df=1, p=0.059).

In addition to behavioral choice data, we also collected latency data. These results revealed differences in response time when making a selection in the 2-choice olfactometer (Figure 2-6). Comparing the latency to selection for all *D. citri* during single stimulus tests using a GLM, there was a significant effect of sex (females compared to males) and treatment (naïve insects compared to experienced insects) on the time needed for selection (Table 2-2). All of the interactions among sex, treatment, and the sensory modality of the tests (olfactory tasks compared to visual tasks) were insignificant at $\alpha < 0.10$ and were consequently removed from the model. Time to selection was significantly greater in female *D. citri* as compared to males (Figure 2-6ab). Additionally, experience was associated with a small but significant decrease in response time compared to naïve insects (Figure 2-6b). No significant differences were observed between the olfactory and visual tasks (Figure 2-6a).

Discussion

The host plant preference tests in Experiment 1 compared the behavioral response of *D. citri* to familiar or novel host plant species. We found that female *D. citri* displayed host plant preferences based on experience, such that these insects initially preferred the host plant species on which they were reared. Similar cases of induced host preference have been well documented in *Manduca sexta* (Yamamoto & Fraenkel, 1960; Stadler & Hanson, 1978) and *Heliothis zea* (Jermy et al., 1968), where moth larvae reared from egg on a specific host species display feeding preferences for the species on which they were reared. However, the results of a second experiment showed that those initial developmental preferences were subject to change after adult *D. citri* were moved to an alternative host plant species. *D. citri* preferences shifted to the alternative host plant species within 24-48 hrs following transfer. This suggests that although developmental experience may influence mature adult insect behavior, adult experiences may be more significant in affecting *D. citri* host selection, such that the most recently fed upon plant is preferred.

The single stimulus conditioning tests in Experiment 2 investigated the parameters surrounding learning in *D. citri* in terms of environment-host plant associations, and showed that male and female *D. citri* can associatively learn cues in both olfactory and visual contexts. This reinforces the hypothesis that both olfactory and visual stimuli are important in *D. citri* host selection (Patt et al., 2011) and confirms previous findings of multimodal learning in an artificial feeding assay (Patt et al., 2014). Research on multimodal sensory perception in the hawkmoth, *Manduca sexta*, suggests that both forms of sensory information act synergistically, likely aiding in communication between the environment and the foraging insect such that visual cues in combination with olfactory stimuli may provide contextual information (Raguso & Willis, 2002; Couvillon & Bitterman, 1998; Goyret et al., 2008). In *D. citri*, the capacity for visual and olfactory learning may increase the likelihood of detecting suitable hosts when migration and dispersal is required. *D. citri* are known to disperse up to 2 kilometers, particularly in search of flushing host plants suitable for oviposition (Martini et al., 2014; Lewis-Rosenblum et al., 2015; Hall & Hentz, 2011).

In Experiment 3, we investigated the relative associative strength of olfactory and visual stimuli in terms of biological relevance to *D. citri* host plant selection behavior. We predicted that the more salient stimulus would show the greatest rate and proportion of learning (Rescorla & Wagner, 1972; Miller et al., 1995). The results suggest that female *D. citri* learn the individual components of the compound stimulus separately, as well as the compound stimulus as a whole. In addition, while naïve females display strong preference for the olfactory stimulus compared to the visual stimulus, after conditioning, the two stimuli appear to share similar strength of attraction. These findings suggest that female *D. citri* actively acquire visual and olfactory information about their host plants, similar to that found in previous work (Patt et al., 2011; Patt et al., 2014). Interestingly, after the compound presentation of the stimuli, we were unable to demonstrate learning in male *D. citri*. Unlike the females, males appeared to show lower capability of learning each stimulus individually and some degree of learned aversion to the compound stimulus. The reasons for this finding are unclear, but it may indicate that the biological significance of the information, or perhaps the way information is stored neurologically, varies depending on the sex of the insect. More experiments are required to better understand these findings and explore those hypotheses.

Along with choice, Experiment 3 also examined the latency prior to behavioral response, which is often used as a measure of decision-making, and in some cases, its own measure of learning (Morris, 1981; Wessnitzer et al., 2008). We found that experienced *D. citri* make target selections faster than naïve insects. The most significant difference was dependent on the sex of the insect, with males making more rapid selections than females regardless of task or experience. This may reflect differences in overall selection strategy between the sexes and may suggest that male psyllids have weaker discriminatory abilities than females, which may help explain the sex differences in learned response rate. This is not surprising considering the reproductive role of females and the importance of oviposition site selection for successful nymph development.

Our investigation was not without some limitations. While our olfactory stimulus, vanillin, was truly novel and is not found in association with the *D. citri* host environment, our visual stimulus was not novel. Blue light appears to provoke a strong innate repellent response (Wenninger et al., 2009; Paris et al., 2015; Coutinho-Abreu et al., 2014). In addition, some *D. citri* possess a “blue-green” abdominal color-morph and there is increasing evidence that abdominal color is associated with important behavioral differences among psyllids with respect to dispersal and reproduction (Martini et al., 2014; Wenninger & Hall, 2008). However, we avoided strongly attractive colors such as yellow, orange, red, or green (Paris et al., 2015; Setamou et al., 2014), since previous work has indicated that there is little learned increase in *D. citri* behavior when the innate response to the stimulus is positive (Patt et al., 2014). Therefore, although the blue light stimulus used in this study does not conform to the characteristics of traditional stimuli used in most learning experiments, we believe it was the appropriate choice for our subject animal. In fact, it revealed a rather interesting result - *D. citri* appeared capable of significantly changing their responses, with blue no longer acting as a repellent after conditioning. This suggests that while psyllids may possess intrinsic aversions to certain stimuli, likely evolutionarily selected to help them avoid non-hosts, those aversions are not so hardwired as to be immuTable (Huang & Renwick, 1995; Szentesi & Bernays, 1984; Gill, 1972). The data shown here suggest that short-term exposure to those stimuli within the context of a reinforcing stimulus, such as food, may override the innate responses of these insects, allowing them to maximally benefit from a complex environment of potential hosts, even those which are on the fringes of their oligophagous range.

Based on these data, we conclude that *D. citri* not only learn, but learning may be partially responsible for local host plant preference phenomena (Wenninger et al., 2009; Patt et al., 2010; Chan et al., 2011). To our knowledge, the findings presented here represent the most extensive study of learning in a Sternorrhynchan species. This is significant because sternorrhynchans are an important group of disease vectors and understanding the behavioral ecology involved in host plant preference and selection in these insects is potentially beneficial in the design and implementation of pest monitoring and management programs. Although this work is limited to fairly fundamental questions regarding learning in *D. citri* (eg., what types of stimuli are learned, how long memories are retained), and may at this point in time have limited application, we believe that it lays the foundation for further study of learning of this insect and other related Sternorrhynchans. It appears likely that *D. citri* acquire olfactory and visual information about the host plants with which they have experience. Similar to other psyllid species, visual information appears to be as biologically relevant to *D. citri* as olfactory cues in terms of orientation towards a target and association with host plants (Farnier et al., 2014; Farnier et al., 2015). Given the observed plasticity in response to visual stimuli seen in these experiments, even brief visual experience with a novel, non-traditional, and initially deterrent host may increase the likelihood of future selection for that species, despite repellant visual properties. This could facilitate vector dispersal by providing temporary refuge for psyllids during migration, or even reduce intraspecific competition by introducing novel host plant options; however, further investigation is needed to fully test this hypothesis. For that reason, future *D. citri* traps may benefit from increased attention to the visual aspects of design, possibly tailoring color, as well as semiochemical lures to the most prevalent host plant varieties within each citrus growing region.

Table 2-1. Compound conditioning experimental test groups. Each group differs only in stimulus presentation during testing. Each single stimulus is the presentation of the olfactory or visual stimuli alone. The compound stimulus is presentation of the olfactory and visual stimuli simultaneously.

Test Group	**Arm 1**	**Arm 2**
a	Olfactory	Blank
b	Visual	Blank
c	Olfactory	Visual
d	Olfactory +Visual	Blank
e	Olfactory +Visual	Olfactory
f	Olfactory +Visual	Visual

Table 2-2. Results from the GLM with Gaussian distribution. Results based on the latency data associated with the single stimulus tests in the compound conditioning experiment. All non-significant interactions were removed (α >0.10).

Factor	***df***	***F***	***p*-value**
Sex	1, 402	22.32	<0.0001
Treatment	1, 402	3.94	0.048
Test	1, 402	2.36	0.125

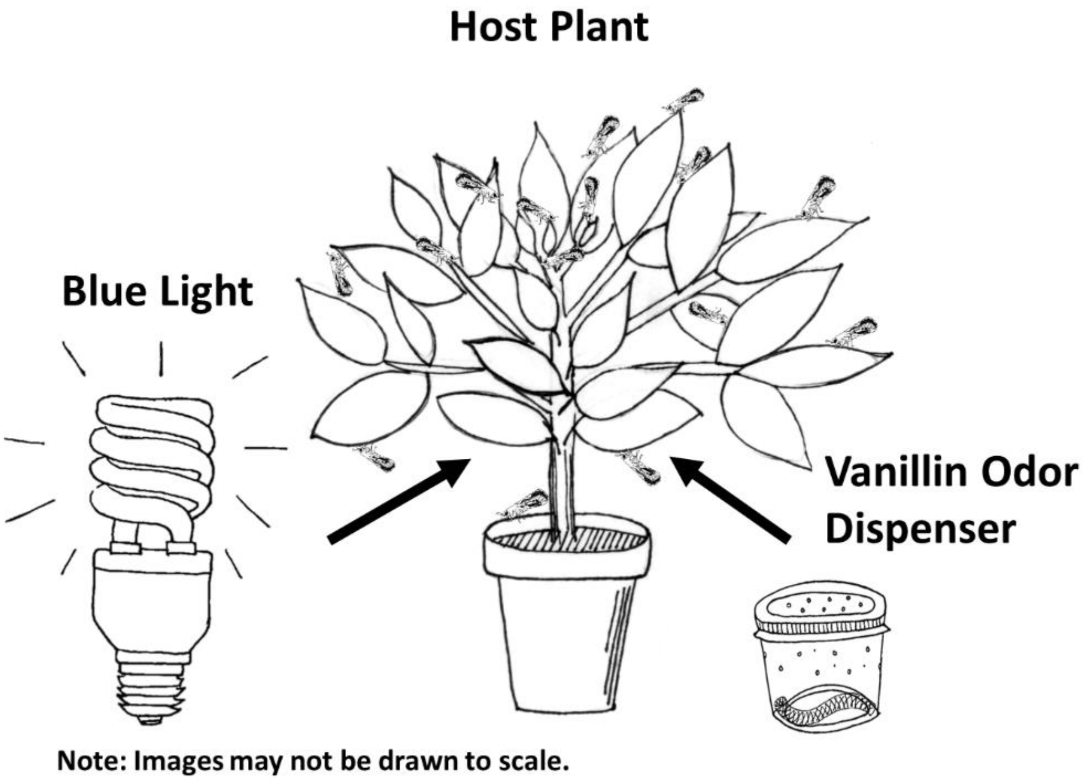

Figure 2-1. Experimental conditioning procedure. In single stimulus tests, either blue light or vanillin were paired with the host plant. In compound conditioning, all test groups received simultaneous presentation of the visual and olfactory stimuli.

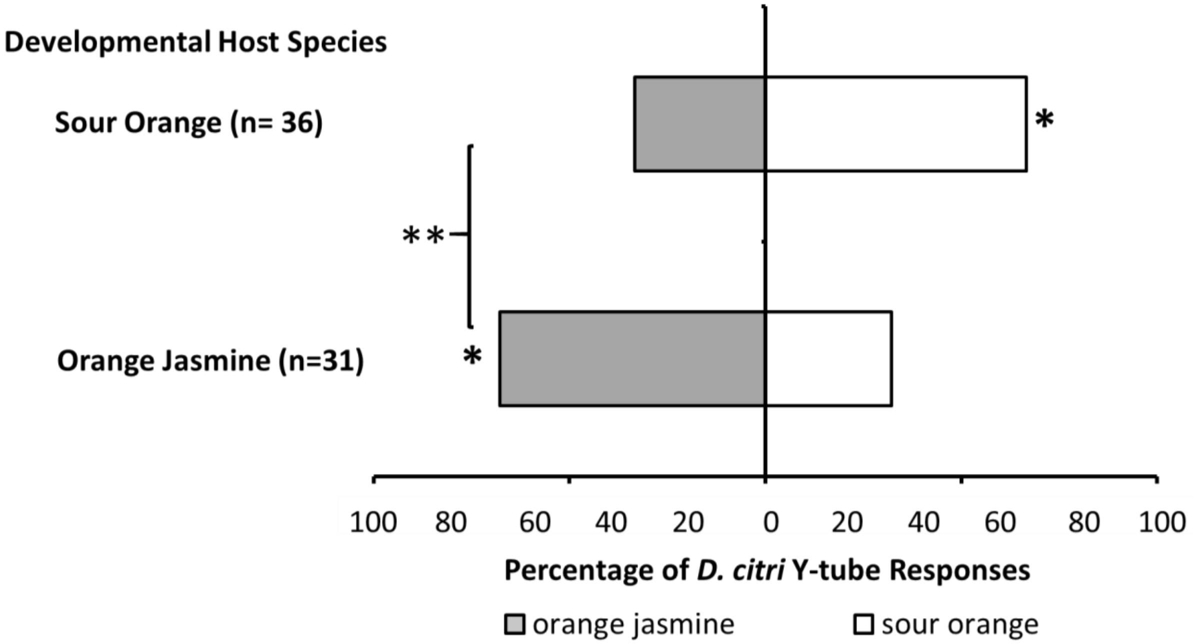

Figure 2-2. Developmental host plant preference. Differences in female *D. citri* preference based on natal host plant species. Asterisks within bars indicate statistically significant differences within groups, while asterisks associated with a bracket indicate differences between groups (χ^2 test, *: < 0.05, **: < 0.01).

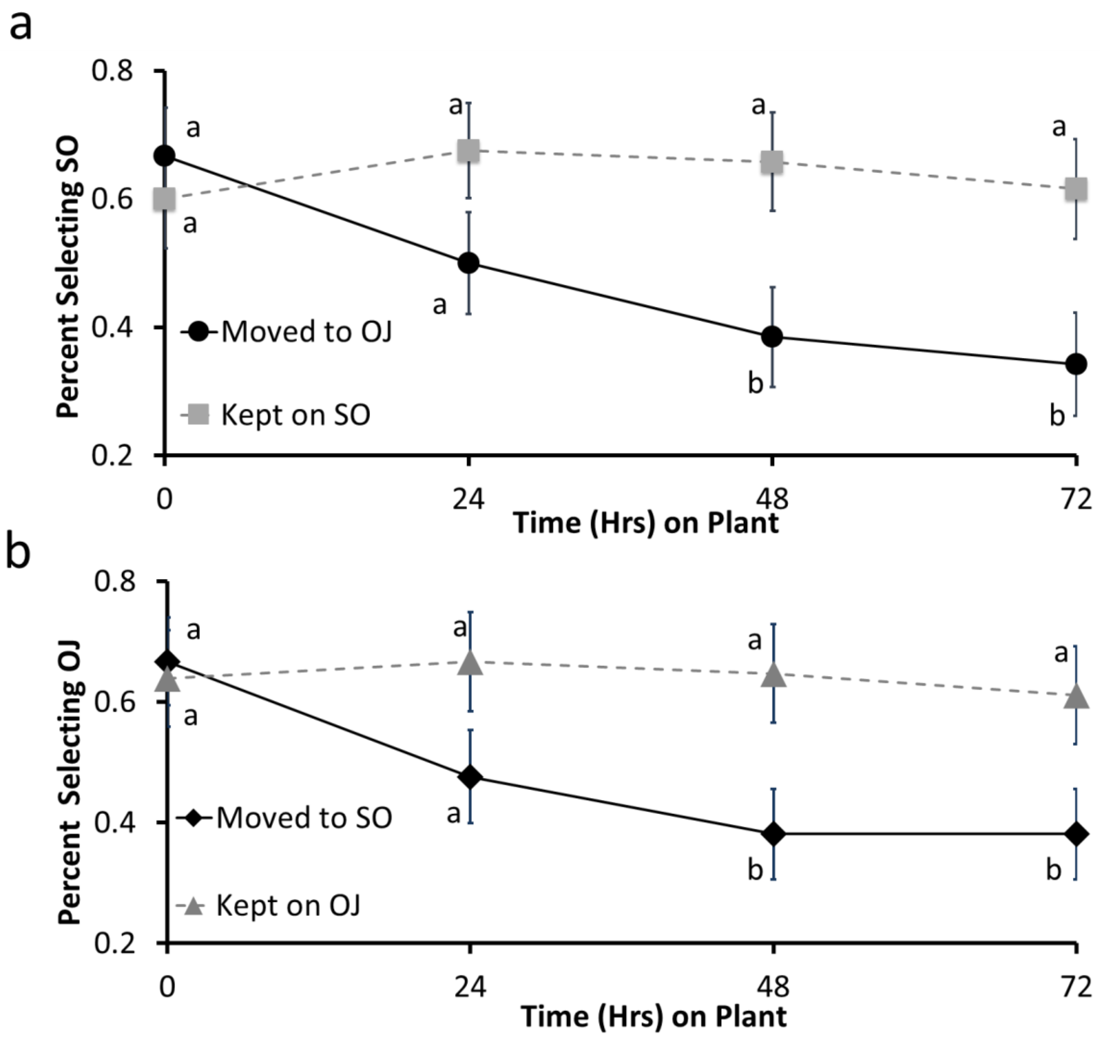

Figure 2-3. Experience dependent host plant preference. Shifts in preference after short-term adult experience feeding on the alternative host plant species. The labels “Kept on SO” (sour orange) or “Kept on OJ” (orange jasmine) represent the control groups - *D. citri* maintained on a single host plant species for the duration of the study. The experimental groups are labeled as “Moved to SO” or “Moved to OJ.” Significant differences between groups at each time point are indicated by different letters (χ^2 test, α≤0.05).

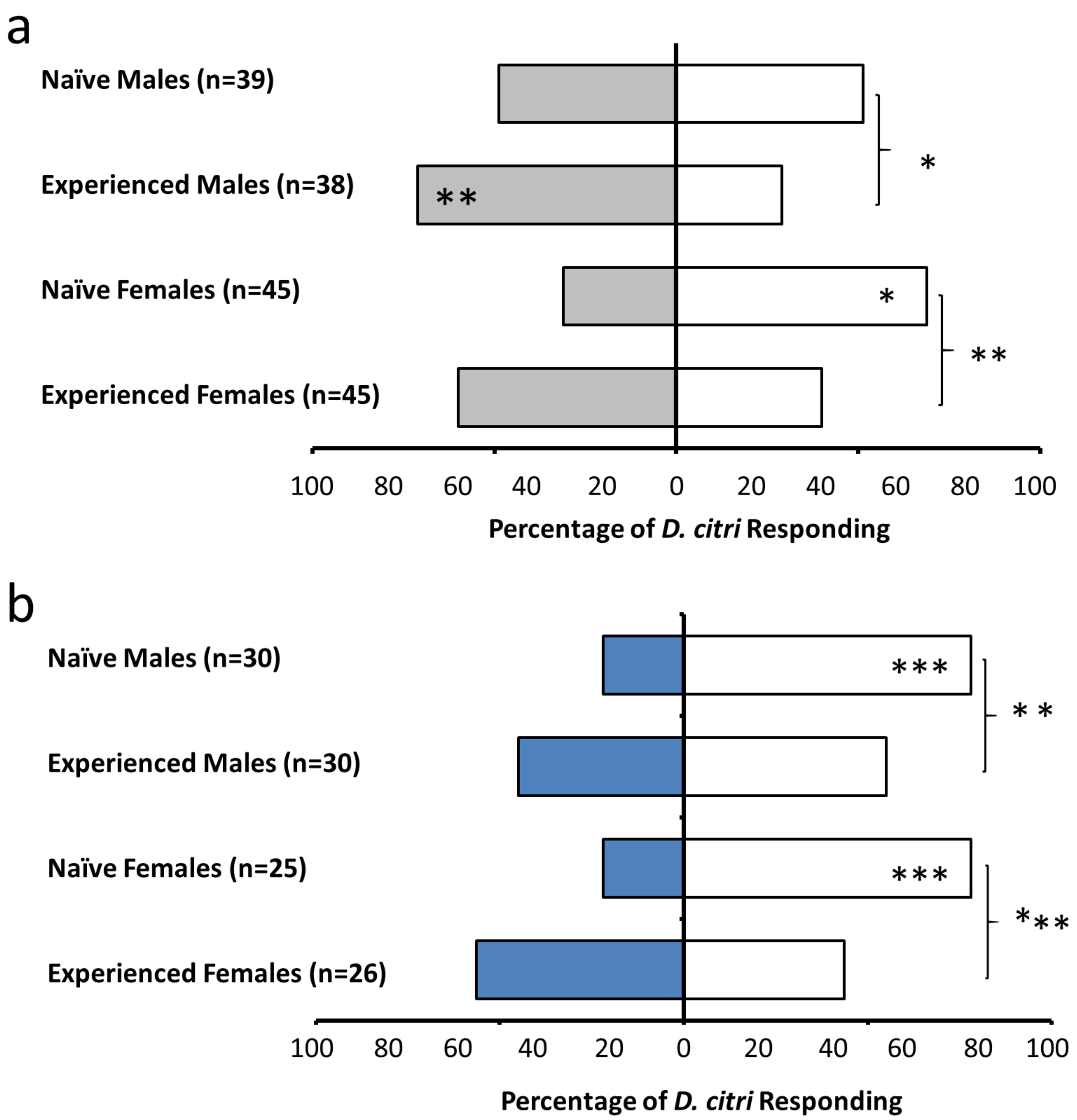

Figure 2-4. Learned response to a novel olfactory stimulus (a) and a visual stimulus (b). (a) Light gray bars indicate responses to a novel olfactory stimulus (vanillin odor). (b) Blue bars indicate the responses to a novel visual stimulus (blue light). (a, b) White bars indicate responses to the blank control. Asterisks (*) within bars indicate statistically significant differences within groups selecting arm A or arm B, while asterisks (*) outside bars indicate differences between naïve and experienced insects (χ^2 test, *: < 0.05, **: < 0.01, ***: <0.001).

