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Reproductive Biology of Mealybugs (Pseudococcidae) and Practical Applications of
Their Sex Pheromones in California Nurseries

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Entomology

by

Rebeccah Anne Waterworth

June 2010

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The Dissertation of Rebeccah Anne Waterworth is approved:

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Acknowledgements

I would like to acknowledge the assistance that I have received from my dissertation committee: Dr. Jocelyn G. Millar (chair), Dr. Marshall Johnson, Dr. Timothy Paine, and Dr. Richard Redak. Dr. J. Daniel Hare assisted me with statistical analyses and experimental design questions. Dr. Mark Hoddle was generous in allowing me to use his microscope and recording system for reproductive biology studies. Dr. Kent Daane and Mr. Walter Bentley provided mealybugs to start colonies. Mr. James A. Bethke helped me locate production nursery study sites in Riverside and San Diego Counties. Mr. Todd Birchler, general manager at Milfelds' Nursery, Inc., Riverside, CA graciously donated plant and insect material during my work and was helpful in coordinating experiments with the nursery's schedule. Mr. Juan Paz and Mr. Ken Taniguchi, managers at Mellano and Company, Bonsall, CA, and owner, Mr. Mike Mellano, Sr., also were generous in allowing me use of space and plant material in their nursery for over three years. In addition, collaborators at both nurseries, including Mr. 'Buzz' Uber, a crop protection specialist, were very patient in answering my endless questions regarding nursery production. I am grateful to the UCIPM Grants program (Project number 06DS006), the American Vineyard Foundation, the Viticulture Consortium West, and the Oregon Wine Board for providing funding in support of my work.

I would also like to acknowledge the assistance of current and past members of the Millar research group, including Dr. J. Steven McElfresh, Mr. Joshua Rodstein, Dr. Yunfan Zou, Ms. Carmen Romero, Mr. Ian Wright, Dr. Ann Ray, Dr. Jardel Moreira, and Mrs. Mariana Krugner.

Dedication

This work is dedicated to past teachers, mentors, advisors, and professors, who were all an inspiration. In addition, I would like to dedicate this dissertation to my boyfriend, Jason Mottern, sisters, Lisa, Allison, and Sarah, and my parents, Howard and Pam, who all encouraged me, from long distance or short, to do my best.

ABSTRACT OF THE DISSERTATION

Reproductive Biology of Mealybugs (Pseudococcidae) and Practical Applications of Their Sex Pheromones in California Nurseries

by

Rebecca Anne Waterworth

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, June 2010
Dr. Jocelyn G. Millar, Chairperson

Traps baited with sex pheromones were used to monitor field populations of longtailed and obscure mealybugs. Lures loaded with 25 microgram doses of racemic pheromones remained effective for at least 3 months. Pheromones could also be combined to make lures that attracted several species simultaneously. The numbers of insects caught in traps were correlated with mealybug abundance, indicating that pheromone traps can be used in place of laborious manual sampling to monitor populations. The efficacy of using pheromone traps to monitor seasonal fluctuations in mealybug populations was demonstrated.

Obscure, vine, and longtailed mealybugs were shown to reproduce only through sexual reproduction. Copulatory behavior for both sexes was stereotypical across all species. Female and male mealybugs were capable of multiple copulations in a single day and over multiple days. Female reproductive output did not increase with multiple copulations. Male longevity and activity levels were minimally affected by constant

exposure to pheromone, and males typically lived 4-5 days after emergence as adults.

This better understanding of mealybug reproductive biology has implications for the use of pheromones for monitoring and control of mealybugs.

Several pieces of evidence suggest that the sex pheromone is produced somewhere on the hind pair of legs, probably from translucent pores that are only present on the hind legs of adult females. Males were attracted to body sections of females with the hind pair of legs, and extracts of these legs strongly attracted males. Scanning electron microscopy confirmed the presence of the pores on the hind coxae of adult females. Elucidating the site of pheromone production provides the key baseline data required for studies of the biosynthesis of the irregular terpenoids that comprise the sex pheromones of various species.

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Chapter One: Introduction

Mealybugs (Hemiptera: Pseudococcidae) are widely distributed phytophagous insects, often with broad host ranges. Worldwide, there are approximately 2,000 described mealybug species (USDA 2007a), with a few species considered serious pests of economically important plants (McKenzie 1967). There is little published information about either the specific losses caused by mealybugs or the costs associated with their control. To give a few examples from what literature is available, losses and costs of controlling mealybugs in Georgia in 1996 were estimated at about \$9.8 million (from Chong et al. 2003). Damage and costs of controlling the pink hibiscus mealybug in the United States were recently estimated at \$700 million annually (Ranjan 2006). In South Africa, costs for control of vine mealybug in vineyards were estimated at around \$100 per hectare per season (Walton et al. 2004).

Economic damage and mealybug management. During feeding, mealybugs pierce a plant's phloem with needle-like mouthparts to extract photosynthates (Gullan and Martin 2003). Their feeding results in distorted plant tissues, leaf yellowing, defoliation, reduced plant growth, and potentially the death of a plant (McKenzie 1967, Culik and Gullan 2005, Culik et al. 2006, Walton et al. 2006). Phloem feeding is mostly restricted to insects in the order Hemiptera. These types of phloem feeders have evolved to feed on high-sugar diets through the use of gut enzymes that transform excess ingested sugar into longer-chain oligosaccharides that are excreted from the animal as honeydew (Douglas 2006). The sticky honeydew contaminates foliage and in large quantities, becomes a cosmetic problem for ornamental plants. In vineyards, honeydew from the vine

mealybug, *Planococcus ficus* Signoret, encrusts leaves, canes, and grape clusters, leading to defoliation, bunch rot, and a reduction in the marketability of the crop (Daane et al. 2006, Walton et al. 2006). Possibly more important than the cosmetic problem is the growth of black sooty mold and related organisms on honeydew. These organisms growing in and on the honeydew reduce photosynthetic activity (Gullan and Martin 2003, Zada et al. 2004, Daane et al. 2006, Walton et al. 2006). White mealy wax and other insect residues that remain on plant material after mealybugs have dispersed or died, especially on fruits and vegetables, are also a cosmetic problem.

Cosmetic and aesthetic damage are not usually life threatening to the plant, but they are a major concern for the floriculture industry. Studies have shown that less than 10% distortion, defoliation, or discoloration of woody plants (Raupp et al. 1992, Sadof and Raupp 1997) and annual plants (Sadof and Sclar 2002) is usually sufficient to render these plants unacceptable to the majority of the public when compared to undamaged controls. As a result of low public tolerance of arthropod damage, there is pressure on nurseries to produce marketable plants with minimal levels of damage.

Insecticides are the predominant tools for mealybug management in agricultural systems, including vineyards (Bentley et al. 2009) and ornamental crops (Bethke 2009). Nonetheless, the physical and behavioral attributes of mealybugs render them difficult to control with insecticides. The waxy covering on immatures and adults enables water-soluble, contact insecticides to run off their bodies (McKenzie 1967, Arnett 1993, Walton et al. 2004). Eggs are protected by the ovisac's waxy filaments and also are not easily killed by insecticides. In addition, insecticide treatments do not effectively penetrate into

many of the plant tissues where mealybugs feed, such as bark cracks, undersides of leaves, and developing leaves and flowers (McKenzie 1967, Daane et al. 2003, Godfrey et al. 2003). Systemic insecticides, such as acetamiprid, dinotefuran, imidacloprid, and thiamethoxam are recommended for mealybug management (Bethke 2009). However, only 17%, 17%, 30%, and 9% of California nursery operations in 2006, respectively, utilized these compounds to manage pest infestations (USDA 2007b).

In any case, heavy reliance on insecticides is unsustainable in the nursery and floriculture industries, because a number of important pests of ornamentals have developed resistance to insecticides, including western flower thrips (Immaraju et al. 1992, Jenson 2000), aphids (Kerns and Gaylor 1992), mites (Ramdev et al. 1988, Fergusson-Kolmes et al. 1991), whiteflies (Prabhaker et al. 1985), and leafminers (Sanderson et al. 1989). Whereas there are no documented cases of mealybugs becoming entirely resistant to insecticides, it is critical that insecticides are used more judiciously (and target one specific group of pests at a time) to delay or avoid onset of resistance. A report by Flaherty et al. (1982) described how it was becoming more difficult to manage grape mealybug, *Pseudococcus maritimus* (Ehrhorn), infestations in vineyards with existing chemicals (e.g., parathion) and label rates. Mealybugs remained susceptible to chemical applications, though not to the extent that they were in the 1950s and 1960s (Flaherty et al. 1982).

To shift from calendar-based prophylactic cover sprays to integrated pest management tactics, growers must have a reliable method of monitoring key pests so that corrective actions are taken as soon as a problem is detected, and pesticides are applied

only when necessary. Until recently, there were no simple and effective methods to monitor most mealybug species in vineyards (Geiger and Daane 2001, Daane et al. 2002), because mealybugs conceal themselves under plant material or in the soil (McKenzie 1967). The only method to monitor for mealybugs has been time-consuming and laborious examination of plant material for live mealybugs (Geiger and Daane 2001). Similarly, visual inspection of plant material is the only method currently in use for monitoring mealybugs in production nurseries.

Mealybug biology. Male and female mealybugs have divergent life cycles. A generalized life cycle for a female mealybug consists of an egg stage, three nymphal instars, and the adult (Fig. 1.1). Mature adult females have a vulva and distinct segmentation, and females of the largest species can grow to a length of 9 mm (McKenzie 1967). Adult females are flightless, and females of species that reproduce sexually use a sex pheromone to attract conspecific males (Tremblay et al. 1980, Bierl-Leonhardt et al. 1981, Zhang and Amalin 2005). A mated female usually will lay eggs in a waxy or felt-like ovisac containing ca. 100 - 500 eggs (McKenzie 1967, Chong et al. 2003, Daane et al. 2006). Females of some species (e.g., *Pseudococcus longispinus* (Targioni Tozzetti)) bear live young. These ovoviviparous females protect the first instar crawlers for a brief time period by covering them with their abdomen (McKenzie 1967). Unlike their scale relatives, immature stages and adult females can move around on host plants by walking, but overall, their ability to spread is limited by their relatively poor and slow dispersal abilities. Thus, most dispersal, especially over longer distances, is dependent on the movements of infested plant material or debris (e.g., on fallen leaves

carried by wind), contaminated agricultural machinery, or by wind-borne crawlers (Lo et al. 2006).