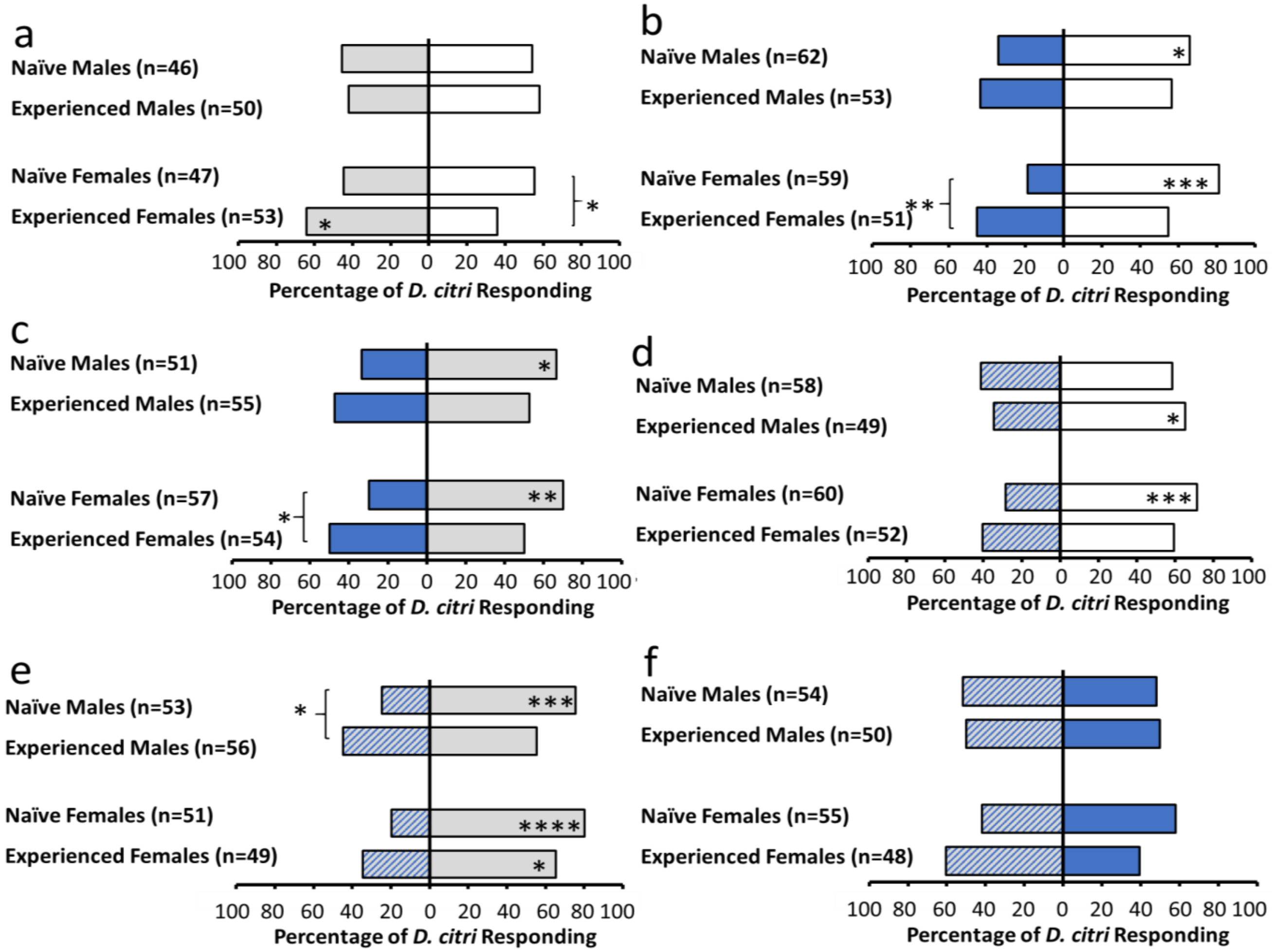

Figure 2-5. *D. citri* responses after compound conditioning to six test conditions (a-f). Gray bars represent the olfactory stimulus (vanillin odor), blue bars represent the visual stimulus (blue light), striped blue and gray bars represent the compound stimulus (vanillin odor + blue light), and white bars indicated a blank control. Asterisks (*) within the bars indicate statistically significant differences within a group selecting arm A or arm B (χ^2 test, *: < 0.05, **: < 0.01, ***: < 0.001), ****: <0.0001. Asterisks (*) outside the bars indicate differences between a set of naïve and experienced insects for a given test condition (χ^2 test).

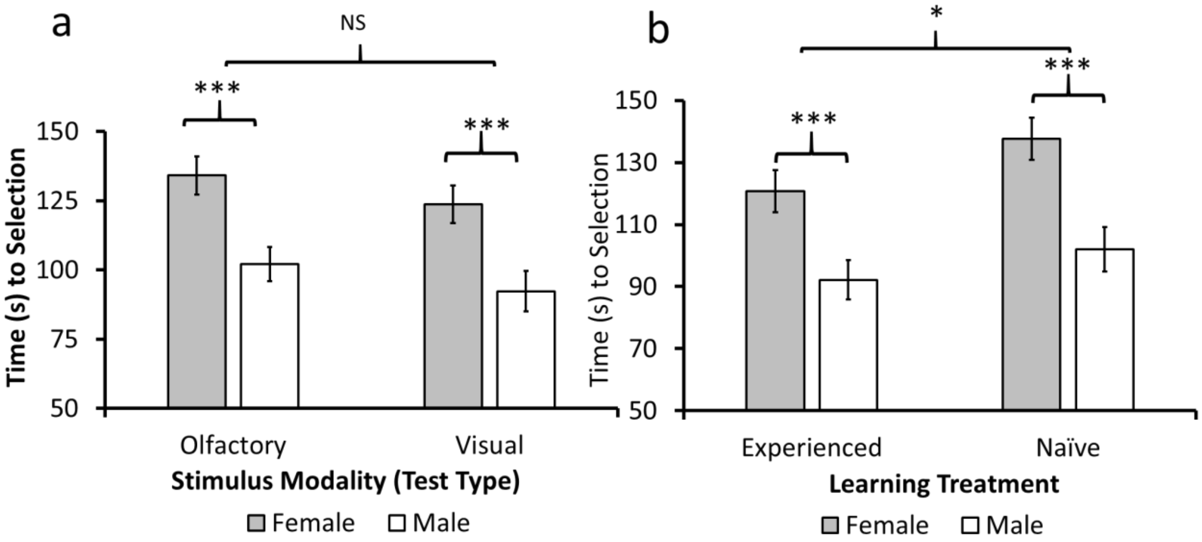

Figure 2-6. Latency to selection for *D. citri*. Differences in selection time in female (gray bars) and male (white bars) psyllids based on (a) the stimulus modality of the test (olfactory or visual test) and (b) the learning treatment applied to the insects (naïve or experienced). Asterisks (*) indicate significant differences *: < 0.05, **: < 0.01, ***: < 0.001).

CHAPTER 3
INDUCED PREFERENCE AND PERFORMANCE

Chapter Abstract

Alternative host plant genotypes may differentially affect the preference and performance of female insects and their offspring. Learning plays a role in modulating maternal preferences, where experience with a particular host increases the likelihood that females will select it for future feeding and oviposition. In the Asian citrus psyllid, learning facilitates host recognition through complex visual and olfactory associations; however, it is unclear whether learning is used in female reproductive strategies and confers fitness benefits to her offspring. We investigated the potential fitness benefits of learned host preference by examining the influence of natal host experience on adult settling and oviposition site preference when reared on either *Citrus* x *sinesis* or *Murraya paniculata*. We then determined the relative fitness of juvenile psyllids when moved from one host species to the other. Our results indicate that females prefer an oviposition environment most similar to their natal host species when the natal host plant is associated with fewer plant defense compounds than the alternative host. When the non-natal host species was associated with higher allelochemical defenses, transferred nymphs displayed decreased fitness. They were smaller in size than insects reared on the less-toxic natal host and required more time for development. In contrast, fitness increased when moved from the more toxic to less toxic host species. We conclude that induced oviposition preference may provide an important mechanism of adaptive plasticity in psyllid reproductive strategies, allowing females to discriminate among potential host species in favor of those to which her offspring are most adapted.

Background Information

Phenotypic plasticity allows organisms to adapt to the environment (Agrawal, 2001). This can occur as polymorphisms in physical appearance, physiology, or behavioral variation within a population. For example, in response to chemical cues produced by predaceous fish, the water flea *Daphnia lumholtzi* develops defensive exoskeletal spines, whereas those without exposure do not (Green, 1967). Although the capacity to develop a trait may be genetically fixed within a population, expression of those features depends of the environment in which the organism develops. In contrast to physical modification, behavioral plasticity occurs when organisms demonstrate varied behavioral responses to a stimulus based on environmental change (Keiser & Mondor, 2013) or past experiences (Menzel, 1985). In some cases, when the latter is responsible for such change, that plasticity can be attributed to learning.

Many well-documented examples of behavioral plasticity demonstrate adaptive significance, such that past experience modifies future behavior to improve reproductive success and offspring fitness. In the fruit fly *Drosophila melanogaster*, female mate choices may reflect early experiences she has with courting males, where a preference for male size is determined by the courtship encounters early in her adult development (Dukas, 2005a). Similarly, male fruit flies appear to use failed courtship attempts to refine their selection of conspecific females and effectively avoid heterospecific species (Dukas, 2005b).

In addition to mate choice and courtship behaviors, learning may influence other areas of reproduction such as oviposition rate and substrate selection (Papaj & Prokopy, 1989). Depending on maternal diet and natal host experience, offspring may demonstrate differential host preference and compatibility (Stoyenoff et al., 1994).

Indeed, many female insects prefer to oviposit onto plants similar to those of their natal environment to avoid the risks associated with a novel environment (Agrawal et al., 2002; Singer et al., 1988; Papaj & Prokopy, 1986; Jaenike, 1983). Such preference for plants similar to the maternal natal host appear to directly benefit offspring. When forced to feed on a novel host plant species with which they are not acclimated, some arthropods show significant declines in performance such as decreased oviposition, longevity, and size associated with inadequate detoxification of defensive allelochemicals (Egas et al., 2003; Agrawal et al., 2002; Egas & Sabelis, 2001; Traxler & Joern, 1999; Stoyenoff et al., 1994; Karowe, 1989).

Previous research has indicated that learning plays a role in host plant selection and reproduction for an invasive citrus pest, the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Stockton et al., 2016; Patt et al., 2014). This phloem-feeding insect represents an important model system of agricultural disease transmission, as it is a vector of *Candidatus* Liberibacter species, the putative causal agents of huanglongbing (HLB) in citrus (Grafton-Cardwell et al., 2013; Bove, 2006; Halbert & Manjunath, 2004). This bacterial disease kills citrus trees and has significantly limited citrus production worldwide (Wang & Trivedi, 2013). *D. citri* associate olfactory and visual stimuli with specific host plant genotypes (Stockton et al., 2016), and demonstrate learned mate choice, where females avoid immature males after mating experience (Stockton et al. unpublished data). In addition, males and females prefer familiar host species, particularly those on which they were reared (Stockton et al., 2016). However, the extent of that learned host preference, and whether it confers benefits to the offspring of *D. citri*, is unclear.

Our goal was to investigate learning in this agricultural phytopathogen vector in the context of oviposition and offspring survival. While previous research has identified preferred oviposition hosts of *D. citri* (Teck et al., 2011; Patt & Setamou, 2010; Wenninger et al., 2009; Nava et al., 2007), those studies did not account for the possible influence of the natal host on adult host preference. Therefore, we evaluated whether female oviposition substrate choice varied based on the developmental host species. Subsequently, we tested the hypothesis that female choice affects the development and survival of offspring. Immature 1st instar *D. citri* were collected from two host plant species. They were transferred from the maternal oviposition host plant to the alternative host. Thereafter, the development time and relative mortality of those nymphs was quantified. To assess whether host fidelity affected psyllid fitness, adult size and activity were measured using the next generation of emerging *D. citri*.

Methods

Insect Colony and Host Plant Maintenance

D. citri were obtained from a greenhouse colony maintained at the University of Florida Citrus Research and Education Center in Lake Alfred, Florida (Stockton et al., 2016). Insects were reared and collected from species-isolated colonies on ‘‘Valencia’’ orange (*Citrus sinensis* (L.) Osbeck) or orange jasmine (*Murraya paniculata* (L.) Jack.). All colonies were maintained at 28^{o}C with a standard L14:D10 light cycle. To maintain *D. citri* reproduction and ensure plant health, host plants were rotated out of the colony cages once per month and replaced with new plants.

Female Settling and Oviposition

To investigate the effects of juvenile host species experience on adult female host preference, females reared on either orange jasmine or ‘‘Valencia’’ orange were assayed in a two-choice test to determine settling and oviposition rates on flush clippings of both species. To avoid cavitation and subsequent wilting, leaf flush shoots were clipped from plants underwater and immediately transferred to individual 15 ml vials filled with a 2.5 % sucrose solution. One clipping of ‘‘Valencia’’ flush was paired with one clipping of orange jasmine flush, comparable in size (~6 cm) and amount of leaf tissue (~1 g), and placed in a small cage. The cages measured 28 x 10 cm (height x diameter). Psyllids were confined to the upper 14 cm of each cage using a cardstock divider. This facilitated easy observation of the insects within the cages and caused the insects to move onto the flush clippings. Five female and five male psyllids were released per cage. Half of the cages contained psyllids reared on orange jasmine plants (n = 40 cages), while the other half were reared on ‘‘Valencia’’ orange (n=40 cages). The number of psyllids settling on each clipping was recorded at 24, 48, and 72 hrs. At 72 hrs, the number of eggs laid on each clipping was counted with a dissecting microscope.

The settling experiment was replicated 7 times with 12 cages observed per replicate. Of those 12 cages per replicate, 6 contained psyllids reared on ‘‘Valencia’’ orange and 6 contained psyllids reared on orange jasmine. After removing 4 cages due to flush decline during the course of the experiment, data were collected from 80 cages total. The oviposition data were collected from the final 5 replicates because initial observations did not include egg counts. Oviposition data were collected from 60 cages total; 30 cages with psyllids reared on ‘‘Valencia’’ and 30 cages with psyllids reared on orange jasmine.

Nymph Development Time and Survival

Juvenile psyllids were collected from leaf flush as 1st instar nymphs. They were transferred in groups of 100 to new plants. Nymphs collected from ‘‘Valencia’’ orange flush were transferred to orange jasmine flush and vice versa. Two control conditions were also included in this study: ‘‘Valencia’’ orange nymphs were transferred to a new ‘‘Valencia’’ orange plant, and orange jasmine nymphs were transferred to a new orange jasmine plant. The development time, from the 1st instar stage at the time of transfer to adult eclosion, was observed daily. Three replicates were conducted.

Nymph survival was similarly quantified, although 20 3rd instar nymphs were transferred per treatment. Survival was calculated as the total number of nymphs transferred minus the total number of adult psyllids that subsequently emerged. Two replicates were performed.

Morphometric Analysis

Adult psyllids were reared under conditions identical that used in the *Nymph Development Time* study. However, immediately after emergence, psyllids were euthanized in ethanol and measured according to five body features with the aid of dissecting microscope at 10x magnification and an ocular with a 10mm ruler. Body length, abdominal length, wing length, femur length and head width were recorded. Three replicates were conducted across the 4 treatment groups, ‘Valencia’ → ‘Valencia’ (n=104); ‘Valencia’ → Orange jasmine (n=195); Orange jasmine → Orange jasmine (n=155); Orange jasmine → ‘Valencia’ (n=296).

Activity Level

Adult psyllids were reared under conditions identical that used in the *Nymph Development Time and Survival* studies. Adults from each of the four treatments previously described (‘Valencia’ → ‘Valencia’; ‘Valencia’ → Orange jasmine; Orange jasmine → Orange jasmine; Orange jasmine → ‘Valencia’) were used to assess the activity level of psyllids reared on non-natal hosts. Adult female psyllids (n = 40) from each treatment were compared for activity level using a y-maze apparatus thoroughly described in Mann et al. (2011). Aged, virgin 5-7 day-old female psyllids were released into the base of the y-maze and were given a maximum of 5 minutes to reach a target arm. Both arms contained a stimulatory odor target, 0.01ng/ml *E*-*β*-caryophyllene (Sigma-Aldrich, St. Louis, MO, 98% purity) pumped at a rate of 0.5 LPM (liters per minute) using an ARS Inc. (Gainesville, FL) flowmeter with charcoal filters and humidified air. The time to reach a target arm, or the failure to leave the inlet arm, were recorded. The y-maze was replaced with a clean apparatus every 5 trials. Every 10 trials, the y-maze was rotated 180º to control for possible orientation towards non-target environmental stimuli.

Statistical Analysis

Differences in *D. citri* settling were analysed with paired *t*-tests. Oviposition data were analysed with non-parametric Wilcoxon tests because of the non-normal distribution of the data. Overall differences in oviposition rate were compared with a generalized linear model with binomial distribution. Differences in development time among the four nymph treatments were analysed with Kruskal-Wallis and Wilcoxon sum-rank tests due to positive data skew. Differences in morphometric characters among the four treatment groups were compared with multivariate analysis of variance

(MANOVA) and Tukey HSD for multiple comparisons. Activity levels among groups were compared with ANOVA. Analyses were performed in R i386 (Version 3.0.2; the R Foundation for statistical software R; Vienna, Austria).

Results

Female Settling and Oviposition

There were minor differences in settling frequency among psyllids reared on either ‘‘Valencia’’ orange or orange jasmine plants (Figure 3-1ab). Psyllids reared on ‘‘Valencia’’ orange initially preferred to settle upon ‘‘Valencia’’ plants rather than orange jasmine (paired *t* test: t_{39} =3.3, P=0.002); however, that preference was no longer statistically significant at 48 and 72 hrs (Figure 3-1a). In contrast, psyllids experienced on orange jasmine plants showed little preference at 24 hrs post-release, but significantly increased preference for orange jasmine plants at 72 hrs (paired *t* test: t_{39} = 2.71, P=0.009; Figure 3-1b).

We also observed differences in oviposition preference depending on host plant experience (GLM with binomial distribution: χ^2_1=182.83, P<0.001; Figure 3-1c). Females reared on ‘‘Valencia’’ orange plants (Wilcoxon sign-rank test: V=72, P=0.005) laid significantly more eggs on ‘‘Valencia’’ flush (M=30.27, SD=29.28) than on orange jasmine flush (M=13.93, SD=13.99); whereas, there were no statistical differences in oviposition rate between the two host species when psyllids were reared on orange jasmine (Wilcoxon sign-rank test: V=253, P=0.126). Mortality was compared with chi-squared tests.

Development Time and Survival

Development time from 1st instar nymphs to adult psyllids varied significantly depending on the natal host species and host transfer treatment (Kruskal-Wallis rank sum test: $\chi^2_3 = 113.1$, $P<0.0001$; Figure 3-2, Table 3-1, Table 3-2). Of the 4 treatment groups, ''Valencia'' → ''Valencia'' psyllids developed 1-2 days faster than all other groups (Table 3-1). Orange jasmine → orange jasmine psyllids developed the slowest (Table 3-1).

When ''Valencia'' psyllids were transferred to orange jasmine, development time increased, and was statistically similar to the orange jasmine → orange jasmine group (Table 3-2). However, when orange jasmine psyllids were transferred to ''Valencia'' orange, development time decreased, but remained slower than ''Valencia'' → ''Valencia'' psyllids (Table 3-2).

Although mortality was low for all host transfer treatments, those maintained on their natal hosts species' experienced the lowest morality (Figure 3-3). Psyllids transferred from orange jasmine to ''Valencia'' orange and those transferred from ''Valencia'' orange to orange jasmine, experienced the greatest mortality. ''Valencia'' psyllids transferred to orange jasmine showed significantly greater mortality than psyllids maintained on ''Valencia'' plants ($\chi^2 = 4.35$, $P = 0.037$). However, there was no difference in mortality between orange jasmine reared psyllid maintained on orange jasmine and those transferred to ''Valencia'' orange plants ($\chi^2 = 1.58$, $P = 0.21$).

Post-hoc analysis (Tukey's HSD) showed significant differences in almost all comparisons of the control to the experimental group (Figure 3-4). Consistently, orange jasmine psyllids increased in size when moved to 'Valenica' orange plants, while

‘‘Valencia’’ psyllids decreased in size when moved to orange jasmine. There was one exception - ‘‘Valencia’’ psyllids did not display decreased abdominal size when moved to orange jasmine. There was also a significant effect for sex (Table 3-3). Female psyllids were consistently larger than males on all measurements

Activity Level

The amount of time taken to make a selection in a Y-maze varied by treatment. Psyllids transferred to ‘‘Valencia’’ orange plants took longer to make a selection than those transferred to orange jasmine plants regardless of the natal host (Figure 3-5). Orange jasmine reared psyllids reached the target 33% faster than those psyllids transferred to ‘‘Valencia’’ orange (Wilcoxon-sum rank test: *W*=398.5, *P*=0.01). Similarly, ‘‘Valencia’’ orange psyllids transferred to orange jasmine reached the target 40% faster than those maintained on ‘‘Valencia’’ orange plants (Wilcoxon-sum rank test: *W*=600.5, *P*=0.055).

Discussion

Our results demonstrate the adaptive significance of learning in the context of reproduction, and illustrate how experience-based maternal decisions benefit offspring fitness. First, we found that adult *D. citri* prefer plants with which they are experienced. Adult psyllids preferred to settle on their natal host species slightly more than the alternative host, with ‘‘Valencia’’ psyllids preferring ‘‘Valencia’’ plants and orange jasmine psyllids preferring orange jasmine plants with varying levels of significance over the 3-day observation period. However, species performance differed depending upon the host species with which the insects were experienced. While psyllids reared on

‘‘Valencia’’ orange displayed aversion to orange jasmine plants in favor of their natal host, orange jasmine psyllids oviposited on both host species equally.

The discrepancy between settling and oviposition preference may reflect differences in the risks of changing resources relative to feeding versus oviposition. With regard to feeding, adult *D. citri* demonstrate short-term natal host plant preferences, which may be overridden by additional experience on an alternative host plant (Stockton et al., 2016). Although more research is needed to confirm this observation, adult *D. citri* do not appear to be significantly affected by movement between hosts. Adult psyllids can move between host species without apparent fitness costs. However, the data presented here show that oviposition preferences acquired during development may last longer, and are more resistant to change. This is possibly because immature *D. citri* are more sensitive than adults to differences in plant-defense allelochemicals, which are known to vary greatly among potential host plants species (Hijaz et al., 2016; Robbins et al., 2012). If this is the case, rigid oviposition preferences may reflect a maternal ability to recognize potential threats to their offspring.

For the common sulphur butterfly, *Colias philodice* Latreille, host switching during late larval development is associated with decreased feeding, digestion, and larval weight due to difficulty acclimating to a novel host (Karowe, 1989). Similarly, the two-spotted spider mite (*Tetranychus urticae* Koch) can be reared on either bean or tomato plants, but display differential preference and performance depending on the host species with which they are acclimated (Agrawal et al., 2002). Tomato plants are more toxic than bean plants, and as a result, mites reared on tomato display up-regulated p450 enzymes (Agrawal et al., 2002). This increased detoxification ability allows mites reared on tomato to reproduce easily on both tomato and bean plants; whereas, mites reared on bean plants show deceased fecundity on tomato. We suggest that a similar effect may occur in *D. citri*.

Analysis of the volatile profiles of both *Citrus* and *Murraya* has shown that relative to orange jasmine and curry leaf plants, 'Valenica' orange emits lower concentrations of defensive sesqueterpenes, particularly β-caryophyllene, germcrene-D, and β-cubebene, (Robbins et al., 2012). In addition, psyllids reared on *Murraya* (orange jasmine and curry leaf) exhibit upregulated cytochrome p450 monooxygenase and glutathione s-transferase activity, which are both detoxification enzymes that allow psyllids to process defensive chemicals allelochemicals that would otherwise reduce herbivory (Liu et al., 2015). Insecticide resistant psyllid populations have been found with similar upregulated enzymes (Tiwari et al., 2011). Although direct investigation of host plant experience on insecticide resistance remains to be investigated, it is possible that insecticide resistance increases in psyllids reared on *Murraya* spp. This "preadaptation hypothesis" supposes that there are similar pathways involved in detoxifying both plant-produced allelochemicals and insecticides (Dermauw et al., 2013).

Our data also suggest that host fidelity affects offspring fitness. While orange jasmine nymphs transferred and reared on ''Valencia'' orange plants consistently increased in size, ''Valencia'' nymphs transferred to orange jasmine plants decreased in size. In addition, while orange jasmine nymphs did not show a significant effect of host transfer on mortality, ''Valencia'' nymphs were more likely to die if transferred to orange jasmine plants. This is consistent with our hypothesis that psyllids exhibit preference for less toxic hosts. While psyllids that developed on orange jasmine were ‘prepared for’ and even benefited from the change to a less toxic plant species, those reared on ‘‘Valencia’’ orange and transferred to orange jasmine exhibited reduced fitness, possibly because they were less effective in metabolizing the array of defensive chemicals characteristic of this new host. As a result, those nymphs developed into smaller adults. However, forced movement between hosts did not appear to affect psyllid activity level once they had completed adult development. Rather, our measure of activity level showed a decline in energetics associated with the host plant species rather than an effect of natal host experience. It is possible that the olfactory stimulant (β-caryophyllene) used in this assay was more attractive, and therefore more motivating, for psyllids recently exposed to orange jasmine plants. Previous research has shown that short-term adult experience with specific host plant volatiles can increase preference for those volatiles and override natal-derived preferences (Stockton et al., 2016).

By selecting the host plant species on which they themselves have successfully developed, female *D. citri* may avoid the risks associated with unknown species. As proposed by Beltman and Metz (2005), we suggest that maternal host preference is guided by an interplay among three key players: 1) genetic preference, 2) learned preference, and 3) varying levels of adaptedness to certain host plants depending on the natal environment. Although females may have innate feeding preferences, those preferences may be modified by experience to encourage alternative resource selection. In addition, the natal environment shapes maternal and offspring adaptedness to certain host species, further influencing future host selection. When the decision is solely based on feeding, females may be more likely to select hosts due to learned preference. However, for oviposition, females may be more likely to select hosts based on their own levels of adaptedness in an effort to protect unprepared offspring from plant defense allelotoxins. Due to incomplete natal host controls used in previous studies, it is unclear to what extent genetic preferences influence *D. citri* beyond general restriction to host plants in the family Rutaceae. It is possible that genetic preference in *D. citri* is limited to the selection of plants in the family Rutaceae, while selection within the family, particularly among plants within the genera *Citrus* and *Murraya*, is more experiential. However, future studies may clarify how this 3-way interaction shapes psyllid preferences such that, for this devastating disease vector, more reliable movement and transmission models can be developed to combat the spread of HLB.

Table 3-1. Psyllid emergence patterns by treatment. Descriptive statistics showing the total number of psyllids completing development (total emergence = *n*), as well as the mean ($\bar{x}$), median (*M*), and interquartile range (*IQR*) of the number of days required to complete development for each host transfer treatment.

Natal Host	**Transfer Host**	***n***	**$\bar{x}$**	***M***	***IQR***
‘‘Valencia’’ Orange	‘‘Valencia’’ Orange	216	9.84	10	11-9
‘‘Valencia’’ Orange	Orange Jasmine	161	11.42	12	13-10
Orange Jasmine	Orange Jasmine	210	11.73	11	13-10
Orange Jasmine	‘‘Valencia’’ Orange	248	10.92	11	12-10

Table 3-2. Wilcoxon sum- rank test comparisons of emergence time for each pair of host transfer treatments (natal host – transfer host). Host species denoted as ‘‘Valencia’’ (Val) and orange jasmine (OJ). Significant differences highlighted in bold. Bonferroni correction applied for multiple comparisons, $\alpha = 0.0016$.

Group 1	**Group2**	***W***	***P***
Val → Val	Val → OJ	26510	**<0.001**
Val → Val	OJ →Val	37659	**<0.001**
Val → Val	OJ → OJ	33400	**<0.001**
OJ → OJ	OJ →Val	29444	**0.014**
OJ → OJ	Val → OJ	16709	0.847
OJ → Val	Val → OJ	16324	**<0.001**

Table 3-3. MANOVA comparisons of five psyllid body characteristics by treatment and sex. Asterisks indicate statistically significant differences; * 0.05, ** <0.01, *** <0.001, ****<0.00001.

Body Characteristic		***df***	***F***	***p***
Wing				
	Sex	1	162.02	<0.0001****
	Treatment	3	143.74	<0.0001****
	Sex : Treatment	3	3.03	0.03*
	Residuals	742		
Femur				
	Sex	1	1.21	0.27
	Treatment	3	54.04	<0.0001****
	Sex : Treatment	3	1.30	0.27
	Residuals	742		
Head				
	Sex	1	53.90	<0.0001****
	Treatment	3	54.31	<0.0001****
	Sex : Treatment	3	0.27	0.85
	Residuals	742		
Abdomen				
	Sex	1	128.89	<0.0001****
	Treatment	3	18.15	<0.0001****
	Sex : Treatment	3	0.27	0.85
	Residuals	742		
Body				
	Sex	1	145.83	<0.0001****
	Treatment	3	32.01	<0.0001****
	Sex : Treatment	3	0.54	0.65
	Residuals	742		

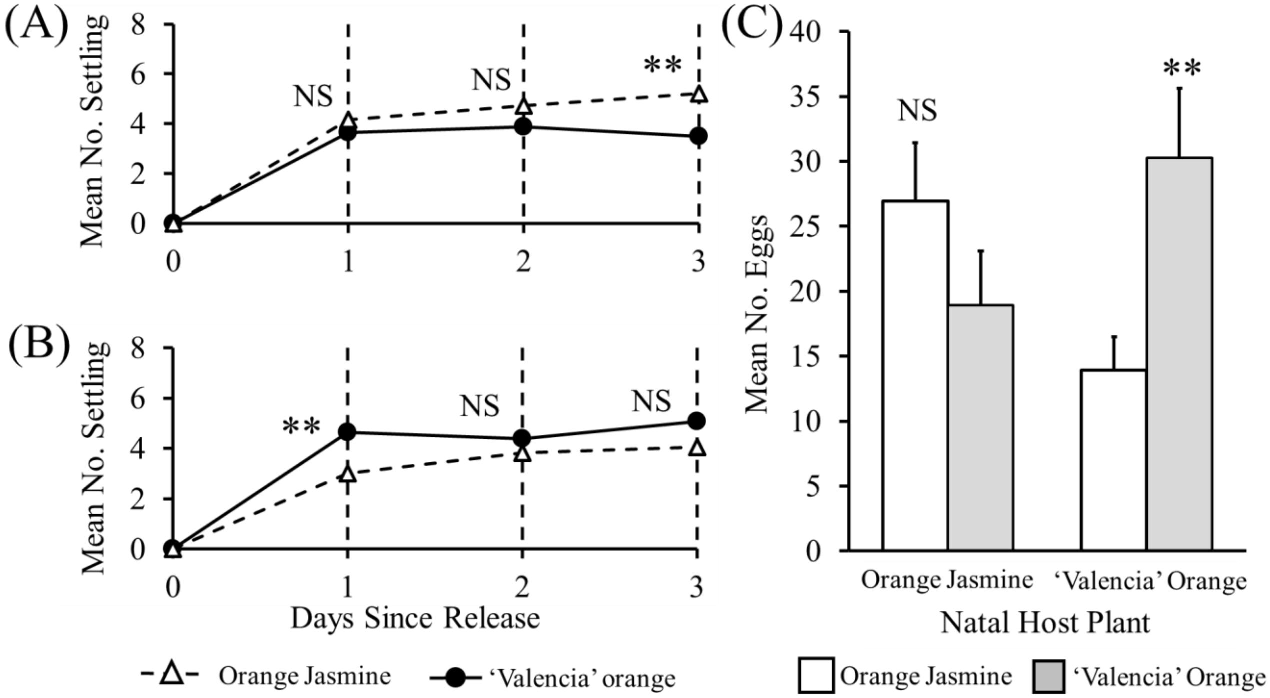

Figure 3-1. *D. citri* settling (a, b) and oviposition (c) choices on host plants based on previous experience. (a) Settling preference among psyllids initially reared on ''Valencia'' orange plants (*N*=40), and (b) psyllids initially reared on orange jasmine plants (*N*=40) are indicated by asterisks at each time point (t-tests: *≤ 0.05, **≤ 0.01, "NS" = non-significant). (c) Differences in oviposition rate (Mean ± SEM) in a two-choice test (''Valencia'' orange = grey bars; orange jasmine flush = white bars) among psyllids reared on either ''Valencia'' orange (N=30) or orange jasmine (*N*=30; Wilcoxon tests: *≤0.05, **≤0.01, "NS" = non-significant).

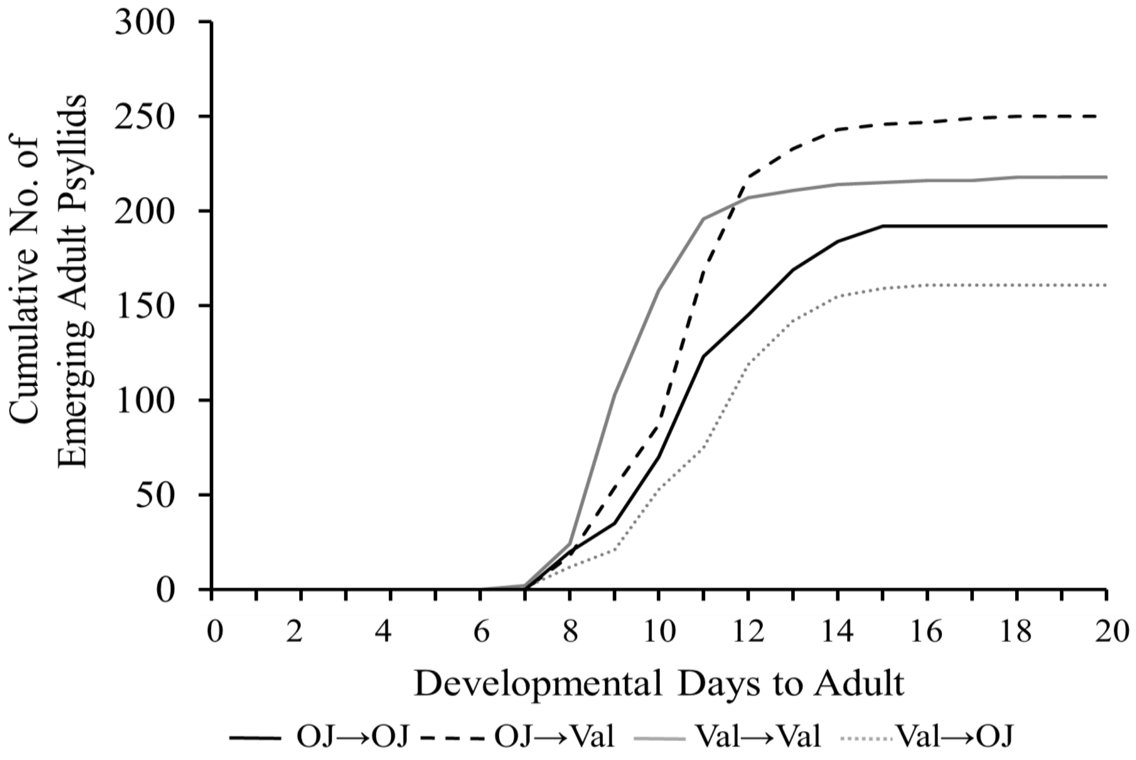

Figure 3-2. Cumulative emergence of adult psyllids over time depending on host treatment. ‘‘Valencia’’ orange (Val) and orange jasmine (OJ) psyllid transfer treatments are shown as natal host → transfer host in the Figure legend.

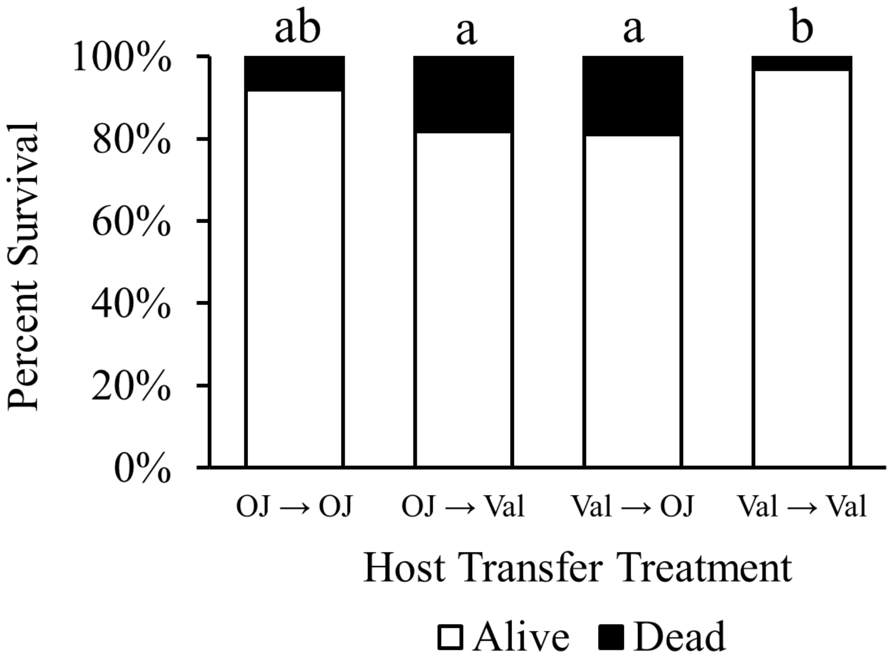

Figure 3-3. Mortality following host transfer. The number of living (white bars) or dead psyllids (black bars) after five days were compared with χ^2 tests. Different letters indicated statistically significant differences (α = 0.05).

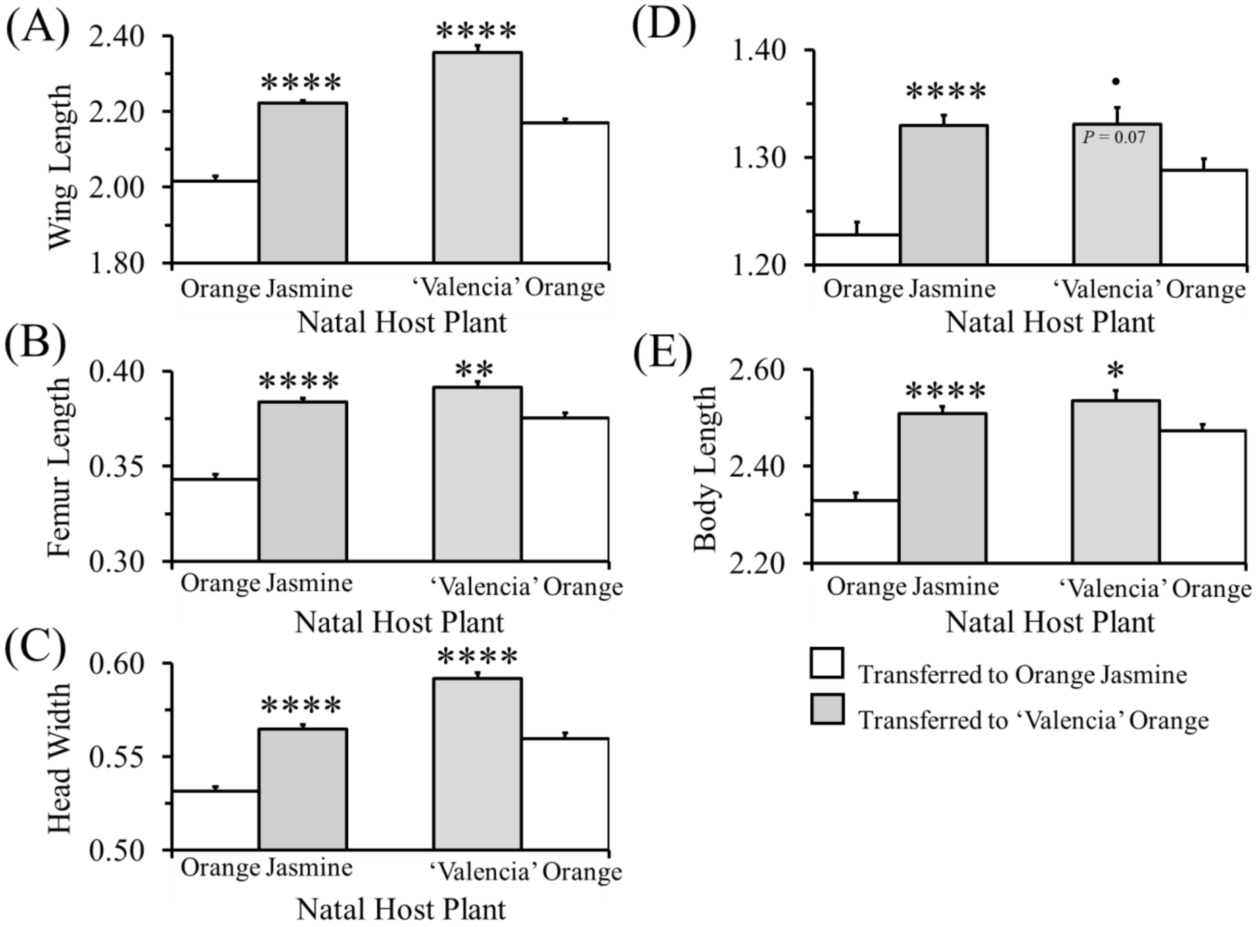

Figure 3-4. Morphometric comparisons of five adult psyllid body characteristics, wing length (a), femur length (b), head width (c), abdominal length (d), and body length (e) within each host transfer treatment. Asterisks indicate significant results from post-hoc multiple comparisons of structure size (Mean ±SEM) among psyllids reared on orange jasmine or ''Valencia'' orange (x-axes) and transferred to ''Valencia'' orange (grey bars) or orange jasmine (white bars) using Tukey HSD, NS = non-significant, •<0.1, * 0.05, ** <0.01, *** <0.001, ****<0.00001.

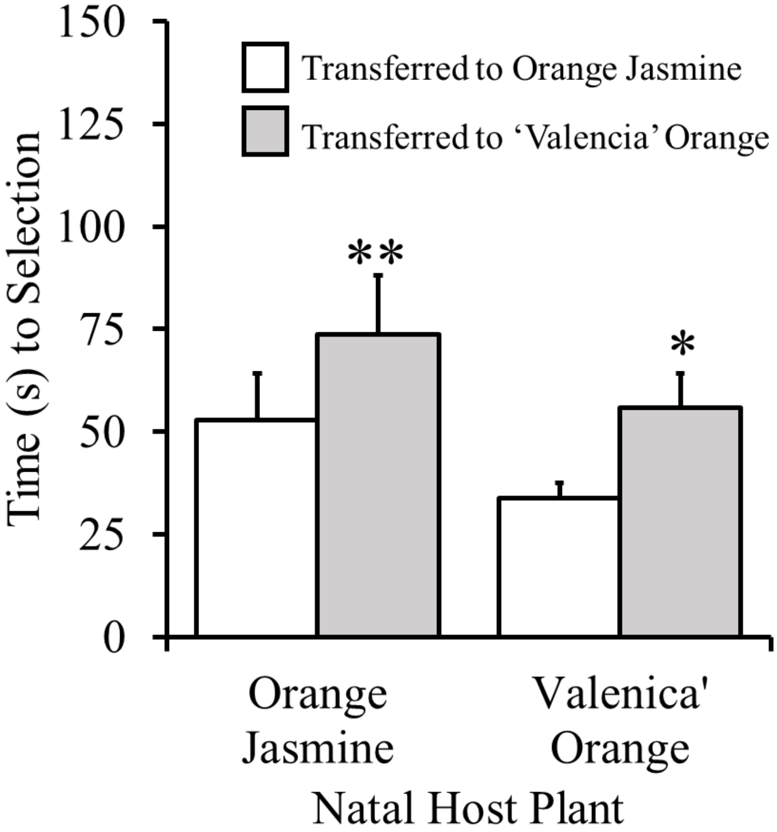

Figure 3-5. Latency (s) to target selection for psyllids reared on orange jasmine or ''Valencia'' orange plants (x-axis) and transferred to either orange jasmine (white bars) or ''Valencia'' orange (grey bars).