Of the economically important mealybugs, the citrus mealybug, *Planococcus citri* (Risso), has been studied most extensively in terms of general life history parameters such the number of generations per year and longevity of each life stage. *Planococcus citri* overwinters predominantly in the egg stage (Bartlett 1978, Godfrey et al. 2002). Godfrey et al. (2002) reported that in vineyards, depending on temperature, there are two to five overlapping generations of *P. citri* per year, whereas on citrus grown in California, there are four or five overlapping generations per year (Bartlett 1978). Laflin et al. (2004) reported that in conditions typical of California cut-rose production, *P. citri* females developed from first instar to adults in a median of 32 and 30 days at 18.3°C and 20.3°C. The time period from egg to adult was a little longer (median of 39 days) at both 18.3°C and 20.3°C.

Less information is available on life history parameters of *Pseudococcus viburni* (Signoret). Depending on temperature, in Californian vineyards, there are two to three generations per year (Godfrey et al. 2002, Varela and Smith 2006); however, there are four or five generations of *P. viburni* in California citrus (Bartlett 1978). Under summer conditions, sexual maturity is attained 42 days after egg hatch (Bartlett 1978). All life stages overwinter, with lower population growth in cold weather (Bartlett 1978).

Pseudococcus longispinus has four to six overlapping generations per year in California (Bartlett 1978, Godfrey et al. 2002). Its life cycle is completed in 29 days (Bartlett 1978). In Australian pear orchards, there are three or four distinct generations of

P. longispinus per year (Barass 1993). There are no reports in the literature that discuss how this species overwinters. From personal observation in commercial ornamental nurseries in southern California, *P. longispinus* are more abundant during the cooler winter months; apparently all life stages overwinter under these mild climatic conditions.

For sexually reproducing mealybug species, males have a life history that can be regarded as holometabolous (e.g., possessing a pupal life stage), though they are far removed from the majority of holometabolous insects (Chapman 1998). During the late second instar, males secrete waxy filamentous tests (similar to cocoons) prior to molting (McKenzie 1967; Chong et al. 2003). Inside these tests, males develop into the third (prepupal) and fourth (pupal) instars (Fig. 1.1). They are non-feeding in the last two instars (Chapman 1998). Males emerge from these cocoons as winged, non-feeding adults. There is only one pair of wings, on the mesothoracic body segment.

Sex pheromone-baited traps.

The economic pressures that confront growers when managing mealybugs, especially when using insecticides, provide incentives for growers to adopt more environmentally benign integrated pest management tactics. The overall goal of this research is to determine whether better methods of monitoring and management of mealybug infestations can be developed through the use of pheromones. Some specific areas where improvements on current practices would be beneficial include:

1. The development of tools to decrease the time spent monitoring,
2. The development of more sensitive methods of monitoring to allow early detection of mealybugs, and

3. More accurate timing of control measures to decrease the amount of insecticides used.

A possible alternative to visual sampling that may both decrease monitoring time and increase the sensitivity of mealybug detection methods is the use of pheromone-baited traps. Based on general experience with mealybug pheromones, it is clearly possible to use such traps to detect male mealybugs (Millar et al. 2002, Walton et al. 2004, Zada et al. 2004, Bell et al. 2005). However, to be really useful to growers, it is also necessary to show a robust correlation between trap counts and the abundance of mealybugs on plants. An additional benefit of pheromone-baited traps is that all experience to date indicates that they are species-specific (Millar et al. 2002). Thus, no taxonomic expertise would be required to determine the species of males present in the trap and presumably found on plant material adjacent to the trap.

One possible limitation to the feasibility of using pheromone traps to monitor mealybugs, based on the physiology and life history of male mealybugs, is that pesticide applications may be more effective at killing males than females. Previous work has shown that males of California red scale, *Aonidiella aurantii* (Maskell), are more susceptible to pesticides than females, including the pesticides dichlorvos (Shaw et al. 1973) and pyriproxyfen (Zalom and Morse 1991, Rill et al. 2007). Male red scales, like male mealybugs, have holometabolous development (Chapman 1998), and male mealybugs are also more susceptible to insecticides than females (Zhang et al. 2004, Hinkens et al. 2001, Millar et al. 2005). As a result, insecticide treatments that

selectively kill males may result in artificially low trap catches in pheromone-baited traps and an underestimation of mealybug populations.

The sex pheromones of some economically important species of mealybugs have been identified and synthesized, primarily during the last decade. These include the Comstock mealybug *Pseudococcus comstocki* (Kuwana) (Negishi et al. 1980), citrus mealybug *Planococcus citri* (Bierl-Leonhardt et al. 1981), vine mealybug *Planococcus ficus* Signoret (Hinkens et al. 2001), citriculus mealybug *Pseudococcus cryptus* Hempel (Arai et al. 2003), pink hibiscus mealybug *Maconellicoccus hirsutus* (Green) (Zhang et al. 2004), obscure mealybug *Pseudococcus viburni* (Millar et al. 2005), grape mealybug *Pseudococcus maritimus* (Ehrhorn) (Figadère et al. 2007), passionvine mealybug *Planococcus minor* (Maskell) (Ho et al. 2007), Japanese mealybug *Planococcus kraunhiae* (Kuwana) (Sugie et al. 2008), longtailed mealybug *Pseudococcus longispinus* (Millar et al. 2009), Madeira mealybug, *Phenacoccus madeirensis* Green (Ho et al. 2009), and citrophilous mealybug *Planococcus calceolariae* (Maskell) (El-Sayed et al. 2010). Three of these species (*P. citri*, *P. longispinus*, and *P. viburni*) are key pests in production nurseries in California, and my research focused on developing and utilizing pheromone-based traps to monitor these three species.

Previous work with mealybug pheromones/pheromone-baited traps:

Pseudococcus viburni. Pheromone-baited traps have been used in apple orchards in New Zealand to detect *P. viburni*. *Pseudococcus viburni* infestations are a phytosanitary problem for fruit shipped to some export markets (Bell et al. 2005). Management strategies work best in spring when mealybugs are most difficult to locate. The inability

to determine spring densities of mealybugs results in poor predictability of infestation levels at harvest (Bell et al. 2005). Pheromone-baited traps were used to facilitate monitoring and were placed in several orchards in two different apple-growing areas of New Zealand. Male *P. viburni* mealybugs were found in baited traps in all of the study orchards, with low catch rates of males in unbaited control traps. Unfortunately, trap catches were highly variable in one area, whereas only a single mealybug was caught in another. The authors concluded, based on these trap counts and the assumption that there is an association between counts and density of mealybugs, that *P. viburni* populations were variable within the region (Bell et al. 2005).

Planococcus citri. This species is a key pest in greenhouses and citrus in California and Texas (Bierl-Leonhardt et al. 1981) and of citrus and other subtropical fruit trees in the Near East (Zada et al. 2004). Field and laboratory bioassays showed that the synthetic (+)-enantiomer of the pheromone was as attractive to adult males as the natural material extracted from females. Higher doses of pheromone (1 – 10 µg) caught higher numbers of males compared to lower doses (0.1 µg) (Bierl-Leonhardt et al. 1981). Mealybug feeding does not directly damage citrus fruit, but infested fruit become covered with honeydew and black sooty mold, byproducts of mealybug feeding. As with *P. viburni* in apple orchards and *P. ficus* in vineyards, manual sampling to detect and quantify mealybugs in citrus is laborious and ineffective in early spring (Zada et al. 2004). Pheromone-baited traps offer an alternative to existing monitoring strategies in citrus groves. Zada et al. (2004) evaluated the effects of various parameters on trap catches of *P. citri*, including different formulations of the synthetic pheromone (racemic vs chiral,

and highly purified vs less pure pheromone), different trap types, and different doses of pheromone. They detected no differences in catch rate for either racemic or chiral as well as pure vs less pure “technical” pheromones. Delta traps were recommended for monitoring purposes because they were easiest to use in the field, even though they did not capture the most mealybugs. Male mealybugs responded to a range of pheromone doses (50-1,600 µg) and were not repelled by high pheromone doses (Zada et al. 2004). A rubber septum (West Co., Lititz, PA) loaded with 200 µg of pheromone could be used for monitoring for as long as 16 weeks. Hefetz and Tauber (1990) demonstrated a weak but significant correlation between counts of adult *P. citri* females counted on citrus trees and grass with counts of male mealybugs attracted to pheromone-baited traps.

Planococcus ficus. This species is the key mealybug pest of vineyards in all of California’s grape-growing regions. Field testing of pheromone-baited traps has consisted of evaluating different blends of two possible pheromone components, different doses of the pheromone, field longevity of lures, pheromone range, and a comparison of trap catches with densities of mealybugs on vines (Millar et al. 2002). Only one component (lavandulyl senecioate) of the two components identified from compounds produced by females attracted male *P. ficus*. The racemic synthetic pheromone was highly attractive to male mealybugs. Lures loaded with a range of pheromone doses (10-1,000 µg) were equally attractive, and lures loaded with 100 µg of racemic pheromone were attractive for at least 12 weeks under field conditions. Like the work with *P. citri* in citrus groves, delta traps were recommended for monitoring. Pheromone-baited traps were demonstrated to have an effective range of 50 meters. Lastly, a positive correlation

between trap catches and the abundance of mealybugs counted on vines during visual inspections was demonstrated (Millar et al. 2002).

A similar study conducted in South Africa (Walton et al. 2004) also showed a positive and significant relationship between the average percent stem infestation to the number of *P. ficus* adult males caught in pheromone-baited traps. Here too, a significant correlation between the numbers of males caught in traps early in the season with late season percent stem infestation was demonstrated (Walton et al. 2004). The authors also brought up a potential problem with pheromone-baited traps for predicting *P. ficus* mealybug damage and setting economic thresholds. Specifically, pheromone traps were more sensitive than visual methods for detecting mealybug infestations, and traps may have attracted males from nearby infested fields, skewing the perception of a high-density, local infestation (Walton et al. 2004). Lastly, *P. ficus* mealybugs exhibit a clumped distribution in the field, and the authors state that higher trap densities provide better estimates of mealybug densities (Walton et al. 2004).