CHAPTER 4
LEARNED MATE CHOICE

Chapter Abstract

In some arthropods, females learn in the context of reproduction to refine their mate choices and avoid males displaying traits associated with impotency. Previous studies have shown that male psyllids (*Diaphorina citri*) associate female odor with copulatory rewards. However, it is unclear whether females similarly learn about male traits. We compared mate choice in females previously mated to either blue or orange males and found that females may associate male color with reproductive success, and avoided blue males after previous experience. Orange males mated more frequently than blue males and appeared to be more sexually aggressive in their mating attempts. In addition, females mated to orange males laid approximately twice as many eggs as those mated to blue males. We dissected male psyllids and measured the size of their reproductive organs to determine whether abdominal color was associated with reproductive development. Our morphometric analysis showed that blue males were not physiologically immature compared to orange males. Instead, blue males displayed larger testes and seminal vesicles than orange males, indicating that differences in male virility were likely behavioral. Based on our data, we suggest that females learn about the appearance and mating behaviors of blue and orange males from early mating experiences, and subsequently avoid blue males in future matings. Although the roles of blue males within a population are unclear, it is possible that blue morphs may specialize in other functions such as dispersal.

Background Information

Polyphenisms occur when two or more behavioral or physiological phenotypes are produced from a common genotype. This allows organisms to adapt to variability within the environment, such as seasonal changes requiring different physiological preparation to cope with temperature extremes, or in response to population pressure such that some individuals are well suited for the costly task of migration (Lancaster et al., 2014; Simpson et al., 2011; Calsbeek et al., 2010). In grasshoppers (Locusta migratoria), density-dependent polyphenism is responsible for the occurrence of migratory locusts, which although genetically identical to grasshoppers, display different coloration, enlarged wings and suppressed reproductive systems (Pener & Simpson, 2009). Similarly, when population density and seasonal shifts signal the need for overwintering, female aphids, which are normally wingless and reproduce parthenogenically, produce winged offspring for migration and become sexually reproductive (Moran, 1992). In the Asian citrus psyllid, *Diaphorina citri* Kuwayama, there has been an ongoing debate as to the biological significance of abdominal polyphenisms present in both males and females. These small invasive insects feed on citrus and related Rutaceae and transmit a pathogen that has caused severe economic losses in citrus worldwide (Grafton-Cardwell et al., 2013; Bové, 2006; Halbert & Manjunath, 2004; Garnier et al., 2000). For this reason, there is significant need to understand the biology and ecology of this pest species.

It is common for psyllids to display polymorphisms. In the pear psylla, *Cacopsylla pyricola*, a distinct winterform morphotype possesses longer wings with dark patches and undergoes reproductive diapause (Krysan, 1990). Similar changes occur in *Cacopsylla bidens* in response to seasonal variation in temperature and photoperiod

(Soroker et al., 2013). However, unlike the pear psyllid, *D. citri* is a tropical-subtropical species, and there is little variation in their appearance with respect to seasonal differences (Hall et al., 2011). In *D. citri*, the three commonly observed abdominal color phenotypes in field and laboratory populations appear to occur independently of abiotic seasonal factors: blue/green, yellow/orange, and gray/brown (Husain & Nath, 1927; Wenninger et al., 2008; Wenninger et al., 2009a). Although more research is needed to understand the function of these forms, such phenotypic variation may represent different stages of adult maturation (Wenninger et al., 2009a), differences in energetics/migratory potential (Martini et al., 2014), and/or physiological ability to detoxify allelotoxins and insecticides (Tiwari et al., 2012).

To our knowledge, the influence of color phenotypes on *D. citri* reproduction has been investigated only for blue/green and gray/brown phenotypes, with a large focus on female coloration. Wenninger et al. (2009a) found that while blue/green and gray/brown females do not differ in fecundity, the oviposition rate in females mated to gray/brown males decreased earlier than in females mated to blue/green males. In addition, egg fertility was lowest in females mated to gray/brown males. It is also commonly observed that yellow/orange females are gravid and that a transition from blue/green to orange is associated with increased egg load in mature mated females (Husain & Nath, 1927; Skelley & Hoy, 2004; Halbert & Manjunath, 2004; Wenninger et al., 2008). However, it is unknown how virility and copulatory success in yellow/orange males compares to blue/green or gray/brown males (Wenninger & Hall, 2008). Because we have observed that yellow/orange males are often found closely associated with oviposition sites in the field and in laboratory colonies, we postulated that orange males may play a distinct role in reproduction in this species. Furthermore, we hypothesized that females could assess abdominal color as a cue to predict the quality of future mates.

Previous research has indicated that learning plays a role in ecology and behavior of *D. citri* (Patt et al., 2014; Stockton et al., 2016). Male and female *D. citri* readily associate novel olfactory and visual stimuli with specific host plant genotypes and appear to prefer the host species with which they have had previous feeding experience (Stockton et al., 2016). Learning also influences mate attraction to female *D. citri* (Stockton et al., 2017). In male *D. citri*, we recently found that response to female cuticular odors was not entirely innate. Rather, mating experience with receptive females encouraged attraction towards female-produced odors (Stockton et al., 2017). However, the role of learning in the reproductive repertoire of female *D. citri* is unknown. Given the importance of vision for orientation in *D. citri* (Patt et al., 2011; Setamou et al., 2012; Setamou et al., 2014; Paris et al., 2015), it is reasonable to suspect that abdominal coloration in this species may serve a purpose in mate selection. A better understanding of mate selection in the context of learning for *D. citri* may allow for improvements in behaviorally based management tools (Mankin, 2012; Mankin et al., 2016; Mann et al., 2013; Wenninger et al., 2009).

Indeed, learning allows organisms to behaviorally adapt to their existing environment, thereby increasing the likelihood of survival and reproduction (Dukas, 2013; Svensson et al. 2010; Egas & Sabelis, 2001; Egas et al., 2003; Papaj & Prokopy, 1989). Learned mate choice may affect sexual selection (Whitte & Nobel, 2011) and speciation at least four ways: through sexual imprinting in immatures (Verzijden & ten Cate, 2007; Immelmann, 1972), learned mate preferences in mature adults (Dukas, 2004, Dukas, 2005a, Dukas 2005b, Magurran & Ramnarine, 2004), eavesdropping via female observations of male-male competition (Doutrelant & McGregor, 2000), and mate-choice copying whereby females observe the mate choices of other females (Dugatkin & Godin, 1992; Laland, 2004). Learned mate choice appears to confer fitness benefits that improve courtship efficiency and mate discrimination by allowing individuals to recognize and avoid costly matings with heterospecific species (Seigel, 1979; Wcislo, 1987; Dukas, 2004; White, 2004; Magurran & Ramnarine, 2004; Witte & Nöbel, 2006; Svensson et al., 2010) and select mates that maximize egg production (Dukas, 2005a). In the banded demoiselle, *Calopteryx splendens*, female preference for conspecific males is a learned adaptation to male variation in sympatric and allopatric populations (Svensson et al., 2010). In *Drosophila*, female preference for mate size is determined by early mating experience with courting males (Dukas, 2005a). Similarly, male flies learn to avoid heterospecific species after early courtship experience (Dukas, 2005b, Dukas, 2004). We hypothesized that adult female *D. citri* may similarly learn about the relative virility of males and use traits associated with reproductive success to refine their future mate choices.

Our goal was to investigate learned female mate choice in *D. citri*, focusing on variation in male abdominal color as a possible indicator of virility in male psyllids. We hypothesized that male abdominal color could serve as an indicator of reproductive maturity since male abdominal coloration was correlated with age in previous studies and in females, abdominal color is associated with gravidity (Husain & Nath, 1927; Wenninger et al., 2009a). Naïve virgin females were mated to either blue or orange males. Thereafter, they were given a choice between blue or orange males to determine learned preference. We quantified the frequency of male mating attempts in order to compare the relative sexual tenacity of orange and blue males. Next, we quantified oviposition rate and spermathecal size in females mated to orange and blue males to determine whether male mate choice caused differences in fecundity following copulation. Male abdominal color was associated with differences in reproductive potential. For that reason, we performed additional experiments to determine whether the observed differences in fecundity were due to behavioral or physiological differences between orange and blue males. We tracked transitions in male color and used morphometric analysis of compare the reproductive organ development of orange and blue males following eclosion.

Methods

Insect Colonies and Host Plant Maintenance

D. citri were obtained from a colony maintained at the University of Florida Citrus Research and Education Center in Lake Alfred, Florida (Stockton et al., 2016). Insects were reared on orange jasmine (*Murraya paniculata* (L.) Jack.) and kept in screen cages. All colonies were maintained in a climate-controlled greenhouse at 28oC and L14:D10 light cycle. To maintain *D. citri* reproduction and ensure plant health, host plants were rotated out of colony cages once per month and replaced with new plants.

To ensure that virgin insects remained unmated, *D. citri* were collected one week prior to use as 4-5th instar nymphs. Those nymphs were transferred onto a clipping of leaf tissue from the larger orange jasmine colony to a separate cage with a single orange jasmine plant. The nymphs were allowed to independently migrate from the clipping to the new plant. As new adult psyllids emerged, they were removed daily, sexed with the aid of a dissecting microscope, and placed into separate cages with clean orange jasmine plants. Virgin male cages and virgin female cages were kept on separate benches within the greenhouse. The age of the colonies was recorded to ensure that the insects used across replicates were of similar age.

Although there are three recognized abdominal color phenotypes in *D. citri* (gray/brown, blue/green, and yellow/orange), the gray/brown phenotype is characterized by lower fitness than the other two, with decreased size, dispersal, fecundity, and longevity (Wenninger & Hall, 2008; Wenninger et al., 2009a). In addition, the gray/brown color morph occurs in lower frequencies in the field compared to blue/green and orange/yellow psyllids (Martini et al., 2016). For these reasons, males with a gray/brown abdominal color, or those not easily classified, were excluded from the experiment. Herein, we refer to the blue/green phenotype as 'blue' (Figure 4-1 A) and the yellow/orange as 'orange' (Figure 4-1 B).

Insect Marking Technique

To distinguish between male and female insects during our experiments without disruption, female insects were marked on the thorax (Figure 4-1 C) with a bright yellow dot composed of 80% water-soluble liquid craft glue (Elmer's® glue-all multi-purpose glue) and 20% yellow powder (#1162Y luminous powder- yellow, BioQuip® Products, Inc., Rancho Dominguez, CA). The glue mixture was carefully applied to the thorax with a minuten pin, such that no contact was made with wings, limbs, or antennae.

Marking did not deter mating attempts by males or inhibit the ability of females to mate.

Female Mate Choice

We observed how initial mating experience affects subsequent mate choices in female psyllids (Figure 4-2). Individual virgin females (4-7 days old) were initially paired with four males of the same color for 24 h. The insects were kept inside 35 x 10 mm polystyrene Petri dishes overnight (Fisherbrand™). This design allowed females to experience courtship and mating from all blue or all orange males exclusively (n blue mated females = 27, n orange mated females = 23). As a control, another group of females (n naïve virgin females = 71) remained unmated, but were similarly confined to a dish for 24 h.

After the 24 hr experience period, the males were removed. To test the effect of experience on future mate choice, all females were then presented with a choice between two blue males and two orange males. Female receptivity to each of the color morphs was recorded, as described above, over a 2 h period. The number of successful matings between females and blue or orange males was compared. This experiment was replicated 3 times.

All adult virgin females were 4-7 days old and displayed ‘blue/green’ abdominal coloration at the time of use. Females that failed to sclerotize within 4 days, displayed abdominal coloration other than ‘blue/green,’ displayed signs of malformation, or appeared otherwise unhealthy were discarded from the study. All males were collected from virgin male cages and were 7 days old at the time of use. This allowed us to study the effect of abdominal color in isolation and remove the effects of age. Males were identified as either blue or orange, based on the color of the abdomen (Figure 4-1 A & B).

We operationally defined mating as the joining of a male and female psyllid for more than 2 minutes. Initial observations, and previous reports (Wenninger & Hall, 2007), indicated that 2 min was sufficient to distinguish between a mating attempt and a true mating. Receptive females will allow the male to remain conjoined for an average of 45 min. We did not interrupt mating within the observation arena. The psyllids were allowed to mate ad libitum for 2 h.

Male Sexual Behavior

To quantify and compare the sexual aggression of the different male psyllid phenotypes, males were observed courting and mating females within a confined arena. All males were 7 days old and were collected from the virgin male colony. After sorting male psyllids according to color phenotype, 2 orange and 2 blue males were placed in a 35 x 10 mm polystyrene Petri dish (Fisherbrand™) along with one marked female (n = 121; Figure 4-1 C). All females used in this experiment were collected from the general orange jasmine colony and were not controlled for mating status. However, females were excluded if they displayed abdominal coloration other than ‘blue/green,’ displayed signs of malformation, or appeared otherwise unhealthy.

For each trial, we observed the mating success of one female paired with four males (2 blue and 2 orange) for 2 h. During the observation period, the psyllids were not interrupted and were allowed to mate *ad libitum*. The number of “attempted matings” and the number of successful “matings” was recorded. Matings were defined as the joining of a male and a female for more than 2 min. Mating that occurred for less than 2 min was counted as a mating attempt. Attempted matings were further categorized as either a “true attempt,” where a male attempted to mate with a marked female; or a “false attempt”, during which a male attempted to mate with another male. The color of the male making the observed behavior was noted. Approximately twenty trials were observed simultaneously per replicate. Two observers were present for all replicates to ensure that all behaviors were recorded. This experiment was replicated 6 times for a total of 121 trials.

Female Oviposition and Reproductive Organ Size

We quantified the number of eggs laid by females after isolated copulation with either blue or orange males. For each replicate (n orange mated = 29, n blue mated = 29), three virgin female psyllids (4-7 days old) were confined to a 10 x 28 cm cage containing two flushing orange jasmine clippings. Five virgin male psyllids (7 days old) were added to each cage. Each cage contained either blue or orange males exclusively. The psyllids were free to mate and feed for 5 days. After 5 days, all of the psyllids were removed and the number of eggs laid on the clippings was counted with the aid of a dissecting microscope at 40X magnification.

Next, we measured the size of the spermathecae from females mated to blue males (n=33), orange males (n=33), and unmated virgin females (n=56). Females from each of the cages were stored in 70% ethanol at 20oC until the dissections were made. The spermathecae were dissected with the aid of a dissecting microscope at 10X magnification, plated on a microscope slide, and photographed with a digital microscope (Dino-Lite ©, New Taipei City, Taiwan). Image analysis was performed with ImageJ software (Version 1.51, National Institutes of Health, Bethesda, MD, USA). We measured the length (a) and the width (b) of each spermatheca. Spermathecal area was calculated as the area of an ellipse, (area = πab), where a = ½ length of the structure, and b = ½ width.

Male Color and Reproductive Organ Size

Male psyllids were observed for 7 days to determine whether color changes as males age. We used this aging duration to directly compare with the mate choice and male sexual behavior experiments. The males in those experiments were also 1 wk old. Prior to use, virgin males were isolated in a cage on orange jasmine plants. Male color was recorded at 3 discrete time points, 0 days (day of emergence; n = 70), 4 days (n = 87), or 7 days post-eclosion (n = 92). The insects were not reintroduced into the colony after their use.

We also measured the size of male psyllid reproductive organs using a digital microscope. Males were collected from orange jasmine plants in the virgin male colony cages. Dissections of testes (Ts, n = 160), seminal vesicles (SV, n = 167), and accessory glands (AG, n = 113) were made at 0, 4, and 7 days post-eclosion with the aid of dissecting microscope at 10X magnification, mounted on microscope slides, and photographed. Photographs of the organs were taken with a digital microscope (Dino-Lite ©, New Taipei City, Taiwan). Image analysis was performed with ImageJ (Version 1.51, National Institutes of Health, Bethesda, MD, USA). Total area of the structures was estimated as the area of an ellipse, area = πab, where a = ½ length of the structure, and b = ½ width.

Statistical Analysis

Female mate choice and male sexual behaviors were analysed with non-parametric Wilcoxon sum-rank tests because of the non-normal distribution of the data. We compared the mean number of eggs laid by females mated to blue or orange males with a t-test. We compared spermathecal area in females mated to blue and orange males using analysis of variance (ANOVA) and student’s t-tests.

We compared the proportion of males displaying orange abdominal coloration at three time points (0 days, 4 days, 7 days) using Chi-squared tests. One-way analysis of covariance (ANCOVA) was conducted to determine the effect of male color (independent variable) on organ size (dependent variable) controlling for male age (covariate). A separate ANCOVA test was conducted for each of the three male reproductive organs: the testes, seminal vesicles, and accessory glands. Student's t-tests were used to compare the mean differences in organ size between blue and orange males. Analyses were performed with the statistical software R (Version 3.0.2; the R Foundation for statistical software R; Vienna, Austria).

Results

Female Mate Choice

Female mate choice varied based on the original mating experience of the female (Figure 4-3). Females originally mated to orange males selected orange and blue males with similar frequencies (W=216.5, P=0.246). However, females mated originally to blue males selected significantly more orange males than blue males (W=259.5, P=0.046). Virgin females with no previous mating experience (negative control) selected orange males and blue males at similar rates (W=2299, P=0.319).

Male Sexual Behavior

Abdominal color had a significant effect on the frequency of observed mating attempts by male *D. citri* (Figure 4-4). Orange males performed more false mating attempts than blue males (W=5357, P=0.0002). Orange males also performed more 'true' mating attempts (heterosexual) than blue males (W=5215.5, P<0.0001). Finally, orange males successfully mated more often than blue males (W=6226, P=0.027).

Female Oviposition and Reproductive Organ Size

The mean number of eggs laid by mated females was significantly greater when mated to orange than blue males (Figure 4-5 A). Comparative dissections of females mated to blue or orange males did not reveal statistically significant differences in spermathecal area (t 64 = 1.50, P = 0.14; Figure 4-5 B, Figure 4-6 A-C). However, spermathecal area was significantly smaller in virgin females than in mated females (ANOVA: F2, 119 = 49.57, P < 0.0001; Figure 4-5 B; Figure 4-6 A-C).

Male Color and Reproductive Organ Size

Observations of male abdominal color prevalence showed that male color changed significantly over time (Figure 4-7). There was a significant increase in the proportion of orange males at 4 days post-eclosion compared to day 0 ($\chi^2 = 17.91$, $P < 0.0001$). The greatest proportion of orange males occurred at 7 days post-eclosion ($\chi^2 = 34.01$, $P < 0.0001$). There was no difference in the proportion of orange male psyllids between 4 days and 7 days ($\chi^2 = 3.66$, $P = 0.056$).

ANCOVA showed a significant effect for post-eclosion age on male reproductive organ size (Figure 4-6 D-G, Figure 4-8, Table 4-1). Over the course of 7 days, all three organs approximately doubled in size. Size of the testes ($F_{3,156} = 44.71$, $P<0.0001$, Figure 4-8 A), seminal vesicles ($F_{3,163} = 45.69$, $P<0.0001$, Figure 4-8 B), and accessory glands ($F_{3,109} = 18.23$, $P<0.0001$; Figure 4-8 C) increased significantly over time. The slopes were not significantly different between the different color morphs for testis size ($F_{1,156} = 0.0388$, $P = 0.844$), seminal vesicle size ($F_{1,163} = 0.316$, $P = 0.575$), and accessory gland size ($F_{1,109} = 0.160$, $P = 0.690$).

We also directly compared the size of those reproductive organs in 7-day-old males using student’s t-tests. Male color phenotype was a significant factor affecting the size of testes, with 7 day-old blue males displaying larger testes (t 55 = 2.91, P = 0.005) than 7 day-old orange males (Table 4-1). Blue males had slightly larger seminal vesicles than orange males, although this difference was not statistically significant (t 63

= 1.75, P = 0.086). There were no differences in accessory gland size between blue and orange males (t 55 = 0.63, P = 0.529).

Discussion

We investigated the influence of mating experience on subsequent mate choice by female *D. citri* among males displaying abdominal color phenotype variations. We hypothesized that females may learn to prefer orange males after mating experience based on findings indicating that orange males were typically more mature than blue males (Wenninger et al., 2009a). We found that indeed, mate experience did influence future female mate choice. Females originally mated to blue males preferred orange males in subsequent matings. In contrast, naïve, unmated females did not discriminate between blue and orange males, suggesting that a difference in subsequent male preference was learned, rather than genetically determined. In addition, naïve females displayed lower rates of mating compared to previously mated females, suggesting that mating experience may increase female receptivity over time.

In other psyllid species, traits such as mate age and mating status have been shown to influence mate attractiveness. In the pear psylla, *C. pyricola*, older summerform females are more attractive to males than younger females (Horton et al., 2008). This preference appears to be associated with differential production of pheromones as *C. pyricola* females age, and mating status does not affect male mate attraction in this species. It is unknown whether female pheromone production varies with age in female *D. citri*. However, *D. citri* mating status does appear to affect mate preference (Stockton et al., 2017). Mated males display increased attraction to female odors after mating experience. This appears to be a learned response and depends on a close association between female odor and mating. Males exposed to the odor in the context of feeding or ambient exposure failed to demonstrate attraction to female odor (Stockton et al., 2017). Other research on male *D. citri* has shown that with regard to appearance, blue females are more attractive than gray females (Wenninger et al., 2008). To our knowledge, our findings on female mate choice are the first to demonstrate learned female mate preference in *D. citri*. Female mating status directly affected female receptivity to male courtship. In addition, male appearance influenced the copulatory success of *D. citri* and affected female fecundity. These results appear consistent with findings in other species, where learned mate preferences are dynamic, adaptive, and often associated with fitness benefits (Verzijden et al., 2014; Dukas, 2013; Verzijden et al., 2012; Kozak et al., 2011; Verzijden et al., 2008; ten Cate & Voss, 1999).

There are several hypotheses that may explain why the females in our experiments avoided blue males after mating experience, and we suggest three possibilities here. The first is that female *D. citri* are genetically predisposed to prefer orange males to blue counterparts. Our data appear to falsify this hypothesis - it was only after mating experience that females displayed male preference. The second is that blue males are physiologically immature and therefore sexually inadequate mates. However, our experiments controlled for male age and showed that male testes were larger in blue males than orange males. The third hypothesis is that blue males are behaviorally less fecund. Although they are physiologically capable of mating and producing offspring like orange males, blue males may be less likely to court females. Our data support the third hypothesis given that blue males attempted to mate and successfully mated less often than orange males. This was further confirmed by our oviposition data, which showed that females mated to orange males laid approximately 2x as many eggs as females mated to blue males. However, it is unclear why blue males were less sexually active.

During our observations, we noticed that male abdominal color appears to be associated with the amount of fat body present in the abdominal cavity. While the internal organs of all male psyllids are orange, the blue fat body in some males obscures the testes and seminal vesicles. In addition, as the size of the reproductive organs increases, the orange coloration of the testes becomes more apparent. To our knowledge, the biochemical significance of the blue fat body in *D. citri* is currently unknown. In other insects, the fat body serves as an energy reserve for migration and other energetically costly tasks (Arrese & Soulages, 2010). In the corn earworm, Helicoverpa zea, the perivisceral fat body is also blue and gains its pigmentation from sequestered lipoproteins and biliverden (Haunerland & Shirk, 1995; Wang & Haunerland. 1992). Previous behavioral and biochemical research on *D. citri* has shown that blue psyllid phenotypes are associated with greater flight capability (Martini et al., 2014) and higher levels of insecticide resistance (Tiwari et al., 2012) than orange psyllids. Unlike orange males, which appear well suited for reproduction, blue males may instead function as a migratory group, better equipped for dispersal flights in search of new host resources (Martini et al., 2014).

There are several questions to address following this investigation. We are uncertain why blue males had larger testes than orange males despite lower levels of fecundity. Testis size is likely proportional to overall body size (Wenninger et al., 2009a;

Martini et al., 2014), although we did not measure male body size prior to our dissections and measurements of the male reproductive organs. It possible that the timeline used in this study was insufficient to fully describe the transitions in abdominal color and organ size. Adults psyllid live approximately 30 days (Wenninger et al., 2009a), and if given more time, a higher proportion of males may have transitioned into the ‘orange’ group. At present, we are also uncertain why the females’ spermathecae were of similar size regardless of mate color. The spermathecal structure in *D. citri* appears similar to laurel psyllid, *Trioza alacris*, with sperm held both loosely and in ordered packets, referred to as spermatodoses (Marchini et al., 2014). Unfortunately, an adequate sperm quantification assay for psyllids is currently unavailable. If such a method were to be developed, we could directly address the relative fertility of blue and orange males.

Previous research on learning in *D. citri* indicates that this species uses experience to refine its selection of resources and mates within the host plant environment (Stockton et al., 2016; Stockton et al., 2017). Males and females are capable of olfactory and visual associative learning, and female host plant preference appears to be dependent on natal host plant experience (Stockton et al., 2016). With regard to reproduction, mate preference also appears to be dynamic, rather than a static response. We previously reported that male *D. citri* appear to learn about female odor such that experienced males are more attracted to females than naïve virgin males (Stockton et al., 2017). The results of this study extend our current understanding of the reproductive behaviors of *D. citri* and suggest that female mate preferences may be influenced by early adult mating and courtship experiences with different male phenotypes, such that females use male abdominal color to discriminate among potential mates. Females originally mated to blue males may learn traits associated with those males and subsequently avoid them in favor of alternative mates. By using experience to modify mate choice, *D. citri* may enhance its reproductive success by selecting the most viable mates with the greatest likelihood of producing fecund offspring. Combined with previous findings on learning in *D. citri*, our data present increasing evidence of behavioral plasticity in this species, which appears to be adaptive and consistent with the behavior of other insect species known to display learned mate choice (Verzijden et al., 2014; Dukas, 2013; Verzijden et al., 2012; Svensson et al., 2010).

Table 4-1. ANCOVA results showing the significance of age and color as factors affecting male reproductive organ size.

Variables	***df***	***F***	***P*-value**
Testes			
Age	1	121.01	<0.0001
Color	1	13.07	0.0004
Age x Color	1	0.04	0.84
Residuals	156		
Seminal Vesicles			
Age	1	133.75	<0.0001
Color	1	3.01	0.085
Age x Color	1	0.32	0.57
Residuals	163		
Accessory Glands			
Age	1	52.59	<0.0001
Color	1	1.95	0.16
Age x Color	1	0.16	0.69
Residuals	109		

Figure 4-1. Representative images of blue males (A), orange males (B), and marked females (C) used in the mating experiments. Photos courtesy of author.

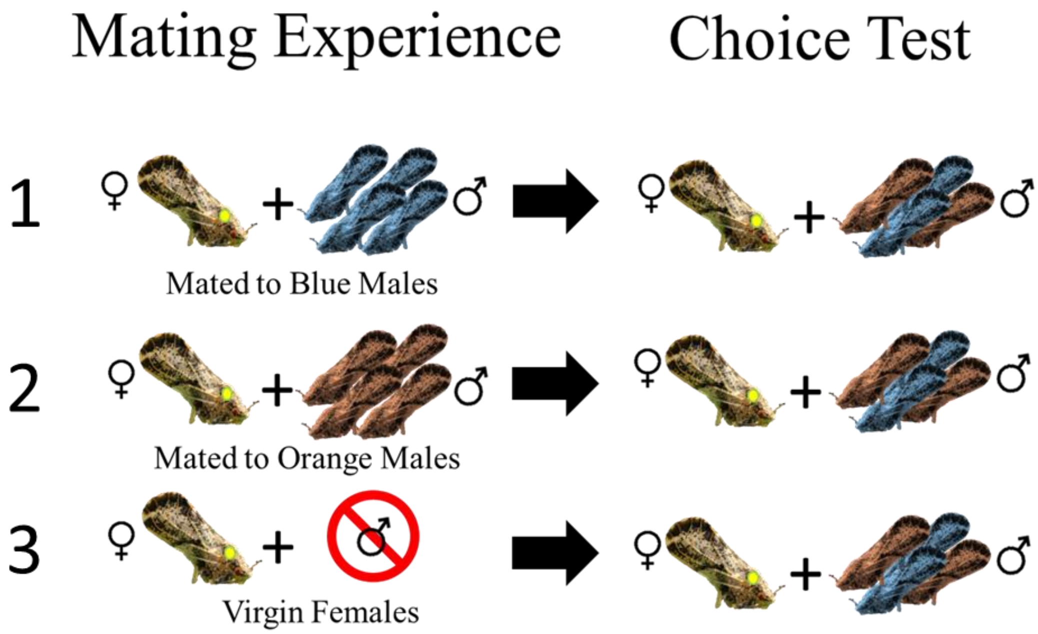

Figure 4-2. Experimental design of the learned female mate choice test. Females were originally mated (left) to either four blue males (1), four orange males (2), or remained unmated (3). After 24 hrs, the females were tested in a 2-choice design (right) to determine whether females displayed preferences based on male color and if those preferences were influenced by previous experience.

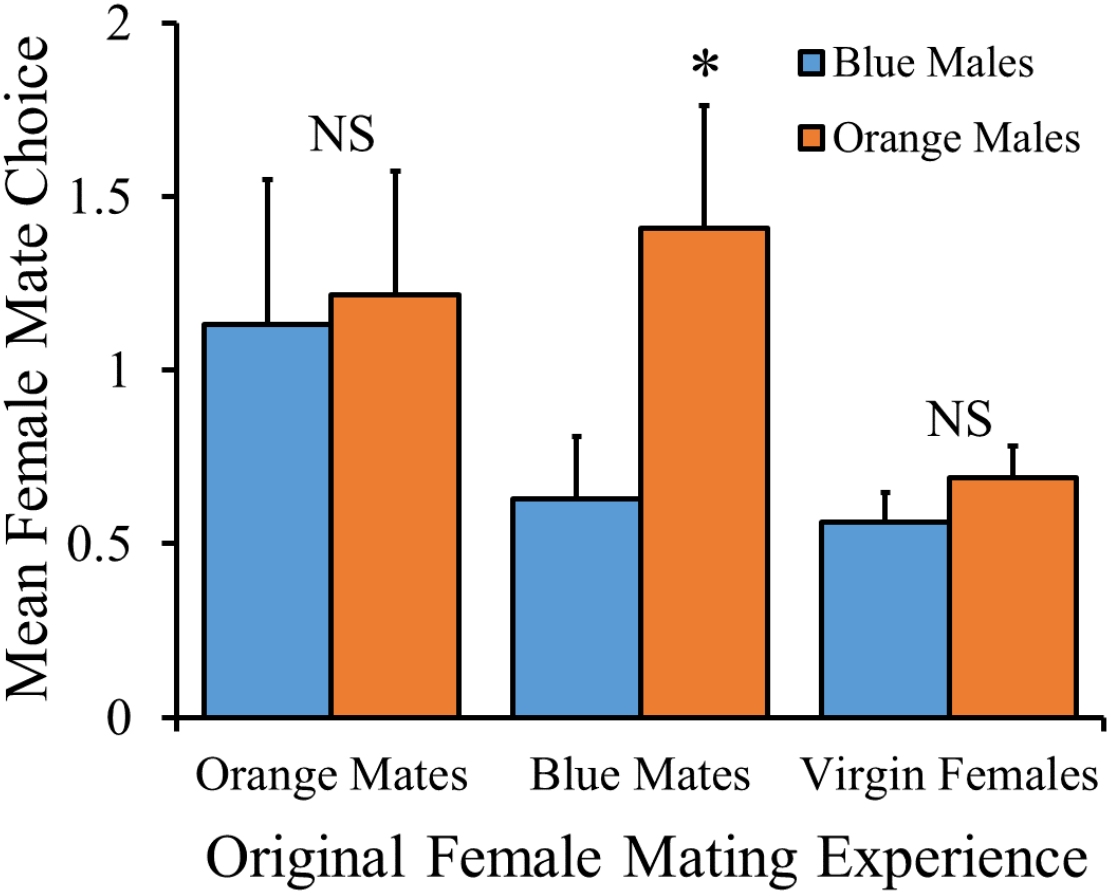

Figure 4-3. Female mate choice depending on experience and male abdominal color. Females were mated exclusively to either blue or orange males for 24 h (x-axis). Virgin females remained naïve/unmated. Thereafter, females selected between blue males (blue bars) or orange males (orange bars) in a choice test. Asterisks indicate statistical differences in the number (Mean ± *SEM*) of matings (Wilcoxon sum-rank test: *=0.05, “NS” = non-significant).

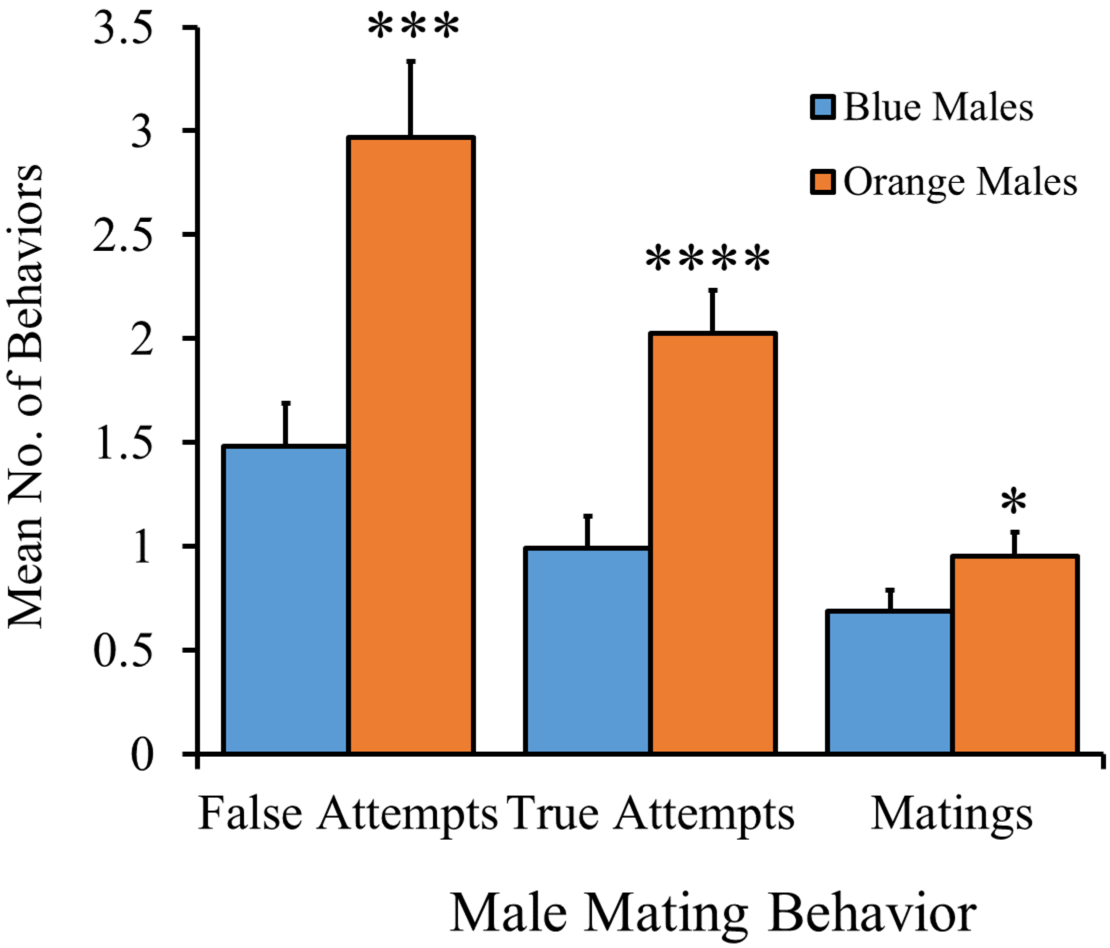

Figure 4-4. Differences in male mating behaviors between orange and blue color morph *D. citri* males. Mean ± *SEM*, Wilcoxon sum-rank test: *≤0.05, **≤0.01, ***≤0.001, ****≤0.0001.

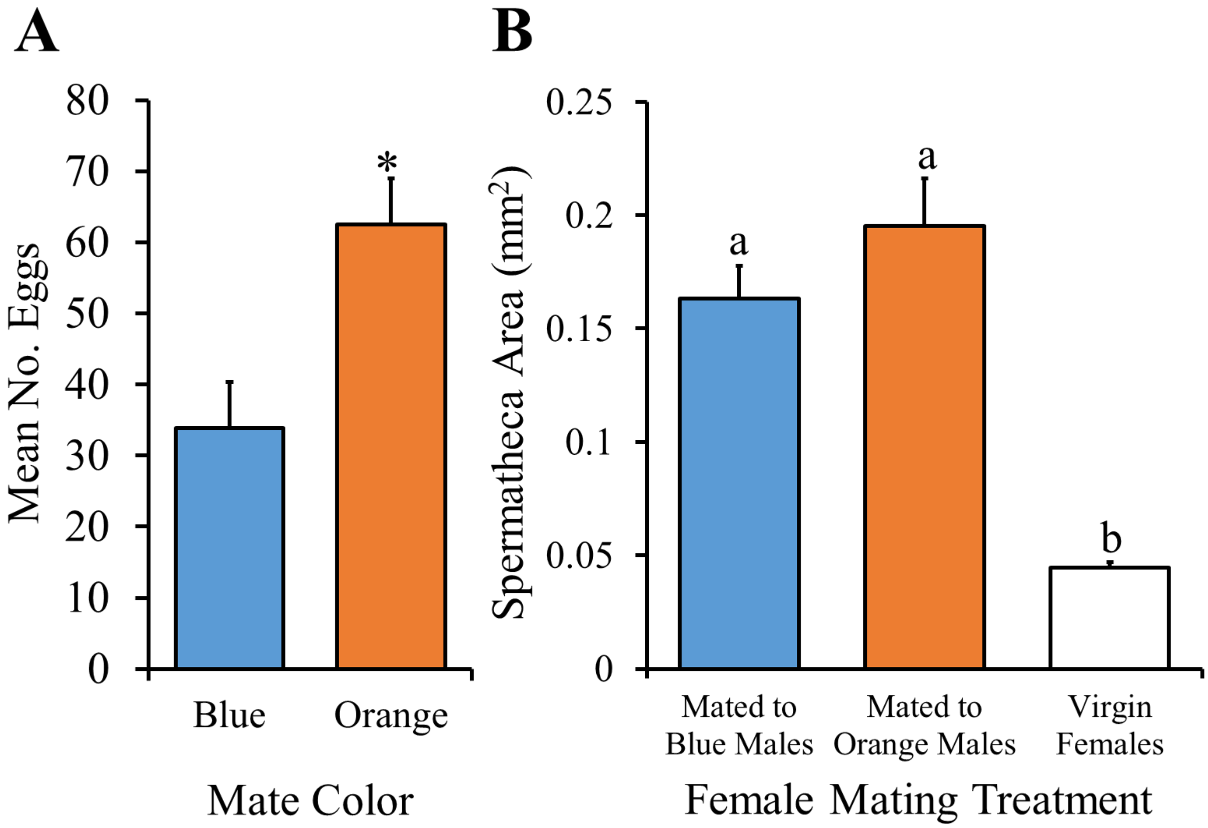

Figure 4-5. Differences in Mean ± *SEM* female *D. citri* oviposition, Student's *t*-test: *≤0.05, * ≤ 0.05 (A) and Mean ± *SEM* spermathecal area (mm^2), Student's *t*-test: α = 0.05 (B) depending on male mate color. Blue bars indicate females mated to blue males, orange bars indicate females mated to orange males and white bars indicate virgin females. Different letters indicates statistical differences in spermathecal area.

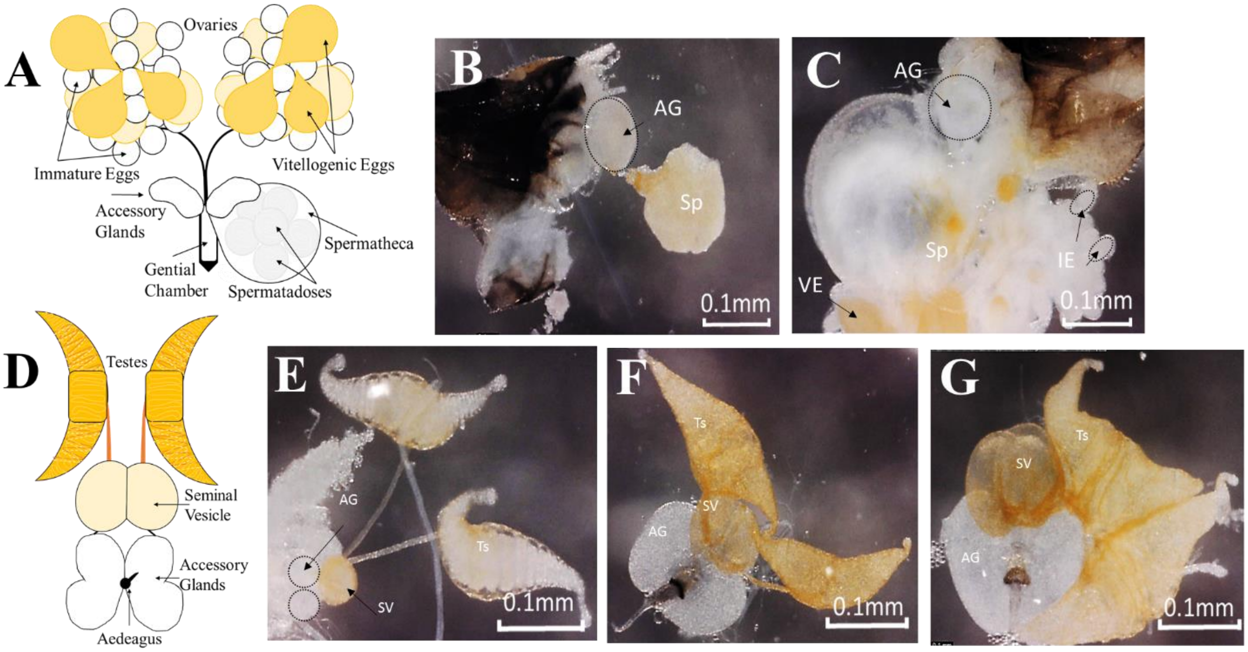

Figure 4-6. Female (A-C) and male (D-G) psyllid reproductive anatomy. Reproductive organs in a virgin female (B) and a recently mated female (C) highlight the difference in spermathecal (Sp) size after sperm reception. Also shown are the female accessory glands (AG), vitellogenic eggs (VE) and immature eggs (IE) (A-C). Male testis (Ts), seminal vesicle (SV), and accessory gland (AG) size is shown at 0 days (E), 4 days (F), and 7 days (G) post-eclosion. Photographs courtesy of the author.