Maconellicoccus hirsutus. Unlike the aforementioned species of mealybugs, the female's sex pheromone consists of a blend of two compounds, and the stereochemistry of each compound is critically important to the biological activity (Zhang et al. 2004, Zhang and Amalin 2005). The optimal dose of *M. hirsutus* pheromone was 1 µg (Zhang and Amalin 2005), much lower than *P. ficus* and *P. citri* (Millar et al. 2002, Zada et al. 2004), and higher doses resulted in reduced attraction. Pheromone-baited traps were used to detect and assess the phenology of populations of *M. hirsutus* in landscape plantings of hibiscus, and several studies have been conducted to evaluate the efficacy of different traps and

field longevity of lures (Vitullo et al. 2007, Francis et al. 2007, Hall et al. 2008). Similar to Millar et al. (2002) and Zada et al. (2004), Francis et al. (2007) recommended the use of Delta sticky traps to capture males and minimize the capture of nontarget species. There is some variability in the literature regarding the field longevity of lures and their attractiveness to males. Zhang and Amalin (2005) determined that pheromone lures (1 – 10 μ g) were active for about 5 months. Residual activity may last for more than 7 months (Hall et al. 2008). Pheromone traps were used to determine that male flight activity peaked around dusk (Francis et al. 2007). Lastly, Francis et al. (2007) reported that traps captured males in areas where there were no visual indications of a mealybug infestation, demonstrating the sensitivity of the traps.

Despite the available information regarding the use of pheromone-baited traps in fruit orchards, citrus groves, or vineyards, to date there has been no research performed on the possible use of these traps in production nurseries. Basic trap parameters (pheromone dose and lure longevity) have been determined in other systems, but these and related parameters have not been tested and optimized in production nurseries. In addition, plant material in orchards, citrus, or vineyard systems remains stationary, with the exception of the harvestable products. In contrast, plant material in production ornamental nurseries is harvested, and containers are shipped at regular intervals ranging from several weeks to months. Containers are also moved to different sites within a nursery depending on the stage of production.

Production nurseries often have multiple species of mealybugs infesting their crops, and management efforts are identical for all mealybug species. Therefore,

identification to the species level is not critical; a grower only needs to know the overall level of mealybug infestation in order to make effective control decisions. It would be beneficial if traps could be baited with the pheromone of more than one species, and preferably the pheromones of all species of concern, thus minimizing the number of traps that need to be deployed, counted, and serviced. Before such a “generic” pheromone lure can be deployed, it is crucial to determine whether the presence of other pheromones antagonizes males of each particular species, resulting in artificially low trap catches. There is little precedent in the literature for combining sex pheromones to make lures that will simultaneously attract two or more species. In one of the few published examples, Jones et al. (2009) recently demonstrated that a combination of the sex pheromones of two moth species did not diminish the attraction of males of either species when compared to traps with single pheromones.

With the exception of work by Daane and Walton in vineyards in California and South Africa (Millar et al. 2002, Walton et al. 2004, Daane et al. 2006), much of the published work describing pheromone-based monitoring of mealybug populations has not progressed to associating trap catches with insect abundance on plants. In commercial nurseries, it is unknown whether there are relationships between trap catches of males, the abundance of mealybugs on plants, and economic thresholds. If such relationships can be shown to exist, pheromone traps would then be useful tools for growers to more effectively time management actions for mealybug populations. Nonetheless, even if positive correlations between trap catches and abundance of mealybugs on plants were determined for a few plant species, the relationship may not hold for other host plants or

cropping systems. Many production nurseries in Southern California grow a huge diversity of plants. For simplicity, a single relationship is desirable but may not be feasible.

Pheromone-based control of insects.

Pheromone-based monitoring for mealybugs has been used effectively in several cropping systems, including pome fruit (Bell et al. 2005), vineyards (Millar et al. 2002, Walton et al. 2004), and citrus (Zada et al. 2004). To date, pheromone-based control by disruption of mating has only been attempted in vineyards for *P. ficus* (Daane et al. 2006). Possible pheromone-based control tactics include mating disruption, attract and kill, and mass trapping (Jones 1998). All three methods are predicated on decreasing mating, either by killing or trapping a large percentage of one sex, or by interfering with mate location. This approach, theoretically, results in lower reproductive success and a subsequent decline in the population. Examples in the literature of successful pheromone-based control include a number of moth, beetle, and fruit fly species (reviewed in Witzgall et al. 2010). There has been a varying degree of success of control measures for each of these insect pest groups (Cardé and Minks 1995). One important factor contributing to the efficacy of these tactics is the number of possible copulations for either sex. In the case of most moths, males can fertilize more than one female and a very high proportion of males must be removed to markedly decrease population growth (Jones 1998).

Despite the chronic pest status worldwide of economically important mealybug species, surprising little is known about their reproductive biology. Some species are

thelytokous (only females), such as *Dysmicoccus brevipes* (Cockerell) in Hawaiian pineapple plantings or *Phenacoccus solani* Ferris in California (Beardsley 1959, Nur 1971). Other reproductive biology information is only anecdotally mentioned in the literature. For example, James (1937) reported that the maximum number of copulations for male *P. citri* was 23, with other males mating from zero to 17 times during their lifetimes. *Pseudococcus longispinus* males mated from zero to 20 times (James 1937). No other published literature discusses the number of times that females may copulate, or the duration and frequency of copulation for either males or females. In addition, it is unknown whether males that mate multiple times may continue to fertilize females after a number of prior copulations.

Male life span must also be considered when evaluating pheromone-based management of mealybugs. Because males stop feeding prior to the end of their second instar, their energy reserves are limited, and it is imperative for them to quickly respond to pheromone signals to locate mates before they die. Consequently, exposure to sex pheromone might be predicted to increase the activity level (flying or walking) of males, causing them to expend energy more quickly, resulting in a shorter lifetime.

Mating disruption has been attempted for one mealybug species, *P. ficus* (Walton et al. 2006), with results that were sufficiently promising to merit the development of a commercial mating disruption formulation marketed by Suterra LLC, Bend, OR. Walton et al. (2006) reported that a sprayable, microencapsulated formulation of the pheromone applied multiple times during two growing seasons in vineyards resulted in reduced trap catches of males and reduced egg production by females. The percentage of crawlers

was also lower in plots sprayed with pheromone during the second season. Only vines with the lowest rating of infestation at the start of the trial had a significant reduction in mealybug densities after the pheromone was applied (Walton et al. 2006).

To assess the effectiveness of pheromone-based control of mealybugs in production nurseries, detailed knowledge of the reproductive biology of target mealybugs (*P. citri*, *P. longispinus*, and *P. viburni*) is highly desirable. This information will affect control decisions such as the duration of deployment, (e.g., continuously or at intervals). It is also important to understand male mealybugs' responses to constant pheromone exposure.

Location of sex pheromone pores in mealybugs

Mealybug dermal pores. To date, the location of pheromone glands and pores has not been determined for any mealybug species. This is surprising because mealybugs have been studied extensively since 1840, when Westwood first distinguished them as a family separate from other coccoids (Ben-Dov 1994). Regardless, there have been a number of detailed morphological studies that have revealed that females typically possess six types of dermal pores scattered over their bodies, although, some genera do not possess some pore types (McKenzie 1967, Williams 1985, Cox and Pearce 1983). Cox and Pearce (1983) discuss four of these pore types in detail. Trilocular and quinquelocular pores produce waxy filaments that protect adult female mealybugs from defensive exudates (ostiolar fluid) and honeydew. Scanning electron micrographs have shown that ostiolar fluid and honeydew exudates were covered with broken fragments of wax from these pores. *Planococcus* mealybugs do not possess quinquelocular pores. Multilocular disc

pores and oral collar tubular ducts produce wax that surrounds the eggs in the ovisac, and the wax protects the eggs from rain, desiccation, honeydew contamination, and natural enemies. Wax from the multilocular disc pores also is used by males to construct their cocoons after their second instar.

Sex pheromone pores. The sites of pheromone production and emission have been determined in many insect orders, but the locations of the glands and release apparatus differ for each group. Sources of pheromone usually consist of glandular epithelium (Jefferson et al. 1966, 1968, Barnes et al. 1966, Marsh 1975, Moreno and Fargerlund 1975), where epithelium is tissue composed of cells lining cavities and surfaces of structures throughout the body. For example, Barnes et al. (1966) examined the sex pheromone gland of female codling moth, *Cydia pomonella* (L.), and found it to be an invaginated area within the body cavity, lined with columnar glandular cells. When squeezed, the female moth's abdomen extrudes the otherwise concealed 8th and 9th abdominal segments and the pheromone gland is apparent between the intersegmental folds. Jefferson et al. (1966, 1968) determined that the pheromone glands of a number of other moth species were between the 8th and 9th abdominal segments.

Moreno (1972) determined the site of production of sex pheromone in two scale species related to mealybugs, the yellow scale (*Aonidiella citrina* (Coquillett)) and California red scale (*Aonidiella aurantii*). Pheromones for both species are produced in the pygidial glands, epithelial tissue found within the pygidium. The pygidium is extruded from beneath the hard protective cover of female diaspidid scales. Pheromone moves from the pygidial glands through ducts into the rectum from whence it is released

into the environment. Moreno and Fargerlund (1975) conducted additional histological work that showed the recession of the pygidium and dispersal of pheromone cells after insemination. Females that remained unmated were also studied, and after 60 days, pheromone gland cells had dispersed and receded into the anterior of the pygidium. Sex pheromone production ceases when the ducts connecting sex pheromone cells break apart and the cells disperse.

In another insect group related to mealybugs, Pettersson (1970) determined the site of sex pheromone emission in aphids. Holocyclic aphids spend much of the year as asexual organisms. Sexual individuals appear during the fall months and males locate and mate with pheromone-producing females (Marsh 1975). Females possess circular plaques or pseudosensoria on their swollen hind tibia (Pettersson 1970, Marsh 1975). In bioassays, male aphids (e.g., vetch aphid, *Megoura viciae* Buckton) were only attracted to the third pair of legs or extracts made from those legs, and not to other body parts. Females with the hind legs removed or intact sexually immature females were not attractive (Marsh 1975). Marsh (1973) described the cells beneath the pseudosensoria as having a glandular appearance, though he did not study the attractiveness of individual glands in his 1975 paper, as Moreno had done in 1973 with pygidial glands and scale insects.

To date, by analogy to aphids, researchers have suggested that the site of pheromone emission in mealybugs may be the translucent pores found on the hind pair of legs of adult females (Williams 1985, Watson and Kubiriba 2005); however, there is no hard evidence to support this theory. These pores are found on the posterior surface of

one or more of the hind leg segments, including the coxa, femur, and tibia, but never on the tarsi (McKenzie 1967, Williams 1985). Williams (1985) and Watson (personal communication) had observed that immature females lack translucent pores. Both also note that there are mealybug species in which females do not have the translucent pores but still produce sex pheromones, as demonstrated by strong attraction of males to females (e.g., cassava mealybug, *Phenacoccus herreni* Cox & Williams). These researchers thus conclude that, for these species, other morphological characters that are not apparent with light microscopy must be used for pheromone emission.

Overall, my specific objectives in this dissertation were to:

1. Determine the parameters (e.g., pheromone dose, lure longevity) for effective use of pheromone-baited traps in production nurseries as seen in other cropping systems;
2. Determine whether traps baited with combinations of mealybug pheromones, specifically, the pheromones of *P. citri*, *P. longispinus*, and *P. viburni*, were as attractive to males as traps baited with the pheromone of each individual species;
3. Assess if positive correlations exist between pheromone trap catches and abundance of mealybugs on plants;
4. Develop a detailed understanding of the reproductive biology of *P. longispinus*, *P. viburni*, and *P. ficus*, particularly as it relates to the potential efficacy of pheromone-based control methods; and
5. Determine the site of pheromone emission in mealybugs.

Summary. The overall goal of my research was to determine whether mealybug pheromones may be developed for practical applications in nurseries and ornamental plant crops. My research addressed questions related to both the basic reproductive biology of mealybug species, and to the development of methods and protocols for using mealybug pheromones to assist in detection and monitoring of mealybug population densities and population cycles. Specifically, Chapter 2 presents a determination of optimal pheromone doses for *P. longispinus* and *P. viburni* detection and the field longevity of pheromone lures for both species in ornamental nurseries. The correlation between trap counts and density of mealybugs on plant material is also presented. Chapter 3 focuses on mealybug reproductive biology for three species: *P. longispinus*, *P. viburni*, and *P. ficus*, including sexual or asexual reproduction, the number of copulations for males and females, the length of time between copulations, and the longevity of males when constantly exposed to pheromone. Finally, Chapter 4 explores the possible location of pheromone pores in mealybugs.

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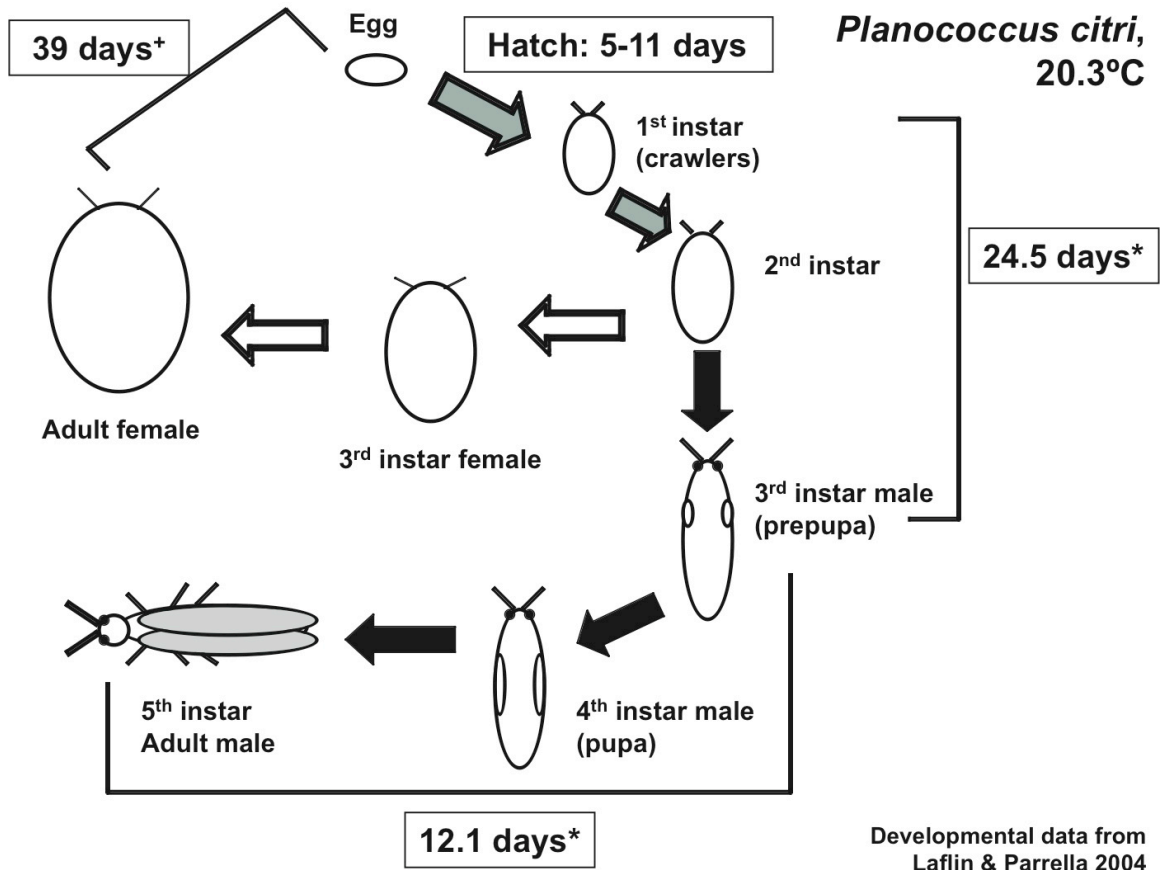
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Figure legend.

Figure 1.1. Generalized life cycle for most mealybug (Hemiptera: Pseudococcidae) species. Life stages connected by gray arrows signify similar development and appearance of females and males. Life stages connected by black arrows signify the developmental path of male mealybugs, whereas white arrows denote the developmental stages of females. Developmental data is from Laflin and Parrella (2004) for *Planococcus citri* (Risso) at 20.3°C. (†) Median number of days between life stages. (*) Mean number of days between life stages.

Figure 1.1.



Chapter 2: Operational parameters of pheromone-baited traps used to assess seasonal activity and population densities of mealybug species (Hemiptera: Pseudococcidae) in nurseries producing ornamental plants

Abstract

Operational parameters of traps baited with the pheromones of three mealybug species were optimized in nurseries producing ornamental plants. Traps were used to detect infestations of mealybugs season-long and to track population changes in the field. In dose response trials with each species, all doses tested attracted *Pseudococcus longispinus* (Targioni Tozzetti) and *Pseudococcus viburni* (Signoret) males, with the lowest dose (1 µg) attracting the fewest males for both species. Doses of 3.2 – 100 µg were as attractive to male *P. longispinus* as the highest dose tested (320 µg), whereas for *P. viburni* males doses from 10 – 320 µg were equally attractive. Lures containing 25 µg doses of either pheromone had effective field lifetimes of at least 12 weeks. With *P. longispinus*, fresh or 1 wk old lures were significantly less attractive than older lures (8 and 12 wk), suggesting that this species may be deterred by high release rates of pheromone. We also tested whether the pheromones of different species could be combined into a single, generic lure to attract several species simultaneously. Lures loaded with a blend of the pheromones of *P. longispinus*, *P. viburni*, and *Planococcus citri* (Risso) were as attractive to *P. viburni* and *P. citri* as single component lures containing their pure pheromones. However, responses of *P. longispinus* to the blend were decreased by 36% when compared to the total counts of males attracted to individual pheromone treatments. A subsequent trial with 2-component blends showed

that the pheromone of *P. citri* was responsible for the decrease. For operational purposes, the 36% decrease in attraction of male *P. longispinus* to the combined lure should not affect the overall efficacy of using these lures for monitoring the presence of all three mealybug species simultaneously. When pheromone-baited traps were compared with manual sampling methods, trap counts of male *P. longispinus* were correlated with mealybugs counted on plants in the vicinity of the traps. This relationship suggests that pheromone-baited traps can be used in place of laborious manual sampling to monitor mealybug populations in nurseries producing ornamental plants.

Introduction

The wholesale value of California nursery and floriculture crops in 2007 and 2008 was \$1 billion for nurseries with more than \$100,000 in sales (California Agricultural Production Statistics 2009-10), which translated into a market value of \$3.6 billion in 2007 (Census of Agriculture 2007). California has the largest nursery and floral industry in the United States (Carman 2002, 2007, California Agricultural Production Statistics 2009-10). Jerardo (2007) reported that the wholesale value of California's floriculture crops (cut flowers, annuals, perennials) accounted for 26% of the nationwide value followed by Florida at 20%. California also led in cash receipts for greenhouse and nursery crops (plants with woody stems) at 22.5% of the national total with Florida a distant second at 10.4% (Jerardo 2007). With crop values and revenue so high and a low tolerance by the public for aesthetic damage to plants (Raupp et al. 1992, Sadof and Sclar 2002), there is intense pressure on nursery managers to control pest arthropods and minimize the damage inflicted on production-stage plants. Classification of nursery products by major categories, including cut flowers and cut greens, nursery stock and other ornamentals, potted plants and flowering foliage, and bedding plants reveals that several of these categories are each valued at close to or more than \$500 million annually (Carman 2007).