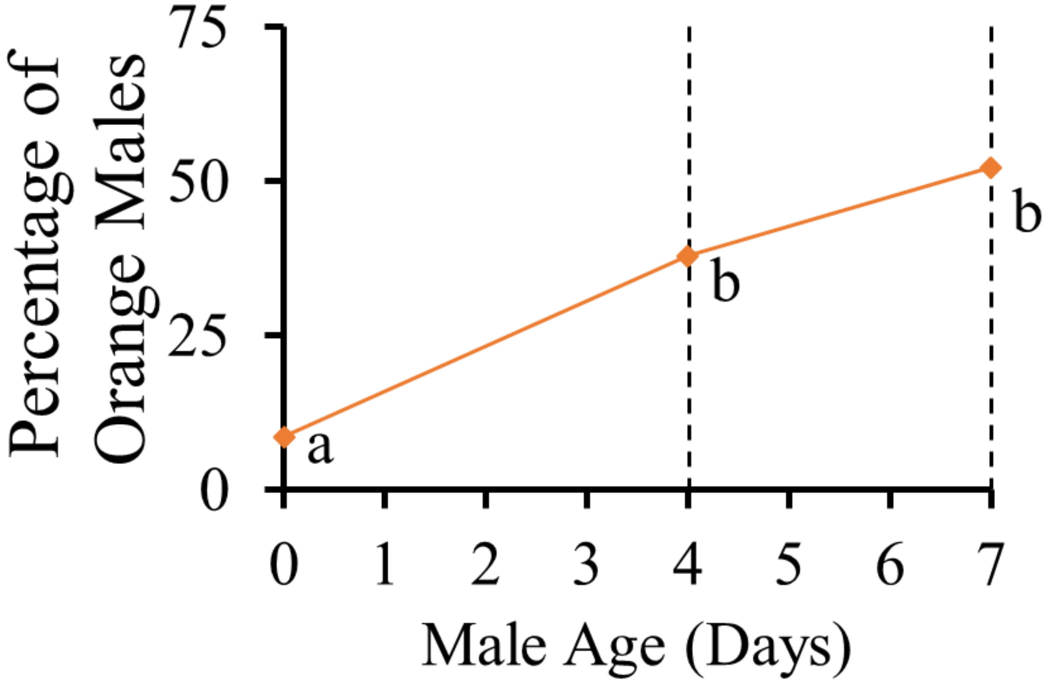

Figure 4-7. Proportion of males displaying orange abdominal color at each time point within the sample. Different letters indicate statistical differences in the proportion of orange males at 0 days, 4 days, and 7 days post-eclosion, χ^2 test, $\alpha = 0.05$.

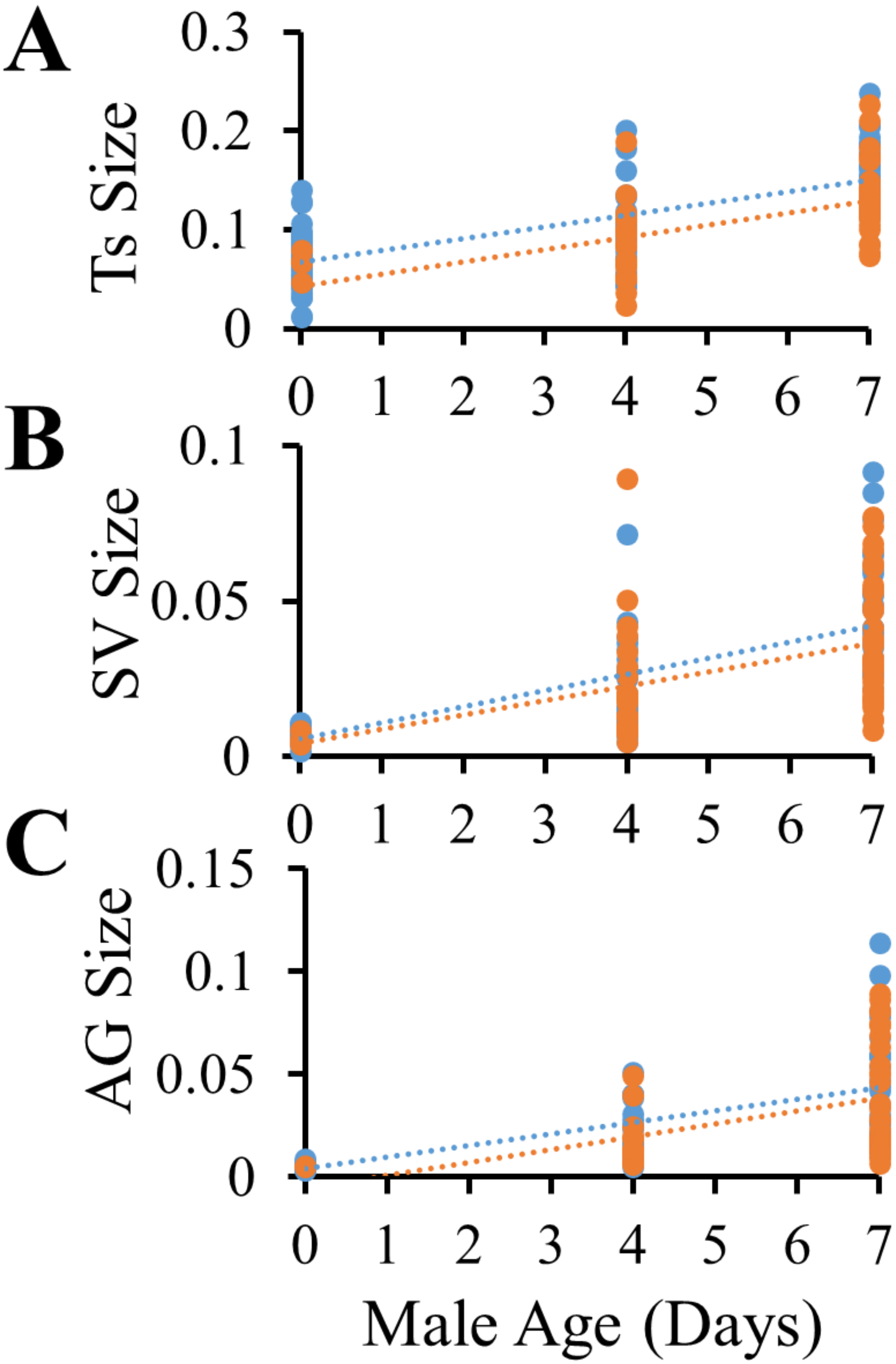

Figure 4-8. Rate of male reproductive organ development over 1 week post-eclosion by color phenotype (A-C). Blue dots indicate blue males and orange dots indicate orange males. Organ area (mm^2) is shown for the testes, Ts (A); the seminal vesicles, SV (B); and the accessory glands, AG (C).

CHAPTER 5
LEARNING IN MALES AND COPULATORY REWARDS

Chapter Abstract

In the Asian citrus psyllid, *Diaphorina citri* Kuwayama, stimulatory cuticular hydrocarbons act as sex pheromone attractants. Male psyllids locate aggregations of females using those olfactory cues, as well as vibrational communication on the plant surface. Although previous research has indicated that learning plays a role in modulating female reproductive behaviors in psyllids, it is unknown whether males similarly use learning to increase the likelihood of copulatory success. We used an olfactometer-based bio-assay to study the effects of experience on male response to female odor. First, we compared male attraction to female odor in virgin and previously mated males. Second, we tested the effect of several modes of experience with a novel odor, vanillin, to determine whether mating, feeding, or general environmental exposure elicited a learned response. We found that male attraction to female odor significantly increased after mating experience. In addition, we found that males learn about odor specifically in the context of mating, rather than feeding or general exposure. Electrophysiological measurements of antennal response to odorants confirmed that mating status did not affect the sensitivity of the peripheral nervous system to volatile stimuli implicating learning at the level of the central nervous system. These results suggest that male response to female odor is not an entirely innate behavior. Males may require mating experience with female conspecifics to develop attraction to those

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Stockton, D.G., Martini, X., Stelinski, L.L. (2017). Male psyllids differentially learn in the context of copulation. *Insects,* 8: 10.3390/insects8010016.

olfactory cues produced by the female and in association with the female's habitat. This adaptive plasticity may allow males to detect females in an ever-changing environment and promote diversification and further specialization on different host genotypes.

Background Information

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, is a sap-sucking hemipteran that transmits *Candidatus* Liberibacter asiaticus, the putative causal agent of huanglongbing (Grafton-Cardwell et al., 2013, Bove, 2006, Halbert & Manjunath, 2004). In an effort to slow the spread of the disease in citrus growing regions worldwide, considerable research on the behavior and ecology of *D. citri* has focused on the reproductive biology and mate finding behavior of this devastating agricultural pest species. Psyllids are known to use multi-modal communication to detect mates within a complex host plant environment (Wenninger et al., 2009a; Lubanga et al., 2014). Vibrational communication is widespread among Hemiptera (Tishechkin, 2005; Tishechkin 2007; Virant-Dobrelet & Cokl, 2004) and in addition to functioning as an independent communication method, appears to modulate other forms of communication including long-range attraction and short-range courtship pheromone production (Lubanga et al., 2014; Virant-Dobrelet & Cokl, 2004; Borges et al., 1987).

In the southern green stink bug, *Nezara viridula*, male-produced sex pheromones first attract conspecifics to a host plant, then vibrational signals are used to specifically locate mates along the plant surface (Borges et al., 1987; Todd, 1989). Similarly, psyllids appear to use both forms of communication to locate mates within the complex host plant environment (Wenninger et al., 2009b). During migration or movement between hosts, *D. citri* first uses herbivore-induced plant response volatiles, and the release of methyl-salicylate, to locate aggregations of conspecifics (Martini et al., 2014).

Once on the host plant, substrate-borne vibrational communication is used by male *D. citri* to call to females on the plant surface via rapid wing movements (Wenninger et al., 2009b; Martini et al., 2014). Females then reply, facilitating their location by males on the host plant (Wenninger et al., 2009b; Rohde et al., 2013). This appears common among most psyllid species (Lubanga et al., 2014) although the characteristics of their vibrational calling is likely species specific (Percy et al., 2006). In an effort to exploit the dueting behaviors of reproductive *D. citri*, artificial female calling devices are being developed for use in the field as a form of mating disruption (Rhode et al., 2013, Mankin et al., 2013; Mankin & Rhonde, 2015; Mankin et al., 2016).

Once in close proximity, female-produced cuticular hydrocarbons, which appear to function as sex pheromones, attract males to engage in courtship and copulation (Martini et al., 2014; Moghbeli et al., 2014; Mann et al., 2013). It is likely that these pheromones function as short-distance cues used after vibrational communication has allowed long-distance detection along the plant surface. At least three other psyllid species are known to use female produced sex-pheromones to attract mates including *Cacopsylla bidens* (Lubanga et al., 2014), *C. pyricola* (Guédot et al., 2009), and *Bactericera cockerelli* (Guédot et al., 2010). However, there is some evidence to suggest that different psyllid species use semiochemicals in a manner that is species specific (Lubanga et al., 2014). In male *C. pyricola*, cuticular hydrocarbon pheromones direct male-male aversion behaviors as well as male-female attraction (Lubanga et al., 2014; Guédot et al., 2009). In *D. citri*, however, it appears that the use of cuticular hydrocarbon semiochemicals is unidirectional to facilitate male-female attraction

(Wenninger et al., 2008). Males of this species do not display aversion to the odor of other males.

Research on the biology of psyllid reproduction and olfactory sex attractants is of particular interest due to the potential impacts on pest management practices. Recent advances in disease vector pest management have highlighted the importance of understanding the effects of experience on insect behavior (Vinauger et al., 2016; McCall & Kelly, 2002; Prokopy & Lewis, 1993). Human disease vectors, such as mosquitoes (Vinauger et al., 2014; Menda et al., 2013; Stanczyk et al., 2013; Tomberlin et al., 2006; Kaur et al., 2003), kissing bugs (Vinauger et al., 2012; Abramson et al., 2006), tsetse flies (Bouyer et al., 2007; Bouyer et al., 2005), and sandflies (Guerra-Silva et al., 2013), demonstrate ecologically significant learning events, driving host selection and oviposition site preferences via visual, olfactory, and even thermal stimulus experiences (Vinauger et al., 2016). Similarly, *D. citri* behaviors, ranging from host preference to mate choice, may be guided by experience-dependent visual and olfactory associations with the host environment and conspecifics (Stockton et al., 2016). In human disease vectors, new techniques such as “zooprophylactic” measures aim to reduce harmful disease spread by exploiting the inherent learning mechanisms used by the vector to shift preference to non-human targets, such as cattle (Vreysen et al., 2013). Similar approaches may be possible for phytopathogen vectors if more information is gathered about the nature of learning in these pest species.

With respect to the reproductive behavior of phytopathogen vectors such as *D. citri* it is important to determine whether male attraction to conspecific female odors is innate or experience-dependent. Contrary to the idea that sex pheromone responses are generally sTable and not subject to changes with experience, there is evidence suggesting that sex pheromone responses can be surprisingly plastic (Anderson et al., 2003; Anderson et al., 2007). Neurobiologically, exposure to sex pheromone is known to induce changes in the insect brain associated with olfactory recognition (Anderson et al., 2007). After frequently observing psyllid mating behaviors in the context of female mate choice, we hypothesized that males may learn about female odor such that male response to females is enhanced after early courtship and mating experiences. If male attraction is experience-dependent, it may facilitate population suppression technologies that manipulate male behavior with the purpose of disrupting mating. Indeed, there is sufficient evidence of male reproductive learning in other insect species to predict similar occurrences in male psyllids. In *Drosophila*, males discriminate among heterospecific and conspecific females through trial and error courtship experiences and young sexually immature males use courtship experience to refine their technique in lieu of future successful matings (Dukas, 2004; Dukas, 2005; Dukas, 2006; Dukas, 2009; Kujtan & Dukas, 2009). In the sweat bee, *Lasioglossum zephyrum*, males learn to avoid odors associated with unreceptive females (Wcislo, 1987), as well as closely related females due to selective pressures promoting outbreeding (Smith, 1983). These learned responses to female traits appear to confer fitness benefits, where copulatory success is more likely in males that refine their ability to detect and court appropriate females.

To address this question in *D. citri*, we designed three experiments to test whether males learn about female odor, specifically in the context of mating, where copulation acts as a biologically significant unconditioned reinforcer. First, we compared the responses of mated and virgin males to female odor (proxy for volatile cuticular hydrocarbons thought to function as sex pheromone). If male attraction is experience-dependent, we expected only mated males to show preference for female odor. Then, we compared the acquisition of response of a novel olfactory stimulus, vanillin, in males exposed to the odor under different conditions: as an environmentally derived odor, an odor associated with females directly, and an odor associated with a food source. If the learned responses were explicitly linked to mating experience, we speculated that only the males exposed to vanillin associated with females directly should demonstrate a learned response. Finally, we compared the electrophysiological antennal responses of virgin and mated males to female whole body extracts to determine if changes in male response to female odor are caused by peripheral sensitization (Anderson et al., 2007; Stelinski et al., 2003) or true learning.

Methods

Insect Colony

Insects were obtained from a greenhouse colony at the University of Florida, Citrus Research and Education Center campus in Lake Alfred, FL, USA (Stockton et al., 2016). Psyllids were collected from two to five year-old potted Valencia (*Citrus* x *sinesis*), orange jasmine (*Murraya paniculata*), or curry leaf plants (*Bergera koenigii*), maintained at 28 °C with a 14L:10D light cycle. Plants were rotated out of colony cages once per month to ensure plant health.

Virgin males were obtained weekly throughout the course of the study. Clippings from the general greenhouse colony were collected when there were aggregations of fourth to fifth instar nymphs. The clippings were transferred to cages with a single potted orange jasmine plant and monitored daily. Newly emerged adults were removed from the cages and sexed. Males were then re-released into a male only cage until use in experiments. To ensure the insects were reproductively mature (Wenninger et al., 2007), all males were one to two weeks old at the time of use. Males greater than two weeks old were discarded from the study.

Prior to use in the experiments described below, all psyllids were collected via manual aspiration from their respective colony cages. The psyllids were then removed individually from the collection vial with a paintbrush and carefully transferred to individual 1/4 dram glass vials with cork stoppers (Bio-Quip Products, Inc., Rancho Dominguez, CA, USA). The psyllids were then sexed (in the case of the general colony collections for females) using a dissecting microscope, or were confirmed as males, and inspected for signs of poor health. Psyllids were discarded if they displayed grey-brown coloration, which has been linked to poor fecundity (Wenninger et al., 2009c), or if they were abnormally small in size or showed physical injury due to handling. In this way, only healthy psyllids were used in the study.

Effect of Mating Status Test

To test the effect of mating status on male response to female odor, we compared virgin and mated male response to female odor in a T-maze similar to that previously reported (Stelinski et al., 2013). A vertically mounted, glass T-maze received charcoal filtered, humidified air pumped at 0.2 LPM (liters per minute) from a regulated flowmeter (ARS Inc., Gainesville, FL, USA). One of the chambers was baited with the experimental odor while the other arm remained an odorless control. To control for potential environmental effects, the apparatus was positioned within a white foam-board box. Positional bias was further monitored by rotating the T-maze 180° every 10 trials so that the target odor and control positions were rotated. The pump inputs were alternated every other repetition. Pump-line flow-rate was recorded at the start of every session. To remove the effects of individual tracking from the experiment, the T-maze itself was replaced every five trials (one psyllid per trial) with a clean piece of glassware. Previously, tracking has only been demonstrated in female *D. citri* (Martini et al., 2014; Wenninger et al., 2008). Male psyllids do not appear to modify their behavior in response to olfactory stimuli produced from other males. However, we adopted a system of replacing the glassware after every five trials to avoid any potentially confounding effects of aggregation or dispersal (Stockton et al., 2016). A trial lasted 5 min (300 s) or until each psyllid made a selection. A selection was defined as chamber entry, 2 cm or more past the crux, for a minimum of 30 s. Psyllids were released into the apparatus from a 4 cm long inlet inserted into the base of the T-maze.

To provide female odor, five female psyllids were released into the odor source compartment of one T-maze chamber. The females were collected from the general colony. Only females with blue/green abdominal coloration were used. Female age and mating status was not standardized. Females were discarded if they were newly emerged, teneral adults; if they were grey/brown in coloration (a likely sign of poor health) or orange/yellow (indicating gravidity); appeared abnormally small or were otherwise injured. The other odor source lines pumped unscented air into the adjacent odor chamber. Virgin males (N = 48) were collected from the virgin male colony as previously described. Mated males (N = 52) were collected from a similar colony, although females were present and males were allowed to mate at will. All males were one to two weeks old at the time of use.

Four replicates of approximately 20 trials each were conducted. A trial consisted of a single psyllid. The number of trials varied between replicates due to variation in the number of available psyllids during the time of testing. One replication was performed per week for four weeks. All psyllids were euthanized after use and were not reintroduced into the colony.

Differential Contexts Test

This experiment included four treatment groups in a randomized block design. Male psyllids were exposed to vanillin (CAS# 121-33-5, Sigma-Aldrich Corp., St. Louis, MO, USA) in one of four possible contexts: on a female, on a leaf disk, on the vial wall in which they were kept, or not at all. After 24 h, the males were removed and individually assayed for response to vanillin. Previous research indicated that vanillin was a suiTable volatile for use in learning assays with *D. citri* (Stockton et al., 2016; Patt et al., 2014).

In group 1 "Mating + Vanillin on Female," (N = 47) previously unmated, virgin male psyllids were mated for 24 h with a female that was marked with a 10 μL vanillin-scented dot (50 μL 2.5% ethanolic vanillin/1 mL glue) on her thorax.

In group 2 "Mating + No Odor" (N = 40) males were mated with females marked with an unscented 10 μL dot (50 μL ethanol/1 mL glue) on the thorax. Group 2 acted as a direct control to group 1 and all psyllids in this group were considered vanillin naïve.

In group 3 "No Mating + Vanillin on Vial" (N = 47) males were exposed to the vanillin glue but not in the context of a female. It was applied to the interior vial wall. The same volume and vanillin concentration were used as in group 1. Males in this group were tested for response to vanillin after 24 h to control for the possibility that exposure alone leads to accentuated response to a novel odor.

In group 4, “No Mating + Vanillin on Leaf Disk” (N = 51) vanillin was paired with a nutritional reward (a leaf disk) to determine whether increased response to vanillin was due to pairing of the novel odor with females, or with a general reinforcing stimulus. The vanillin glue was applied to a leaf disk rather than a female. The same volume and vanillin concentration were used as in group 1 and group 3. In this way, male psyllids would encounter the stimulus in the context of feeding rather than mating. The same amount of glue, 10 µL, was applied to the leaf disks, the vials, and the females.

Preliminary observations suggested that 73% of psyllids mate within 2 h in confined conditions. For the two treatment groups paired with females, group 1 and group 2, one male and one female psyllid were placed in a 1/4 dram glass shell vial with a cork stopper for isolation. Paired insects were observed for 2 h to confirm mating. Unmated pairs were discarded from the study. For the other two treatment groups, group 3 and group 4, males were placed in a vanillin marked vial, or in a vial with a vanillin marked leaf disk for 24 h, respectively. Groups 3 and 4 did not have females present.

After 24 h, the males were removed from their individual vials and placed in new clean vials individually. They were then immediately used for testing in the Y-maze. All insects were used within 2 h of being removed from their “experience” vials. For the Y-maze behavior assay tests, one chamber of a glass Y-maze apparatus was baited with unscented air (25 µL 95% ethanol), while the other was baited with vanillin scented air (10 µL, 2.5% ethanolic vanillin; 15 µL 95% ethanol). The ethanol solutions were applied to a 2 cm cotton wick and were evaporated for 30 min prior to testing in a fume hood.

The Y-maze arms were baited the same way for all treatment groups. The Y-maze set-up was similar to that previously reported (Stockton et al., 2016) and was similar to the T-maze. Only the shape of the assay glassware differed. Rather than a glass cylinder with two adjacent odor chambers, the Y-maze splits from a common base tube into a Y-shape. The two arms of the Y-maze received charcoal filtered, humidified air pumped at 0.2 LMP from a regulated flowmeter (ARS Inc.). A Y-maze was used in this experiment rather than a T-maze because it is a more conservative measure of behavior and provides the insects more distance from the inlet to the crux.