Regardless of how crops are grown, mealybugs are chronic pests of ornamentals throughout temperate regions of the world including California (Laflin et al. 2004). In a survey conducted in 19 of California's production nurseries, 12 mealybug species were found on an assortment of host plants. The most common mealybug species were citrus

(*Planococcus citri* (Risso)), longtailed (*Pseudococcus longispinus* (Targioni Tozzetti)), and obscure (*Pseudococcus viburni* (Signoret)) mealybugs (Laflin et al. 2004).

Planococcus citri was found more often on annual crops, with the exception of *Rosa* species, whereas *P. longispinus* was more common on perennial crops. *Pseudococcus viburni* was found on a mix of both. The remaining nine species of mealybugs listed in this survey were specialists, found on a single or a few host plant species, at one nursery location (Laflin et al. 2004).

Mealybug damage to plant tissues is primarily aesthetic. Their mouthparts pierce a plant's phloem to extract photosynthates (Gullan and Martin 2003) resulting in distorted plant tissues, leaf yellowing, defoliation, reduced plant growth, and possibly plant death. Special adaptations in mealybugs and other phloem feeders allow excess sugar to be processed into longer-chain oligosaccharides that are ultimately excreted as honeydew (Douglas 2006). Accumulations of sticky honeydew not only contaminate plant foliage, but also result in the growth of black sooty mold fungi, greatly reducing marketability of the plant material.

Management strategies for mealybugs primarily involve the use of insecticides. Insecticides registered for use in California against mealybugs include organophosphates, carbamates, insect growth regulators, and the neonicotinoid imidacloprid (Bentley et al. 2009). Many of these chemicals produce mixed results in controlling mealybugs for several reasons. First, mealybugs tend to live in protected areas such as bark cracks and crevices, in grass sheaths, at bases of leaf petioles, on the undersides of leaves, and on roots (McKenzie 1967, Daane et al. 2003, Godfrey et al. 2003). Second, the waxy

covering on mealybug eggs and bodies also offers some protection against insecticides (McKenzie 1967, Arnett 1993, Walton et al. 2004). Eggs are surrounded by waxy, filamentous secretions called ovisacs, and contact insecticides may not penetrate the waxy cover. Insecticides applied to exposed mealybugs also may run off as a result of the waxy secretions covering their bodies. In addition, these insecticides have been shown to impair and kill natural enemies of mealybugs, thus reducing levels of biological control (Walton and Pringle 1999).

For a shift to occur from frequent prophylactic cover sprays to integrated pest management tactics, growers must have reliable tools to monitor key pests in order to facilitate timely pesticide applications. Until recently, there were no simple and effective methods to monitor most mealybug species, for example in vineyards (Geiger and Daane 2001, Daane et al. 2002), because mealybugs conceal themselves under plant material or in the soil (McKenzie 1967). Thus, monitoring usually consisted of time-consuming and laborious examination of plant material for live insects (e.g., vine mealybugs, *Planococcus ficus* Signoret; Geiger and Daane 2001). On pineapple in Hawaii, monitoring of pink pineapple mealybugs, *Dysmicoccus brevipes* (Cockerell), requires destruction of the entire plant to locate the mealybugs deep in the plant's leaf whorls and on the roots (M. Johnson, personal communication).

An important biological characteristic of mealybugs that reproduce sexually is that the sedentary females produce powerful sex pheromones to attract the winged males for mating. Identification and commercial production of *P. ficus* sex pheromone has resulted in the widespread use of sex pheromone-baited traps as an effective tool to

monitor this species in vineyards (Millar et al. 2002, Walton et al. 2004, Flaherty 2008). The sex pheromones of *P. longispinus*, *P. viburni*, and grape mealybug, *Pseudococcus maritimus* (Ehrhorn) are under development for use in monitoring these species (Millar and collaborators, unpublished data), but currently, visual inspection of plant material is the only method used to monitor mealybugs in production nurseries. The pheromone of the other key mealybug pest in California nurseries, the citrus mealybug, *Planococcus citri*, was identified by Bierl-Leonhardt et al. (1981) almost three decades ago, but surprisingly, it has not yet found widespread use in monitoring and management of this species. Thus, the goal of my research was to determine whether the synthetic sex pheromones of *P. longispinus*, *P. viburni*, and *P. citri* can be used as an effective alternative to visual sampling in the detection of mealybug populations in nurseries producing ornamental plants and flowers. My specific objectives were to:

1. Test the use of pheromone-baited traps for detection and tracking of mealybug generations in nurseries, over multiple growing seasons;
2. Optimize the basic operational parameters for use of pheromone traps in nurseries, including effective pheromone dose and lure longevity;
3. Determine whether a combination lure might attract all three target species simultaneously; and
4. Compare the effectiveness of pheromone trapping with visual sampling.

Materials and Methods

Study sites. Mellano and Company of Bonsall, CA, (hereafter Mellano) and Milfelds' Nursery, Inc. of Riverside, CA, (hereafter Milfelds') were the two production nurseries used as study sites. Mellano grows a number of in-field crops for the cut-flower industry, whereas Milfelds' specializes in several woody-shrub, container-grown crops, predominantly azaleas. Both *P. longispinus* and *P. viburni* occurred at Mellano, whereas Milfelds' only had populations of *P. viburni*.

Seasonal trends in mealybug population dynamics. Mealybug infestations regularly occurred on myrtle (*Myrtle communis*) at Mellano. A 1.6 ha field of myrtle was designated as a study site, because it had a history of *P. longispinus* infestations. Twelve delta traps (Pherocon[®] IID, Trécé Inc., Adair, OK) were used at this site with each baited with a gray rubber septum lure (11 mm; The West Company, Lititz, PA) loaded with a hexane solution (1 mg/ml, 25 μ l = 25 μ g per lure) of racemic *P. longispinus* pheromone that was synthesized as previously described (Millar et al. 2009). Traps were positioned uniformly throughout the field beginning on 14 August 2006 and were placed above the plant canopy (Fig. 2.1). As plants grew, trap heights were adjusted accordingly (maximum height = 2.13 m). Plants were cut to within 30 cm of the ground twice during this observation period (mid-January 2007 and early February 2008) and traps were removed during the 7 – 10 day harvesting periods. Male mealybugs were counted in traps every 7 – 14 days until 4 December 2008. Traps were checked less frequently during summer months (May – September), because numbers of *P. longispinus* were

lower than during the cooler months of October - April. Lures in each trap were replaced after approximately 8 weeks. The mean counts per trap period were calculated.

Average high and low temperatures were calculated for the periods preceding trap monitoring with temperature data from University of California Statewide Integrated Pest Management Program's website (ipm.ucdavis.edu). The weather station is located near Escondido, California in San Pasqual Valley (ca. 30 km SE of the nursery), CIMIS station #153 (Escondido SPV).

Effective pheromone dose. Two other large fields of myrtle at Mellano were used for a dose response test of racemic *P. longispinus* pheromone using doses of 0, 1, 3.2, 10, 32, 100, and 320 µg loaded onto gray rubber septa as described above. Within each field, two blocks of delta traps were aligned in a transect from north to south, with prevailing winds from west to east. Traps were spaced 8 m apart in each transect and suspended at the top of the canopy (distance from the ground: 1.52 m – 2.13 m). Traps were collected and replaced every 3 to 4 days starting on 12 October 2007 and removed on 5 November 2007. Treatments were repositioned in the transects at each count so that no dose occurred in the same position more than once.

At Milfeld's, where *P. viburni* was the most prevalent mealybug species, five 37 m × 75 m (ca. 0.4 ha) plots of potted azaleas were used as sampling areas. One diagonal transect was set up so that it incorporated the entire length and width of each plot. Delta traps were separated by 9.4 m along a row and 4.6 m between rows. Traps were suspended from 1.2 m tall wooden stakes so that the traps were level with the plant canopy and oriented from NW to SE, with the prevailing wind. Each dose of racemic *P.*

viburni pheromone (0, 1, 3.2, 10, 33, 100, and 320 µg on gray septa, synthesized as described in Millar and Midland 2007) was represented once in each transect. Traps were deployed on 16 April 2008 and collected and replaced once a week until 29 May 2008. At each count, treatments were repositioned within a transect so that no dose occurred in the same position more than once. Data from both studies were analyzed using SAS version 9.1.3 (SAS Institute, Cary, North Carolina) with $\log_{10}(x+1)$ transformed data. Analysis of variance was performed on transformed data in PROC MIXED with the main treatment effect of pheromone dose (0, 1, 3.2, 10, 33, 100, and 320 µg) and blocks as a component of the RANDOM statement. Four and five blocks for *P. longispinus* and *P. viburni*, respectively, were used. Differences among means were tested using Tukey's HSD test.

Average high and low temperatures were calculated for the periods preceding trap monitoring with temperature data from University of California Statewide Integrated Pest Management Program's website (ipm.ucdavis.edu). The weather station was located on the University of California campus at Agricultural Operations (ca. 9 km NE of the nursery), CIMIS station #44,

Field longevity of lures. Sixty gray rubber septa were loaded with 25 µg doses of either the racemic *P. viburni* or *P. longispinus* pheromones. Ten lures with each species' pheromone were stored at -16 °C immediately after loading. The remaining lures were placed in a greenhouse in an empty wing trap (Pherocon[®] IIC, Trécé Inc.), and 10 lures with each species' pheromone were subsequently retrieved 1, 2, 4, 8, and 12 weeks later and stored at -16 °C until needed. These aged lures, fresh lures, and control septa treated

with solvent only comprised the seven treatments in this study. All septa that contained *P. viburni* pheromone were initially deployed in the greenhouse for aging on 17 April 2008, whereas *P. longispinus* lures were all initially deployed on 10 July 2008.

The attractiveness of fresh, aged, and control lures to male *P. viburni* were compared at Milfeld's. Pheromone-baited traps were laid out in transects as described for the dose response study at Milfeld's, using a total of five 0.4 ha plots of potted azaleas. Traps were installed on 16 July 2008 and collected and replaced every 2-4 days until 1 August 2008. At each count, the treatments were repositioned within a transect so that none occurred in the same position more than once.

The attractiveness of fresh, aged, and control lures to male *P. longispinus* was compared at Mellano, where *P. longispinus* was also a key pest of ruscus (*Ruscus hypoglossum*: Ruscaceae). Ruscus is an evergreen, shrub-like perennial with branched stems bearing numerous flattened, leaf-like stem tissues called cladodes. It reproduces naturally via seed but is propagated commercially primarily by rhizomes. The individual stems are harvested for the wholesale flower market. Mealybugs infest the expanding cladodes, stems, and areas underneath the cladodes where tiny non-photosynthetic leaves form. A 1.62 ha shade house with ruscus was divided into five blocks, with each block containing a diagonal transect with one each of all seven treatments. Traps were separated by 8.5 m along each trap row and rows were separated by 1.5 m with traps suspended 1 m above ground. Traps were installed on 14 October 2008 and collected and replaced every 3-4 days until 28 October 2008. Treatments were repositioned in the block so that no dose occurred in the same position more than once. Trap count data

were $\log_{10}(x+1)$ transformed and analyzed by analysis of variance in PROC MIXED with the main treatment effect of lure longevity (1, 2, 4, 8, and 12 weeks) and blocks as a component of the RANDOM statement. There were five blocks each in the *P. longispinus* and *P. viburni* studies. Differences among means were tested with Tukey's HSD test.

A second set of experiment tested the extended longevity of lures. Lures aged for 12 wk before deployment were left in place for an additional 9 (*P. longispinus*) and 7 (*P. viburni*) wk after the completion of the initial study. For the *P. longispinus* study, fresh lures (25 μg) were deployed once each week for two weeks following the initial study and were not replaced for the remaining seven weeks. Comparisons in trap catches were made between progressively aging 12 wk-old lures and initially fresh and progressively aging newer lures. Fresh *P. viburni* pheromone lures (25 μg) were deployed during each sampling period (ca. 1 – 2 wk) following the initial study, and comparisons were made between trap catches with traps baited with fresh lures and lures aged 12 wk before deployment.

Combination lures. The treatments in the first of these studies consisted of lures loaded with the pheromones of each individual species (*P. citri*, *P. viburni*, and *P. longispinus*), a combination lure containing the pheromones of all three species, and a blank control. The *P. citri* pheromone was synthesized as described by Passaro and Webster (2004). Gray rubber septum lures were loaded with hexane solutions (25 μg doses) of racemic pheromone of each species. The 1.6 ha shade house with ruscus at Mellano was divided into six blocks, with each block containing a diagonal transect that possessed one of all

five treatments. Delta traps in this diagonal transect were separated by 8.5 m along a row and 2.4 m between rows in each block and were suspended at the canopy height, approximately 1 m above ground. Prevailing winds were from the west. Traps were installed on 12 November 2007, collected and replaced every 3-6 days until 3 December 2007, and treatments were repositioned in the transect at each count so that no treatment occurred in the same position more than once. The predominant mealybug species during this study was *P. longispinus*.

Following the initial study testing the efficacy of combining pheromones, two more detailed studies were initiated to assess possible interference among the three pheromones. The first study was conducted at Mellano utilizing the same 1.6 ha ruscus plot. Treatments consisted of combinations of two pheromones (*P. citri* and *P. longispinus*, *P. citri* and *P. viburni*, and *P. longispinus* and *P. viburni*), the combination of all three pheromones, each individual pheromone, and a blank control. All traps were baited with lures that contained 25 µg of racemic pheromone of each species, loaded as described above. Each of the six blocks contained one of all seven treatments in a diagonal transect, as described above in the lure longevity study at Mellano. Traps were installed on 7 August 2009 and collected and replaced every 7-10 days (seven changes) until 28 September 2009. At each count, treatments were repositioned so that no pheromone combination occurred in the same position more than once.

The second study was conducted at Milfeld's. Traps contained one of the seven pheromone combinations listed in the previous paragraph and were laid out in transects as described for the dose response study at Milfeld's. Five plots (0.4 ha) of potted

azaleas were used. Traps were initially deployed on 3 August 2009 and were collected and replaced every 3-7 days (six changes) until 3 September 2009. At each count date, treatments were repositioned in the block so that no pheromone combination occurred in the same position more than once. An analysis of variance using $\log_{10}(x+1)$ transformed data in PROC MIXED was done for each of these experiments with blocks (six at Mellano and five at Milfelds') as a component of the RANDOM statement. Estimate statements in PROC MIXED were used to test for differences between specific pairs of means (three pheromone blend versus the sum of individual pheromone components, *P. citri* and *P. longispinus* combined versus sum of individual pheromone components of *P. citri* and *P. longispinus*, *P. citri* and *P. viburni* versus sum of individual pheromone components of *P. citri* and *P. viburni*, and *P. longispinus* and *P. viburni* versus sum of individual pheromone components of *P. longispinus* and *P. viburni*).

Comparison of pheromone trap catches with mealybug densities. A 0.49 ha plot of ruscus infested with *P. longispinus* was utilized in this study at the Mellano site. The plot had already been divided into seven hoop houses (~ 63 m long × 7 m wide) covered in shading plastic with each house having open ends. The houses were on a hill with the base 10 m lower than the top. The plastic that covered the hoops began 1 m above the plant canopy along the length of these houses so that airflow was not restricted. Ruscus was grown in four rows in each of these houses with each row being ~ 1 m wide. Mealybugs infest the expanding cladodes, stems, and areas underneath the cladodes where tiny non-photosynthetic leaves form. Visual inspection of plant material for

mealybugs usually requires that individual cladodes be separated from one another to locate and identify mealybugs.

Prior to initiation of the study, it was important to determine what growth stage of ruscus was preferred by mealybugs. The stages were classified as post-harvest, harvest, pre-harvest, and newly emerging/expanding stems. Post-harvest stems possessed cladodes that were dark green with a thick, tough texture, and torn cladode margins. Harvest-stage stems and cladodes were also dark green in color but younger (~ 5 months old). Pre-harvest stems were stems that had reached full height with the bright, shiny green cladodes fully expanded. These stems were less than 5 months old. Finally, newly emerged or expanding stems were short and possessed very small cladodes with short inter-cladode distances.

Three of the seven houses were selected at random, and moving from west to east in each house, ruscus stems were collected at approximately 30 m intervals. One stem of ruscus from each stage was cut at or near its base from each of four rows of ruscus in that house. Stems were wrapped in paper towels and returned to the laboratory for inspection of all stem and cladode surfaces. Because ruscus stems were not of uniform height, only the top 30 cm of each stem was inspected for mealybugs. It was determined that both of the youngest stages of ruscus stems possessed higher mean numbers of mealybugs than either harvest or post-harvest stages (data not presented). Consequently, these stem stages were selected for a study comparing pheromone trap catches to mealybug densities on plants.

Each house was divided into three sections (21 m long \times 7 m wide). Then, each section was divided into three subsections (7 m \times 7 m). There were three sampling periods during this study. Wing traps (Pherocon[®] IC, Trécé Inc.) containing standard 25 μ g *P. longispinus* pheromone lures were deployed at random in the middle of one of the three subsections within each section so that at any point during this study there were three traps in each house for a total of 21 traps in the entire growing area. For the second sampling period, wing traps were deployed at random into a previously unoccupied subsection within each section. The final, unused subsection was then utilized for the final sampling period. Wing traps were oriented from west to east, with the opening of the trap being 2.5 cm wide around the trap's perimeter to allow airflow through the trap in all directions. Traps were suspended with zip-ties from 1.2 m metal poles placed in the center of either of the two middle rows. Rows were selected at random within each subsection. The vertex of the bottom of the trap was placed with the top of the crop canopy, approximately 1 m from the ground. Traps were collected, replaced, and moved to a new subsection every 7 days. Lures were not replaced during the course of the 3 weeks.

Once a trap had been deployed for one week, the plants in each subsection that had contained a trap were sampled. A 1 m \times 1 m PVC frame was constructed from 1/2" irrigation pipe with internal pipes dividing the 1 m² area into four equal parts. Copper wire was strung through the main frame and the cross-members to provide an internal grid of 25 cm \times 25 cm squares. The entire grid was placed on the plant canopy with its edge 1 m from the pheromone-baited trap in all 4 cardinal directions. As a result, one

row of ruscus was out of range of the 1 m grid and not sampled. Three ruscus stems of the appropriate stage (discussed above) that were closest to the junction of the copper wires were cut and removed in each of the four 50 cm × 50 cm PVC squares. Therefore, 12 ruscus stems were cut from each grid placement. Although stems were cut at or near the base, stems ranged in length from ~20 – 30 cm. Stems were cut and wrapped quickly in paper towels and returned to the laboratory for counting. All areas of the stem and cladodes were inspected for mealybugs. Stems were stored in a cold room (4 °C) until counted.

Comparison of trap catches to mealybug densities indicated that the slopes of the three regression lines (one for each sampling date, see Table 2.1) were not statistically different from one another ($F = 0.63$, $df = 2, 57$, $P = 0.53$) (methods from Zar 1996). Data were pooled ($n = 63$), and PROC REG was used to analyze pooled sampling dates. Both trap count values (dependent variable) and mean counts of mealybugs found on ruscus stems (independent variable) (48 stems total) were $\log_{10}(x + 1)$ transformed to meet the assumptions of normality and homoscedasticity.

Results

Seasonal trends in mealybug population dynamics. The numbers of *Pseudococcus longispinus* males caught in traps were highest during late fall through early spring months of 2006-2008 (Fig. 2.2). Trap catches decreased into May in both years of the study and remained low through the middle of October. Mean high temperatures during peaks of male mealybug activity were typically between 21.1-23.9 °C. Temperatures consistently greater than 26.7 °C coincided with low counts of *P. longispinus* males.

Traps were removed from 1 December 2006 to 11 January 2007 (42 days) and again from 27 November 2007 to 2 January 2008 (37 days), while the myrtle crop was harvested.

Effective pheromone dose. The numbers of males caught in traps baited with *P. longispinus* pheromone varied significantly with dose ($F = 37.8$, $df = 6, 161$, $P < 0.0001$). Traps baited with *P. longispinus* pheromone caught more male mealybugs than the blank control. The 1.0 µg dose attracted significantly fewer males than the 320 µg dose whereas doses between 1.0 µg – 100 µg attracted similar numbers of *P. longispinus* males (Fig. 2.3). The same lures were used throughout the 3-week duration of the trial.