All of the assay controls were the same in experiments 1 and 2. The Y-maze was rotated every 10 trials, the glassware was replaced every five trials, and the pump inputs were rotated every other repetition. Psyllids were released into the apparatus from a 2 cm inlet inserted into the base of the Y-maze. Psyllids were excluded from the study if they failed to leave the inlet for 5 min, the duration of the trial.

All four treatment groups were tested concurrently. Approximately 10–20 males from each treatment group were run in alternating groups of five on a single day. After five males from each treatment group were tested, another five from each group were run through the assay. The order of the treatment groups was varied for each replicate to ensure that time-of-day did not influence the results. A single day of recording comprised one replicate. The experiment was replicated three times. One replicate was performed per week for three weeks. The one-week time between replicates was necessary to procure mature virgin males, as described previously. During each replicate, each of the four treatments were set up 30 times, meaning 120 males were collected from the virgin colony and placed in individual vials. Of those, 10–20 males were tested in the T-maze per treatment per replicate due to overnight mortality or reduced health. Males were excluded that appeared weakened or unable to move properly through the Y-maze. We did not observe a difference in mortality among the four treatment groups.

EAG Responses

To determine whether male responses to female odor were affected by peripheral sensitization, we evaluated antennal responses by electroantennogram (EAG) of virgin and mated males. Two types of odorant stimuli were tested. First, whole body extracts from females at five concentrations, were prepared as described below to measure male response to the female sex pheromone. Second, linalool (Aldrich Chemical Company, Milwaukee, WI, USA, 98% pure) was used as a known host plant volatile attractant of *D. citri* (Patt & Setamou, 2010).

Whole body extracts were prepared as described in previous research (Mann et al., 2011). Approximately 500 adult psyllid females were agitated in 500 µL of pentane for 10 min in a glass vial. Thereafter, the adults were removed and the solution was strained to remove particulate matter from the stock solution. Various concentrations of female whole body extracts were prepared with a serial dilution in increasing amounts of pentane. Prior to use in the EAG assay, pentane-based extracts were applied to 1.4 by 0.5-cm strips of Whatman No. 1 filter paper (Fisher, Pittsburgh, PA, USA) and solvent was allowed to evaporate under a fume hood for 30 min. The filter papers were then transferred to disposable glass Pasteur pipettes for use as stimulus cartridges in EAG assays. Linalool-loaded EAG cartridges were prepared identically to those described for extracted cuticular hydrocarbon volatiles by diluting neat chemical in pentane on a log scale.

EAG recordings were performed with an IDAC-2 acquisition controller connected to a universal single-ended probe (Type PRS-1) (Syntech, Kirchzarten, Germany) as described previously (Wenninger et al., 2009a). Antennal response was measured following stimulation with the whole body extract at five different concentrations. EAGs were recorded with silver wire electrodes fixed in glass micropipettes containing 0.5 M KCL. The recording electrode was positioned on the tip on one antenna, while the reference electrode was inserted into the back of the psyllid head. Charcoal-filtered, humidified airflow was provided through one arm of a modified glass Y-tube over the recording antenna by a regulated flowmeter (ARS Inc.) at 0.3 LPM. The Y-tube base was positioned 3mm from the antenna. Whole body extracts were manually introduced to the recording antenna through the opposite arm of the Y-tube using an attached 20 mL glass syringe. A 1 mL puff of air through the previously prepared stimulus cartridges delivered the odorant into the airstream of the Y-tube and onto the insect antenna.

The extracted cuticular hydrocarbon volatiles and linalool treatments were delivered in an ascending dosage order beginning with the solvent control per insect. Separate experiments were carried out with female whole body extract collections and linalool. All insects were euthanized after use and were not reused or rereleased in to the colony. We tested the antennal response in 10 virgin and 10 mated males to the extracted whole body wash. Then, another 10 virgin and 10 mated males were tested in response to linalool. EAG data were recorded using a Windows-based computer equipped with an interface card and software from Syntech. The computer was interfaced with a software-controlled amplifier and an A/D conversion circuit; it operated with 12-bit resolution.

Statistical Analysis

Comparisons of male responses in the olfactometer assays were analyzed with Chi-squared tests (Stockton et al., 2016). Within-group comparisons described differences in selection of a particular treatment group for arm A or arm B of the Y-maze. Between-group comparisons described differences in overall selection pattern between different treatment groups using a 2 × 2 chi-squared contingency Table design. EAG data were analyzed with two-way analysis of variance and Fisher's LSD (Least Significant Difference) tests (Wenninger et al., 2009). Analyses were performed in R (Version 3.0.2; the R Foundation for statistical software R; Vienna, Austria).

Results

Effect of Mating Status Test

Mating status significantly affected male response to female odor in a T-maze olfactometer assay (Figure 5-1). Although there was no difference in selection between the female scented and control odor chambers for virgin males ($\chi^2_1 = 0.08$, $p = 0.773$), 69% of mated males selected the female scented chamber ($\chi^2_1 = 7.69$, $p = 0.005$). Comparison of overall selection pattern between virgin and mated males suggested a difference in response to female odor below the 0.1 probability level ($\chi^2_1 = 3.08$, $p = 0.079$), although this did not meet the generally accepted threshold of significance.

Differential Contexts Test

Male *D. citri* displayed differences in response to vanillin based on the type of prior experience (Figure 5-2). Males in group 1 (vanillin on a female) showed a statistically significant increase in selection of the vanillin scented arm than the unscented arm ($\chi^2_1 = 6.1489$, $p = 0.013$). In contrast, males in group 2 (mated to an unscented female) appeared to display aversion to vanillin ($\chi^2_1 = 3.6$, $p = 0.057$), although it did not meet the generally accepted threshold of significance. Males in group 3 (vanillin on the vial wall) showed significant aversion to vanillin ($\chi^2_1 = 4.79$, $p = 0.028$). The insects in group 4 (vanillin on a leaf disk) showed no preference between the vanillin-scented and unscented arms ($\chi^2_1 = 0.49$, $p = 0.483$). Between-group comparisons revealed that group 1 differed significantly from group 2 ($\chi^2_1 = 9.49$, $p = 0.002$), group 3 ($\chi^2_1 = 10.90$, $p = 0.001$), and group 4 ($\chi^2_1 = 5.25$, $p = 0.022$) in overall selection pattern.

EAG Responses

There were no differences in EAG response amplitude to whole body extracts ($F_{5,180} = 0.81$, $p = 0.36$) (Figure 5-3a) or linalool ($F_{5,180} = 0.42$, $p = 0.21$) (Figure 5-3b) among mated and virgin males. Response amplitude increased in virgin and mated male psyllids as the concentration of each odor was increased (Figure 5-3).

Discussion

Our data suggest that male attraction to female odor is not entirely an innate response. Rather, experience with receptive females enhances male attraction to odors associated with those females. Our data are consistent with previous research, which showed that only mated male *D. citri* display significant attraction to female odors (Wenninger et al., 2008). However, at times our results were difficult to interpret. While female produced odors attract males in *D. citri*, they have not been strictly defined as pheromones and innate mate attraction to odors is relatively weak (Mann et al., 2013). In addition, psyllid responses to olfactory stimuli are highly variable and difficult to study due to sensitivity to changes in barometric pressure and other environmental factors, which are difficult to control (Zagvazdina et al., 2015). This may explain why, in at least two cases, our analysis revealed mild to insignificant differences between treatment groups, despite large differences within groups. Our findings are consistent with research on learned male mate choice among insects and some vertebrate species (Wcislo, 1987; Magurran & ramnarine, 2004, Dukas, 2004; Dukas, 2005; Dukas, 2006; Dukas, 2009; Kozak & Boughman, 2009; Takahasi & Watanabe, 2011) and congruent with previous research in *D. citri* demonstrating behavioral plasticity in contexts other than reproduction, including feeding and host preference (Stockton et al., 2016).

Our initial study found that male and female psyllids are capable of single stimulus visual and olfactory associations with the host plant, as well as complex compound conditioning tasks (Stockton et al., 2016). However, we did not rule out the possibility that male psyllids also learn in the context of mating, rather than host feeding alone as a form of reward. Our current data are the first to suggest that male *D. citri* preferentially associate environmental odors and/or female produced odors with receptive females. When conditioned to a novel olfactory stimulus in the presence of a food-based reward, the subsequent associative learning was lower than that observed with a copulation-based reward. Similarly, in the parasitoid wasp, *Leptopilina heterotoma*, female kairomone-host associations are strongest when oviposition is used as a reward (Vet & Groenwold, 1990). Experience had the least effect on subsequent psyllid behavior when the novel odor was associated with the environment alone and lacked a reward. This suggests that the change in behavior observed in our studies reflects true associative learning, such that response shifts are not due to non-associative changes like sensitization or habituation (Anderson et al., 2003; Anderson et al., 2007; Stelinski et al., 2003; Minoli et al., 2012).

In some insects, such as Lepidoptera, non-associative learning phenomena such as sensitization and habituation may account for changes in response to female pheromones (Anderson et al., 2003; Anderson et al., 2007; Stelinski et al., 2003; Bartell & Roelofs, 1973). In the oblique-banded leafroller, *Choristoneura rosaceana*, short-term pre-exposure to female pheromones is sufficient to reduce responsiveness in males (Stelinski et la., 2003), whereas in the cotton leafworm, *Spodoptera littoralis*, pre-exposure results in higher sensitivity to female pheromones (Anderson et al., 2003). To specifically test for the possibility of peripheral sensitization following mating, we used electrophysiological measurements of male antennal response (EAG) to odorants and confirmed no changes in male antennal sensitivity following mating. These results suggest that male experience resulted in true associative learning.

Male attraction to female odor is well documented in other psyllid species (Lubanga et al., 2014). However, some of those studies have failed to account for the influence of experience on male preferences (Soroker et al., 2004; Horton & Landolt, 2007). Males in these studies were commonly kept in cages with females, or were collected in the field. In other cases, virgin insects were used so an assessment of innate attraction is possible. In the potato/tomato psyllid, *Bactericera cockerelli*, virgin males do strongly orient towards females despite a lack of experience, indicating an innate attraction to female-produced pheromones (Guedot et al., 2009; Guedot et al., 2010; Guedot et al., 2013). This is in contrast to our findings with *D. citri* and indicates species specificity with cues and behaviors, such that, along with the composition of vibrational signals across the plant surface, and the constituency of female-produced cuticular hydrocarbons, learning in psyllids is species specific. There are likely differences in the evolutionary pressure faced by each species that would encourage or discourage learned attraction, such a variation in resource availability across generations (Papaj & Prokopy, 1989; Stephens, 1993, Dukas, et al., 2006; Dukas, 2013).

Evolutionarily, there are many potential benefits to selective and learned responses to conspecific pheromones (Papaj & Prokopy, 1989; Dukas et al., 2006; Dukas, 2006; Anderson et al., 2007). Males may use acquired attraction to female odor to detect females in the complex host environment, especially when combined with other sensory modalities such as vibrational communication and visual detection. If experience increases the likelihood that males can locate females, then the likelihood of reproduction increases. Learning may also confer benefits with regard to avoiding predation (Anderson et al., 2007). When attention is focused on female pheromone perception, insects may display reduced perception of predator cues (Skals et al., 2005), although it is unknown if *D. citri* engages in active predator avoidance behaviors.

Another potential evolutionary benefit to male olfactory learning is that learning may facilitate some degree of mate choosiness in male psyllids (Dukas, 2006). In some species, mate choosiness in males is adaptive, where selection of females favors those with high fecundity and low risk of sperm competition (Bonduriansky, 2001; Simmons et al., 1994). Male mate choosiness may also take the form of predicting female receptivity (Dukas, 2004), or avoiding misdirected matings with heterospecific species (Dukas, 2009). In this way, learning in the context of male mate selection may allow males to determine female quality such that resources are not divided among high quality and low quality mates (Dukas, 2006). To accomplish this, males must learn about the relative quality and availability of females in the given area, preferring high quality females based on innate preferences, previous experiences, and the abundance of mating opportunities available (Bonduriansky, 2001; Simmons et al., 1994). Research with *Drosophila melanogaster* has shown that the number of successful matings increases in males with age and experience, presumably because male courtship behaviors and mate choosiness are refined over time (Dukas, 2006). However, because sex pheromone production and overall fertility of *D. melanogaster* increases with age, it is unclear to what extent learning contributes to mating success (Long et al., 1980; Moulin et al., 2001). Future research is needed to determine if male *D. citri* use learning to refine interspecific and intraspecific discrimination among potential mates. Variation in female odor concentration and constituency, as well as abdominal color and size, may serve as potential variables males use to assess female quality.

It is interesting that a species such as *D. citri* would demonstrate learned preference for female odor, rather than entirely innate attraction. These insects are commonly described as having a promiscuous mating system, or scramble polygyny (Thornhill & Alcock, 1983), although females are known to display clear choosiness and refuse the advances of some males (White, 1969; Krysan, 1990). Learning is not thought to be beneficial among males in such species because scramble competition relies on a series of rapid, indiscriminate encounters rather than careful selection among few partners. In addition, mate choosiness is specifically associated with certain male traits, which male psyllids appear to lack, including high male investment in the form of nuptial gifts or limited sperm availability (Lubanga et al., 2014; Kozlowski & Aoxiang, 2006). Indeed, male psyllids are commonly observed attempting to mate with the first psyllid it encounters, regardless of the sex of the insect. However, we do not believe our data are incompatible with those hypotheses. While male psyllid mate choosiness appears greatly reduced compared to other species, learning in male psyllids may function to help detect females, and therefore maintains a beneficial function. Indeed, there is growing body of research on learning in the context of reproduction in *D. melanogaster* which indicates male learning is common and beneficial to insects in cases where promiscuity may be predicted (Dukas, 2004, Dukas, 2005; Dukas et al., 2006).

It is our observation that in nature, male and female psyllids engage in practice courtship and copulation behaviors prior to the age in which they are reproductively mature, which occurs at three to four days post-eclosion (Wenninger & Hall, 2007). It is common to see newly-eclosed, reproductively immature psyllids engage in courtship behaviors. It is during those early encounters that males may become familiar with female traits, specifically female-produced cuticular hydrocarbons. Combined with other forms of communication, early learned attraction to female odor may encourage selective courtship and maximize the number of successful matings. This would not be contradictory to a promiscuous approach to copulation, rather it would likely reduce the number of failed or misdirected mating attempts between males or heterospecific females (Kujitan & Dukas, 2009). In our experience, many mistaken mating attempts occur in artificial settings, such as inside a polystyrene Petri dish, wherein vibrational communication, that may otherwise help psyllids discriminate among potential mates, is compromised. Further research is needed to clarify the role of male mate choice in psyllids and whether male mate choice is refined by experience. It may also be necessary to elucidate the interaction between olfactory and vibrational communication in psyllids mate selection.

Our results may have implications for development of behaviorally based management tools for *D. citri* (Wenninger et al., 2008). Semiochemical-based management tools for *D. citri* are currently under development (Patt & Setamou, 2010). Non-traditional control methods are also being explored, given the importance of this pest. If male attraction to female odor is experience-dependent, it may be possible to manipulate male behavior in such a way that mate detection and reproduction is suppressed. For example, appetitive learning in insects is specifically mediated by octopamine (RS-4-(2-amino-1-hydroxy-ethyl) phenol), a biogenic amine neurotransmitter found almost exclusively in invertebrate species (Farooqui, 2012). While octopamine occurs in vertebrates, it does not appear to have a major role as compared with norepinephrine (Berry, 2004). Indeed, appetitive learning is significantly suppressed in insects when octopamine antagonists are administered (Agarwal et al., 2011, Mizunami et al., 2009; Unoki et al., 2006). It therefore may be possible to introduce a novel form of mating disruption in agricultural settings by disrupting a target species' ability to learn. While the potential for such application is distant and would require significant effort to develop a safe and target-specific method of application, pest control options expand with better understanding of the target species' ecology and behavior.

In conclusion, our data suggest that male *D. citri* become more responsive to the odors of receptive females following previous experience and a copulatory reward. This does not appear to be a peripheral effect; antennal sensitivity to both female-derived and host plant odorants did not change with male mating experience. Rather, the perceived significance of those odors likely changes after successful mating, increasing subsequent attraction towards conspecific females. Furthermore, olfactory learning in *D. citri* males appears to be selectively encouraged by copulatory rewards, where learning was not facilitated by nutritional reinforcement or environmental exposure alone. Such selective olfactory learning in the context of reproduction may allow *D. citri* males to more readily discriminate among heterospecific and conspecific females in the natural environment, and avoid potentially unfavorable matings with immature or otherwise unreceptive conspecific females.

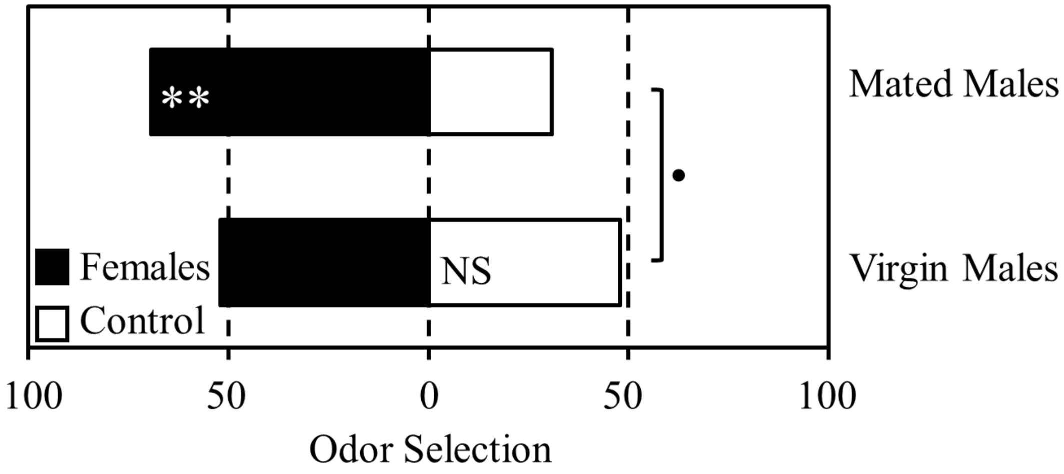

Figure 5-1. The effect of mating status on male response to female odor. Mated or virgin males were given a choice of selecting female odor (grey bars) or an unscented control (white bars) in a Y-maze olfactometer. The x-axis indicates the percentage of psyllids selecting each odor source. Asterisks indicate statistically significant results; • < 0.08, * ≤ 0.05, ** ≤ 0.01.

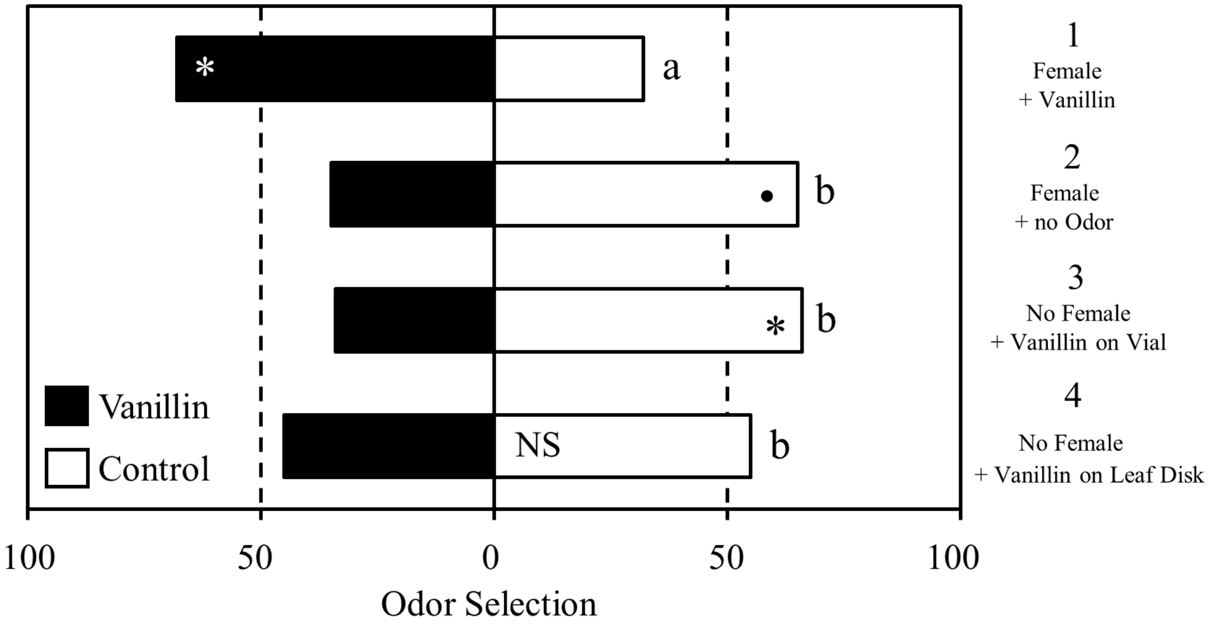

Figure 5-2. Learned response to vanillin depending on the conditioning environment. Significant within-group differences in the selection of vanillin (grey bars) or the unscented control arm (white bars) are indicated by asterisks; • < 0.08, * ≤ 0.05. Significant differences in overall selection pattern between treatment groups are indicated by different letters.