Similar results were observed in the pheromone dose response trials with *P. viburni*. The initial statistical analysis of seven sampling dates over 7 weeks showed a significant interaction between date and pheromone dose ($F = 1.60$, $df = 36, 195$, $P < 0.02$). Removal of the 21 May 2009 data eliminated the interaction ($F = 1.18$, $df = 30, 167$, $P = 0.25$). Removal of the data for this count period from the overall data set was justified on the basis of the inclement weather during this period; mean counts of male mealybugs during the period ranging from 2 – 11 insects per block for doses ranging

from 1 – 320 µg, respectively, much lower than the counts on dates prior to and after this period. The average high and low temperatures in Riverside during this sampling period were 18.8 °C and 10.3 °C, respectively. In contrast, during the periods prior to 21 May, mean high temperatures ranged from 21.7 – 33.9 °C, and the mean high temperature following 21 May was 24.4 °C.

With the removal of the 21 May data, there were significant differences among the pheromone doses ($F = 110.35$, $df = 6$, 197 , $P < 0.0001$) in their attractiveness to male *P. viburni*. The numbers of insects caught with doses of 10, 32, 100, and 320 µg were not significantly different (Fig. 2.4). Similar to the *P. longispinus* study, the lowest dose of 1.0 µg was least attractive whereas the doses of 3.2 and 10 µg were more attractive than 1 µg. Overall, in both studies of effective pheromone dose, male *P. longispinus* and *P. viburni* were attracted by relatively small doses of pheromone, and higher doses were not necessary in order to attract large numbers of males.

Field longevity of lures. There were no significant differences in the numbers of male *P. viburni* captured in traps baited with 25 µg lures that had been field aged for 0 – 12 weeks prior to deployment ($F = 0.71$; $df = 5$, 139 ; $P = 0.61$) (Fig. 2.5). No males were caught in control traps, and therefore the control was excluded from the analysis.

There were significant differences among the treatments in the attractiveness of *P. longispinus* lures aged for different periods ($F = 4.15$; $df = 5$, 110 ; $P = 0.002$) (Fig. 2.6). Unexpectedly, lures aged for 8 and 12 weeks attracted significantly more males than fresh or 1-week old lures and attracted similar numbers of males when compared to 2 and

4-week old lures. Fresh and 1 to 4-week old lures had no difference in their attractiveness (Fig. 2.6).

Aged lures that remained in the field continued to attract males throughout the entire periods of the studies (*P. longispinus* – 9 wks, *P. viburni* – 7 wks). For the *P. viburni* study, there were no significant differences in mean counts of males between traps baited with either the fresh or progressively aging 12 wk lures (Fig. 2.7a). For the analogous study with *P. longispinus*, old lures (aged 12 wks before deployment) were as attractive at 21 wk field age if not more attractive than newer lures. After 21 wk, significantly more males were attracted to traps baited with newer (now 9 wk old) lures (Fig. 2.7b).

Combination lures. The catches in traps baited with the full combination lure treatment (citrus, longtailed, and obscure mealybug pheromones = CLO) were compared to the sum of mealybugs caught in the traps baited with individual pheromones (C+L+O). The predominant mealybug species detected was *P. longispinus*. There was a significant interaction between sampling date and treatment ($F = 3.38$, $df = 4, 49$, $P = 0.016$) so each date was analyzed separately. Traps with combination lures caught significantly fewer males than the sum of males in traps baited with a single pheromone on three of the five dates (15 Nov: $F = 27.9$, $df = 1, 10$, $P = 0.0004$; 26 Nov: $F = 16.41$, $df = 1, 9$, $P = 0.003$; 29 Nov: $F = 18.18$, $df = 1, 9$, $P = 0.002$) (Fig. 2.8).

A follow-up study was performed to determine which heterospecific pheromone component inhibited attraction of *P. longispinus* males. Data from all seven sampling dates over 7 weeks were combined. The combination of all three pheromones (CLO

treatment) attracted fewer males compared to the sum of males in traps each baited with one pheromone (estimate statement: $t = 2.38$, $df = 321$, $P = 0.02$) (Fig. 2.9) as in the previous trial. Significantly fewer males were attracted to traps baited with the blend of *P. citri* (citrus mealybug) and *P. longispinus* pheromone (CL) than to the *P. longispinus* pheromone alone (estimate statement: $t = 2.46$, $df = 321$, $P = 0.01$) (Fig. 2.9), indicating that *P. citri* pheromone was slightly inhibitory to male *P. longispinus*. The numbers of males attracted to the combination of *P. citri* and *P. viburni* (CO treatment) and *P. longispinus* and *P. viburni* pheromones (LO treatment) were not significantly different than the sums of trap catches to the two individual pheromone components, respectively (Fig. 2.9), demonstrating that the *P. citri* pheromone was not antagonistic to male *P. viburni*.

An additional study assessed a population of *P. viburni* to verify that males of this species were not inhibited by the pheromones of the other two study species. There was a statistical interaction between date and treatments ($F = 2.18$, $df = 35, 188$, $P = 0.001$) so data from different dates were not combined (Figs. 2.10a-f). Among all sampling periods, there was only one significant difference between any combined pheromone and the corresponding total of single pheromones. The three pheromones combined attracted fewer males than the total counts of three individual pheromones for 13 Aug. 2009 ($t = 2.19$, $df = 31$, $P = 0.04$) (Fig. 2.10c). Otherwise, there were no differences between treatments, indicating that the presence of other pheromones does not inhibit the attraction of *P. viburni* males (Figs. 2.10a-f).

Comparison of pheromone trap catches with mealybug densities determined by manual sampling. The data from three sampling dates were pooled after determining that the slopes of the three regression lines were not statistically different ($F = 0.63$, $df = 2, 57$, $P = 0.53$) (methods in Zar 1996). There was a significant relationship between the numbers of male *P. longispinus* trapped and the average number of mealybugs on ruscus stems (Fig. 2.11). This indicates that pheromone-baited traps are not only a sensitive tool for detecting mealybugs, but also can be used to assess population densities.

Discussion

Seasonal trends in mealybug population dynamics. *Pseudococcus longispinus* was the most prevalent species during the cooler months of the year at Mellano whereas other mealybugs were more prevalent during the warmer months. This mealybug species is known to have a narrow temperature tolerance, and it is more often a serious problem in glasshouses and indoor plantscapes than in field crops (Godfrey et al. 2002). Peaks and valleys in the trend line for mean mealybug counts appeared to coincide with temperature fluctuations between sampling periods.

Pheromone-baited traps provided a useful tool to detect population trends. Increases in field populations of any mealybug species often remain undetected by growers until the resulting well-established populations are difficult to control. The effectiveness of these traps in attracting male insects year-round demonstrated that there is usually an underlying population of *P. longispinus* in myrtle, although densities at times were too low to merit management. I did not test the effective range of pheromone-baited traps, although this is an area of study essential for their optimal use (see Chapter 5). Overall, pheromone-based monitoring proved to be a sensitive method of following population cycles. Thus, deployment of pheromone traps will help to better time management decisions compared to visual methods of population estimation.

Effective pheromone dose. For both *P. longispinus* and *P. viburni*, relatively small doses of pheromone were sufficient to attract males of both species (Figs. 2.3 and 2.4). Analogous dose-response trends have been observed with other species such as *P. citri* and *P. ficus* (Hefetz and Tauber 1990, Millar et al. 2002, Zada et al. 2004), whereas

the pink hibiscus mealybug, *Maconellicoccus hirsutus* (Green), appears to be acutely sensitive to dose with males being most attracted to lures baited with 1 µg of pheromone (Zhang and Amalin 2005), and with higher doses being inhibitory.

Furthermore, in the studies reported here, trap catches leveled off as the amount of pheromone dose rose above a specific quantity, suggesting that male *P. longispinus* and *P. viburni* are relatively insensitive to dose, and orient and fly to a source once the pheromone surpasses a threshold concentration (Millar et al. 2002, Zada et al. 2004). It should also be noted that the effective doses of 25 µg of racemic pheromone (i.e., 12.5 µg of the natural stereoisomer) or less required to attract *P. longispinus* and *P. viburni* were still considerably lower than the doses typically used for pheromone lures for other types of insects.

Racemic blends of the pheromones were adequate in attracting male *P. longispinus* and *P. viburni* in each pheromone dose trial. These results were similar to those from studies that assessed the biological activity of racemic and chiral *P. ficus* and *P. citri* pheromones (Millar et al. 2002, Zada et al. 2004). In both studies, males were not antagonized by the presence of the other stereoisomer. This trend is not true for all mealybugs; responses of male *M. hirsutus*, and passionvine mealybug, *Planococcus minor*, are inhibited by the presence of unnatural stereoisomers of their pheromones in synthetic pheromone preparations (Zhang and Amalin 2005, Ho et al. 2007). Overall, the cheaper and more easily produced racemic forms of the *P. longispinus* and *P. viburni* pheromones should be entirely adequate for development and production of commercial pheromone products.

Field longevity of lures. Gray rubber septa loaded with 25 µg of racemic pheromone had excellent field longevity; numbers of *P. viburni* males caught were similar in all treatments, regardless of lure age (Fig. 2.5). The fact that traps baited with fresh lures or lures field aged for 1 wk before deployment actually captured fewer male *P. longispinus* than lures aged for 2, 4, 8, or 12 wk (Fig. 2.6) suggested that this species might be more sensitive to dose than we had previously thought, with the initial release rate of the 25 µg dose apparently causing some degree of inhibition. Overall, the effective lifetimes of *P. viburni* and *P. longispinus* lures were similar to those reported for male *P. ficus*, *P. citri*, and *M. hirsutus* (12 wk, 16 wk, and 5 months, respectively) (Millar et al. 2002, Zada et al. 2004, Zhang and Amalin 2005). These long field longevities of lures will lower costs for growers to maintain pheromone traps because lures will need to be changed only every 3 to 4 months, or about twice a growing season when insects are most active.