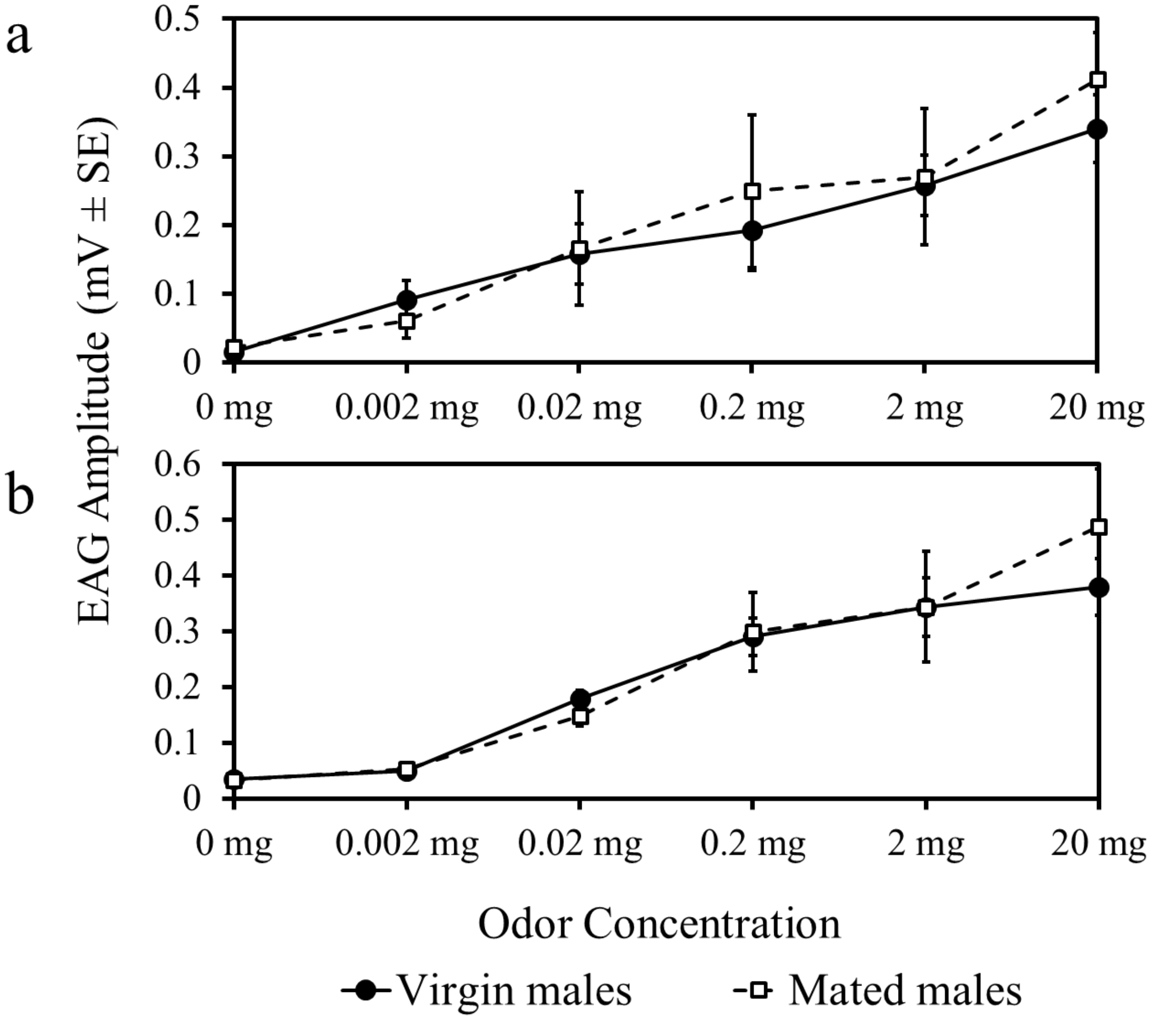

Figure 5-3. Antennal responses to female cuticular hydrocarbon extracts (a) and a stimulatory host-plant volatile, linalool (b), at increasing concentrations compared to an odorless control (0ug).

CHAPTER 6
SUMMARY AND CONCLUSIONS

Associative Learning

Associative learning is an experience-dependent association between a previously novel conditioned stimulus (CS), such as an odor or a color, with an unconditioned stimulus (US) which elicits a behavior. With repeated presentation of the US and CS, the CS then elicits the behavior without presentation of the US. That modification in behavior, evoking a behavioral response where it was previously absent, indicates memory formation and learning at the neurological level. In nature, psyllids must navigate a complex environment to seek out host plants. They must detect the appearance and volatile profile of the appropriate plants, and alight accordingly. Mistakes in host plant identification can be costly and even fatal, depending on the energy expended to locate those plants and the distance traversed when migrating from the original natal host. Learned associations with the natal host, or even host plants previously encountered as adults, would improve the ability of the insect to successfully detect the host among a variety of non-host species.

To investigate the extent of associative learning in *D. citri* we studied the acquisition of memory in response to paired associations with the host plant and two novel stimuli, vanillin and the color blue, both of which are not host plant cues and do not elicit positive responses in the naïve state. In *D. citri*, we found that prior to our experimental learning procedure, vanillin, a novel olfactory stimulus, elicited a negative or neutral response. However, after pairing vanillin with the host plant, psyllids oriented positively towards vanillin in a Y-maze. This occurred in both males and females of the species. Similar effects were seen when we paired the host plant with an artificial blue light. While naïve psyllids showed strong negative response to blue light, after feeding on a host plant illuminated with the same blue light, psyllid response became neutral in selection, indicating that the aversion to blue light was diminished. Again, this occurred in both male and female psyllids. Based on the data collected in these experiences, we were confident that psyllids were able to learn host plant associations to olfactory and visual stimuli.

We also performed a series of tests using compound conditioning, attempting to tease out the relative strength of associations made to olfactory and visual stimuli. These experiments were less conclusive. We paired the host plant with blue light and vanillin odor simultaneously and then measured psyllid response to those stimuli individually and together in different combinations. The data suggested that simultaneous presentation of two novel and slightly aversive stimuli reduced the response to both stimuli individually compared to individually conditioning each stimulus, as was done previously. In addition, it appeared that vanillin preference was greater than blue preference, or a combination of blue and vanillin, however the reasons for this were unclear. It is possible that the aversive nature of the selected olfactory and visual stimuli acted synergistically to suppress response. Due to the greater level of aversion to blue in naïve psyllids, relative to vanillin, it is also possible that our results merely reflect the aversive nature of blue, rather than associative abilities of psyllid for all colors and odors. For that reason, we are still unclear which stimulus modality carries greater associative strength for psyllids.

These questions are important for two reasons. First, there is paucity of research on learning in Sternorrhynchan species. As major agricultural disease vectors, Sternorrhynchan pests pose a serious threat to agriculture here in the US and abroad. If appropriate control measures are to be developed, the biology and ecology of these insects must be studied. Second, if learning plays a role in psyllid host plant selection, it is possible that lure and monitoring devices could be significantly improved by tailoring the design to the host species grown regionally. Although there is an ongoing effort to develop a lure for trapping and monitoring psyllids in the field, current designs have failed to capture psyllids reliably, leaving concerns about the nature of psyllid host plant detection. For instance, Florida is a major producer of navel oranges, while South Texas primarily grows grapefruit. The appearance and volatile profiles of these two cultivars is different enough, that although the genetic populations of psyllids in these two regions are similar, their host preferences are varied based on the cultivar with which they are now accustomed. Psyllids were introduced to the US through Florida in 1998 and hitchhiked to Texas by 2001 (Halbert & Manjunath, 2004). To further explore this topic and investigate whole host plant preferences based on natal experience, we performed more tests, using orange jasmine and ‘‘Valencia’’ orange reared psyllids.

Learned Host Preference

In Chapter 2 and Chapter 3 we demonstrated learned preference for host plants in *D. citri* and the fitness effects of that learned preference in *D. citri* offspring. First we compared olfactory host preference in a Y-maze for psyllids reared in isolated ‘‘Valencia’’ orange or orange jasmine colonies. The results showed a 60% preference for the natal host plant species odor. In a settling test, we compared psyllid preference for the two host species, the natal and alternative host, when given a choice. Psyllids settled on the natal host species predictably, every day for three days. In addition, oviposition preferences were consistent with the natal host species. Orange jasmine psyllids preferred to oviposit on orange jasmine plants, and ‘‘Valencia’’ orange psyllids preferred to oviposit on ‘‘Valencia’’ orange plants.

We were also interested in how long those preference, or memories of the natal host, were maintained over time. In the instance of migration away from the natal host, this would be an important factor in determining whether learned preferences produced ecologically relevant changes in behavior with regard to host selection. When tracked over time, we found that natal host preferences were maintained for approximately 24 hrs when the psyllids were transferred to the alternative host species as adults. After 24 hrs preference for the new host species replaces natal host preference This suggests that memory of the natal host species is maintained until a new host is experienced. That flexibility in response likely enables psyllids to accurately detect the natal host species when migration is required, but accept an alternative when needed or when the natal species is unavailable.

These findings presented new questions. Does induced female host preference confer benefits to the offspring? What are those benefits and what are the consequences of failing to select the natal host for oviposition? To answer those questions, we designed another experiment to study the effects of host choice on offspring development. First instar psyllid nymphs, whose parents were reared long-term in isolated ‘‘Valencia’’ orange or orange jasmine colonies, were either maintained on those host species, or transferred to the alternative host species. We found that the pattern of development differed depended on the transfer group. ‘‘Valencia’’ psyllids developed slower and were smaller when transferred to orange jasmine. However, orange jasmine psyllids developed faster and were larger when transferred to ‘‘Valencia’’ orange plants. To explain these findings, we referred to the literature on psyllid detoxification enzymes, where we found that indeed, different host plant species were associated with differential expression of detoxification enzyme levels in psyllids. Orange jasmine psyllids have higher levels of those enzymes than ‘‘Valencia’’ orange psyllids. This is thought to be a result of varying levels of defensive compounds produced by the plant to deter herbivory. Orange jasmine plants produce greater amounts of terpenoids than orange cultivars (Robbins et al., 2012). As a result, psyllids reared on orange jasmine have greater detoxification abilities which may be passed on the offspring.

The result of this difference in plant defense compound and upregulated detoxification enzyme titre was that while ‘‘Valencia’’ orange psyllids suffered reduced fitness when moved to orange jasmine, orange jasmine psyllid fitness improved. This was likely because orange jasmine psyllids were not only able to easily transition to a less deterrent plant, but were able direct more of their physiological resources to growth. Despite the innate, and often referenced, preference for orange jasmine plants among *D. citri*, orange jasmine appears to be a fairly toxic plant. Our orange jasmine psyllids experienced greater mortality when maintained on orange jasmine, than when transferred to ‘‘Valencia’’ orange.

From the data we can conclude that learned host preference in *D. citri* not only occurs, but may play an important role in host selection particularly with regard to oviposition site selection. Our data suggest real fitness consequences for psyllids which deviate from the natal host species in circumstances where the natal host species is less deterrent than the alternative. This is particularly interesting with regard to those studies citing innate preference for orange jasmine above most other citrus cultivars. We are skeptical that those reports are a true reflection of innate preference. There is likely little data that gives of us a clear indication of innate host plant preference because the natal host species were not controlled for in most studies. Rather, in many cases, those psyllids were reared on one of the test species. One such study claimed that orange jasmine was a preferred host but an examination of their methods showed that all psyllids used in the study were originally collected from orange jasmine plants prior to use (Teck et al., 2011). This example demonstrates the importance of taking learning into account when studying insect behavior.

Learned Mate Choice

The third over-arching topic explored throughout this dissertation was that of mate choice. In our previous topics we examined how natal or early adult experience influences host and oviposition preferences. Then we became interested in the implications for host preference on offspring fitness. Ultimately, those findings led to questions regarding learning in the reproductive biology of *D. citri*, namely, do psyllids show mate preference and if so, are those preferences learned based on early courtship and mating experiences? Our hypotheses were largely based on a body of work studied in *Drosophila* which found males and females demonstrated learned mate choice behaviors. Females prefer male body size based on early courtship experiences and males use early mating experiences to refine their conspecific discrimination abilities and avoid sterile matings with sister species.

First, we focused on females and looked at the mate preference after experience. Virgin females were mated to either blue or orange phenotype males for their original mating experience. Then, they were given a choice of blue and orange males and their preferences were recorded. We found that while females originally mated to orange males selected either male at nearly equal rates, with a slight preference for orange males, females mated to blue males originally strongly avoided those blues males in subsequent choice tests. Physiological and behavioral examination of the two male phenotypes revealed that blue males may differ in the life history role they play in the greater psyllid ecological landscape. Blue psyllids are larger, fly longer, and have greater detoxification abilities which confers greater insecticide resistance, than orange males. However, orange males are more sexually aggressive and females mated to orange males lay more eggs. We suspect that female aversion to blue males after mating experience is largely a behavioral effect. Due to the behavioral impotence of blue males, females learn to avoid them in lieu of the alternative, seeking greater reproductive success. There is no reason to believe, however, that males are physiologically sterile, as their internal reproductive organs were developmentally identical to orange males, if not larger.

Our second course of study, on learning in the reproductive biology of psyllids, focused on male behavior. We examined whether male learning was specific to certain contexts or rewards. To do this, we first compared male response to female odor in a T-maze and found that virgin males were less responsive to female odor than mated males. Then, we compared male response to a novel odor, vanillin, when paired with different rewards. In one group, males were exposed to vanillin painted onto the thorax of a receptive female. The males were then allowed to mate with the females for 24 hrs. In the second group, males were exposed to vanillin on a leaf disk and were allowed to feed on the vanillin scented leaf for 24 hrs. In the third group, the males were only exposed to vanillin on the glass wall of the vial in which they were contained. No food or females were provided. Finally, the fourth group contained naïve males which were unexposed to the vanillin in any context. They were left in a vial to mate with unmarked females for 24 hrs.

The results showed that males indeed learned best in the context of copulation. Although there was a small shift in preference in the “feeding experienced” group, the largest preference for vanillin occurred when it was paired with females. This indicates that male response to female odor is not innate. Males learn attraction to female odor after early courtship and mating experiences. These data also suggest that male learning is strongest when in the context of reproduction. This calls into question some of our previous data from Chapter 2. In those experiments we did not control for the potential acquisition of associations in the context of reproduction versus feeding. Rather, we suggested that the association made between vanillin and the blue light were due to host-plant-stimulus pairings. In fact, females were also in the conditioning cages, so it is possible that the male responses recorded in those data were associations between receptive females and the novel stimuli.

These findings highlight the difficulty studying insect learning, as previously described in Chapter 1. Small, sap-feeding species, such as psyllids, require near constant nutrition to sustain normal patterns of behavior. Although there is evidence to suggest that psyllids do a large amount of their feeding at night, we have regularly observed declines in performance when removed from the host plant for short periods of time. For that reason, the traditional learning trial design used in vertebrate research, and some heartier invertebrates likes *Drosophila* and honey bees, is not possible with psyllids. For that reason, future work should aim to account for all possible interactions within the host plant environment to eliminate confounds. We did accomplish this to some degree with this final experiment. By placing the psyllids overnight in a glass vial with either females or a leaf disk, we were able to tease apart the differences in association between those varying rewards.

In our last experiment, we compared the EAG responses of virgin and mated male psyllids to female odor and a host odor (linalool) at increasing concentrations. We did this to determine whether the previously observed increases in male response to female odor were caused by learning or a non-associative effect like sensitization. The data showed that there was no difference in the peripheral antennal response between virgin and mated males. That means that virgin males can detect female odor as well as mated males but their perception of the odor is changed by experience.

Learning and Speciation

Learned mate choice has direct implications for speciation (Verzijden et al., 2012). In two dimensions, all behavior within a genotype fits along a curvilinear reaction norm. However, learning may alter the slope of the reaction norm, introducing a new range of responses. In some species, mate preferences are determined by early experiences with the parents and siblings (Immelman, 1975; Tramm & Servedio, 2008). In others, those preferences develop in mature individuals after courtship and mating experience (Verzijden et al., 2012). As a result, the individual may learn to avoid heterospecific species (delBarco-Trillo et al., 2010; Magurran & Ramnarine, 2004; Dukas, 2004; Dukas 2009; Svensson et al., 2010), prefer mates most similar to those with which they are experienced (Fincke et al., 2007; Takahashi & Watanabe, 2010), discriminate among high and low quality mates (Hebets, 2003; Hebets, 2007; Bailey & Zuk, 2008; Fowler-Finn & Rodriguez, 2012;), or even develop mate preferences based on the observed preferences of conspecifics, a form of mimicry (Vakirtzis, 2011). Similar results were shown in our own work with psyllids. Female avoidance of blue males was more extreme when females were experienced with those blue males. Compared to the preferences seen in naïve females or females previously mated to orange males, blue mated females showed a greater negative range of behavior as a result of experience.

The effect of mate choice learning is that previously unpreferred traits may become more or less desirable with experience, which in turn affects sexual selection (Aoki et al., 2001; Laland, 1994; Agrawal, 2001b). In wolf spiders, as females learn to prefer a certain trait based on experience with the available male phenotypes, fixation may occur within the population (Hebets, 2007). When females select for certain male traits, those heriTable traits are passed on within the population. With regard to psyllids it is still unknown whether male abdominal phenotype represents a temporally transient stage in development or a genetically fixed trait, although the data currently suggests that abdominal color phenotype is a transient state linked to age. For this reason, the implications for female mate choice on sexual selection in psyllids is unclear. However, because speciation is linked to changes in behavior that influence copulation and offspring fitness (Cocroft et al., 2008), we see our results as an indication that factors such as abdominal color and female mate preference could encourage divergence in this species

In the context of host preference, the impacts of learning on speciation are more direct. As females prefer their natal host for feeding and oviposition, and her offspring are more prepared to cope with the plant defense chemicals they encounter, increased preference for certain host traits occurs within the population. Host fidelity may then, in turn, increase assortative mating. Over time, if two populations remained isolated on differing host species, the adaptations for coping with their unique host environments can drive host race formation and ultimately speciation. Although there is currently no evidence of psyllid host race formation in the field, population divergence is certainly occurring, where we can already detect distinct population haplotypes within the North and South American continents from unique introductions dating back less than two decades, once in North America and twice in South America (Boykin et la., 2012; De Léon et al., 2011). There are differences in endosymbiont communities within different isolated populations of psyllids as well, which has implications for the evolution of immune function and coevolutionary relationship between the insect and the bacterium is transmits (Saha et la., 2012). It is possible that with time, psyllids reared in distinct agricultural regions with one or two predominant cultivars will develop host races similar to that seen with *Rhagolettis pomonella* (Bush, 1969; Prokopy et al., 1988) or the *Enchenopa binotata* species complex (Cocroft et al., 2008; Wood, 1980).

Future Directions

The data presented here, while answering many questions about psyllid host selection mechanisms and reproductive ecology, has also left many more unanswered.

First, our findings on learned host preference suggest the need for field studies testing the efficacy of regionally specific olfactory lure for *D. citri*. We recommend further research into the traits associated different host plants that are most attractive to psyllids. Those traits, such as volatile profile, color, and leaf shape; may be useful in tailoring lures and traps for psyllids found a particular citrus-growing region. For example, lures and traps in regions growing grapefruit as the primary citrus variety may want to use grapefruit traps, whereas traps using the odor and color of orange cultivars may be more useful for psyllid monitoring in orange growing regions.

Just as attractants are important for psyllid behavior, it may also be useful to study the host qualities that are naturally unattractive and determine whether those aversions are subject to habituation are or maintained to such a degree that repellency could be achieved. Although not presented in this dissertation, we did begin a project looking at the effects non-host aversion on psyllid behavior. We were interested in this topic because of the increased use of wind-break crops and bait crops to deter psyllid movement into groves. We were curious if psyllids learned to avoid non-host plants more effectively over time with experience. We housed psyllids in cages with one citrus plant and one non-host plant for 3 days and tested their preference for the non-host plants at 0, 24, 48, and 72 hrs. Our results were variable and did not suggest a clear learn aversion for non-host species. There are many possible reasons for this, including flaws in our experimental design. However, we believe those data suggest that psyllids are not very adept at avoiding non-hosts based on volatile profile, despite experience. Further research is need to confirm our findings on non-host aversion. It is possible that visual stimuli are more readily learned in this context than olfactory stimuli.

Other questions in our research were posed by our data on mate choice learning and male color phenotype biology in the psyllid. More research is needed to understand the role of blue and orange phenotype psyllids in nature and confirm that they serve different life history roles within the population. We also need to understand the physiological function of the blue fat body in psyllids. In some species, the fat body serves as a reservoir for endosymbionts or digestive gut bacteria. However, it is currently unknown if bacteria are present in the psyllid fat body. There was some work done recently (Chia-Ching et al., 2017 unpublished data) that showed a link between the blue fat body coloration and a protein created by a psyllid gut symbiont, *Candidatus* Profftella armatura. This suggests that there may be an evolutionary function for the blue coloration seen in psyllids beyond reproductive biology and mate preference.

Taken together, our work on the Asian citrus psyllid has shown that learning guides important life history functions. This not surprising considering the growing body of work on learning in arthropods and other invertebrate species. Indeed, it is very likely that to live is to learn. All cells, and especially neural cells, have a ‘memory’ for the past which ultimately affects future function. In higher taxa, that cellular memory results in episodic memories, such as the ability to recall the appearance and flavor a meal eaten with friends in the past. In lower taxa, such as arthropods, those memories are likely stored and recalled as a form of procedural memory, a memory that doesn’t not require awareness. There is still much to discover about insect learning and memory, but with every species that is investigated there is greater evidence that they are complex, dynamic organisms that are far from robotic. They interact with the world around them and make complicated, informed decisions that ensure their individual survival and the fitness of their offspring.

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BIOGRAPHICAL SKETCH

Dara Stockton graduated from Tulane University in 2007 with a Bachelor of Science in neuroscience. During that time, she worked in a vertebrate research lab where she was introduced to the study of learning and memory in animal models. In 2010 she was began working in entomology after taking a job with the USDA-ARS in Weslaco, TX. In 2012, she completed her Master of Arts degree in experimental psychology at the University of Texas, Pan American, during which she studied associative learning in the Asian citrus psyllid.

In 2013, she began her doctoral degree under Dr. Lukasz Stelinski at the University of Florida Citrus Research and Education Center in Lake Alfred, FL. Since then she has continued to study learning in psyllids with a focus on how experience influences insect host preference and mate choice. In May 2014, Dara married David Linder in a small ceremony in Lake Alfred. The two met and began dating in 2008 in New Orleans before she left Tulane. In 2015, Dara and her husband welcomed a new addition to their family, John Odysseus “Desi” Linder.

In the future, she and her family are moving to Geneva, NY, where she has accepted a post-doctoral position at the New York State Agricultural Experiment Station through Cornell University where she will study the biology and ecology of spotted wing drosophila.