Combination lures. We did not expect to see inhibition among any of the three pheromones because of the substantial differences in the structures of the pheromones of the study species. Nevertheless, there were indications of slight inhibition of male *P. longispinus* by *P. citri* pheromone, whereas *P. viburni* was unaffected by the presence of either or both of the other two pheromones. Antagonism between pheromones is usually a result of competition for the same pheromone channel, resulting in selection pressure to avoid responding to heterospecifics (Howse 1998). Although many mealybug species now are cosmopolitan with overlapping distributions (Godfrey et al. 2002), the various species originated from different parts of the world. Thus, Bartlett (1978) reported that *P. citri* was likely endemic to China, whereas Miller et al. (2005a) reported that *P.*

longispinus had an Australasian (Australia, Tasmania, and surrounding islands) origin. In contrast, McKenzie (1967), Bartlett (1978), and Ben-Dov (1994) all stated that the origin of *P. longispinus* was unknown. If the historical distributions of both species did indeed overlap, male *P. longispinus* may have evolved to detect *P. citri* pheromone and avoid *P. citri*-infested plants.

Because the level of inhibition of *P. longispinus* males by *P. citri* pheromone was relatively low, it should still be possible to use the combination pheromone to detect and sample all three species of mealybugs simultaneously in nursery settings. In particular, a combination lure provides an opportunity to lower costs and simplify monitoring for multiple mealybug species. With similar mealybug management strategies for all species, identification of mealybugs to the species level is unimportant to growers.

Only a couple of other studies have demonstrated success in combining sex pheromones for detecting multiple insect species. Jones et al. (2009) demonstrated the efficacy of lures loaded with the combined sex pheromones of *Malacosoma disstria* Hübner (Lasiocampidae) and *Choristoneura conflictana* (Walker) (Tortricidae), two lepidopteran forest pests in western Canada. Males of both species were attracted equally to traps baited with combined pheromones or the individual pheromones (Jones et al. 2009).

An earlier study had examined the use of multiple sex pheromones to both monitor and control pests. Qureshi and Ahmed (1989) demonstrated control of three bollworm pests in cotton in Pakistan with a pheromone formulation that incorporated pheromones for pink bollworm, *Pectinophora gossypiella* (Saunders), spiny bollworm

Earias insulana Boisduval, and spotted bollworm *Earias vittella* (Fabricius). There were lower infestations of all three pests in squares and green bolls within areas treated with the combined pheromones (PB/SB-ROPE formulation) compared to areas treated with insecticides. Other studies have used combinations of aggregation pheromones to detect multiple coleopteran pests in orchards (Nitidulidae) (James et al. 2000), forests (Scolytinae) (Miller et al. 2005b), and stored products (Curculionidae) (Wakefield et al. 2005).

Comparison of pheromone trap catches with mealybug densities. We found a strong correlation between mealybug densities on plant material and trap catches of male mealybugs in pheromone traps, as had been found in studies with *P. ficus* in vineyards (Millar et al. 2002, Walton et al. 2004). In the former study, mealybug damage on grape bunches was also correlated to the number of male *P. ficus* in traps (Millar et al. 2002). Hefetz and Tauber (1990) found a weak but significant correlation between catches of male *P. citri* and the mealybugs sampled manually on surrounding citrus trees and grass. Other studies assessing pheromone-baited traps for mealybugs have examined them as “detection only” tools (Zada et al. 2004, Zhang and Amalin 2005, Vitullo et al. 2007), making this study the first to examine the correlation between trap catches and density of insects on surrounding plant material in production nurseries. Our results suggest that it should be possible to replace laborious visual-inspection methods (5-min. timed samples and seasonal stem infestations) by detecting and sampling populations with pheromone traps.

Nonetheless, the situation may be complicated by the fact that male mealybugs are more susceptible than females to insecticides commonly used for mealybug control. In particular, because males undergo complete metamorphosis whereas females do not, males are disproportionately susceptible to insect growth regulators such as pyriproxifen (Hinkens et al. 2001, Millar et al. 2002, Millar et al. 2005, Millar et al. 2009). Thus, heavy insecticide use may kill a larger percentage of males than females, causing a decline in trap catches of male mealybugs even though populations of females may still be significant. To further complicate matters, females that remain unmated for extended periods tend to produce a higher percentage of male offspring once they are mated (Varndell and Godfray 1996), resulting in a male bias in the next generation, and possible overestimation of population sizes from trap counts. This in turn may lead to unnecessary insecticide applications. Thus, it may be important for plant managers to know the insecticide application history of crops when monitoring mealybugs with pheromone-baited traps.

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Table 2.1. Components from the ANOVA in the three regression analyses (three dates) of trap count data regressed against the mean count of *Pseudococcus longispinus* on *Ruscus hypoglossum* stems. Data had been $\log_{10}(x + 1)$ transformed to meet assumptions of normality and homogeneity of variances, and transformed values of the slope and intercept are presented (n = 21 for each date).

Component	Date 1	Date 2	Date 3
Slope	5.48	5.41	10.68
Intercept	2.70	2.64	1.10
Adjusted r^2	0.70	0.64	0.43
F	47.76	35.87	15.85
df	1, 19	1, 19	1, 19
<i>P</i>	< 0.0001	< 0.0001	0.0008

Figure legends

Figure 2.1. Aerial view of myrtle field used to monitor seasonal populations of longtailed mealybugs (*Pseudococcus longispinus*) with pheromone-baited traps. Circles represent the approximate locations of sticky traps.

Figure 2.2. Mean numbers of new male *P. longispinus* (trend line with diamond markers) caught in sticky traps during the 28-month monitoring period in the 1.6 ha myrtle field depicted in Figure 2.1. Average high (square markers) and low (triangular markers) temperatures during the time period preceding the sampling date are shown.

Figure 2.3. Mean numbers (\pm SE) of *P. longispinus* males caught in sticky traps baited with 0, 1, 3.2, 10, 32, 100, and 320 μg of racemic pheromone. Counts were $\log_{10}(x + 1)$ transformed then analyzed by analysis of variance (PROC MIXED in SAS) followed by Tukey's HSD tests for separation of means ($\alpha = 0.05$).

Figure 2.4. Mean numbers (\pm SE) of *Pseudococcus viburni* males caught in sticky traps baited with 0, 1, 3.2, 10, 33, 100, and 320 μg of racemic pheromone. Counts were $\log_{10}(x + 1)$ transformed then analyzed by analysis of variance (PROC MIXED in SAS) followed by Tukey's HSD tests for separation of means ($\alpha = 0.05$).

Figure 2.5. Mean numbers (\pm SE) of *P. viburni* males caught in sticky traps baited with lures loaded with 25 μg of racemic pheromone that were field aged for 0, 1, 2, 4, 8, and 12 weeks before deployment. Counts were $\log_{10}(x + 1)$ transformed and analyzed by analysis of variance (PROC MIXED in SAS) followed by Tukey's HSD tests for separation of means ($\alpha = 0.05$). Control traps caught no males and so were not included in the analysis.

Figure 2.6. Mean numbers (\pm SE) of *P. longispinus* males caught in sticky traps baited with lures loaded with 25 μ g of racemic pheromone that were field aged for 0, 1, 2, 4, 8, and 12 weeks before deployment. Counts were $\log_{10}(x + 1)$ transformed, then analyzed by analysis of variance (PROC MIXED in SAS) followed by Tukey's HSD tests for separation of means ($\alpha = 0.05$). Control traps caught no males and were not included in the analysis.

Figures 2.7a-b. Mean numbers (\pm SE) of male (a) *Pseudococcus longispinus* and (b) *Pseudococcus viburni* mealybugs caught in traps baited with "fresh" 25 μ g lures (white bars) and continuously aging 12-week lures (black bars) from the initial lure longevity experiment (Figs. 2.5 and 2.6). Counts were $\log_{10}(x + 1)$ transformed and analyzed by analysis of variance (PROC MIXED in SAS). After the 6 November 2008 date, "fresh" lures were not replaced for *P. longispinus*. * = significant differences between pairs of means, $P < 0.05$.

Figure 2.8. Mean numbers (\pm SE) of male mealybugs caught in traps baited with a blend of 25 μ g each of *Planococcus citri*, *P. longispinus*, and *P. viburni* pheromones (black bars = combination pheromone) compared to the mean total of males caught in each trap baited with 25 μ g of one pheromone (white bars = sum of pheromones). Because of a date by treatment interaction, sampling dates were analyzed separately by analysis of variance (PROC MIXED in SAS) and statistically different means ($0.05 < P < 0.001$) are noted with an asterisk.

Figure 2.9. Mean numbers (\pm SE) of male mealybugs caught in traps baited with a blend of all three pheromones (CLO) or combinations of two pheromones (*P. citri* + *P.*

longispinus = CL, *P. citri* + *P. viburni* = CO, and *P. longispinus* + *P. viburni* = LO) (black bars = combination pheromones) compared to the sums of males caught in each trap baited with 25 µg of each component pheromone (white bars = sum of pheromones). Sampling dates were combined. Data were analyzed by analysis of variance (PROC MIXED in SAS) using estimate statements, and where statistically different ($P < 0.05$), are noted by an asterisk.

Figure 2.10. Mean numbers (\pm SE) of male mealybugs caught in traps baited with a blend of three pheromones (CLO) or combinations of two pheromones (CL, CO, LO) (black bars = combination pheromones) compared to the sum total of males caught in each trap baited with 25 µg of each pheromone (white bars = sum of pheromones).

Because of a date by treatment interaction, sampling dates were each analyzed separately by analysis of variance (PROC MIXED in SAS). Differences in means were assessed by estimate statements, and where statistically different ($P < 0.05$), are noted by an asterisk.

Figure 2.11. Numbers of male *P. longispinus* caught in pheromone-baited traps regressed against the average count of *P. longispinus* on 48 *Ruscus hypoglossum* stems. Both trap and count data were $\log_{10}(x + 1)$ transformed. Pheromone-baited trap data catches were significantly and positively correlated to mealybug numbers estimated by visual sampling ($y = 0.97x + 1.73$, $r^2 = 0.40$, $P < 0.0001$).

Figure 2.1.



Figure 2.2.

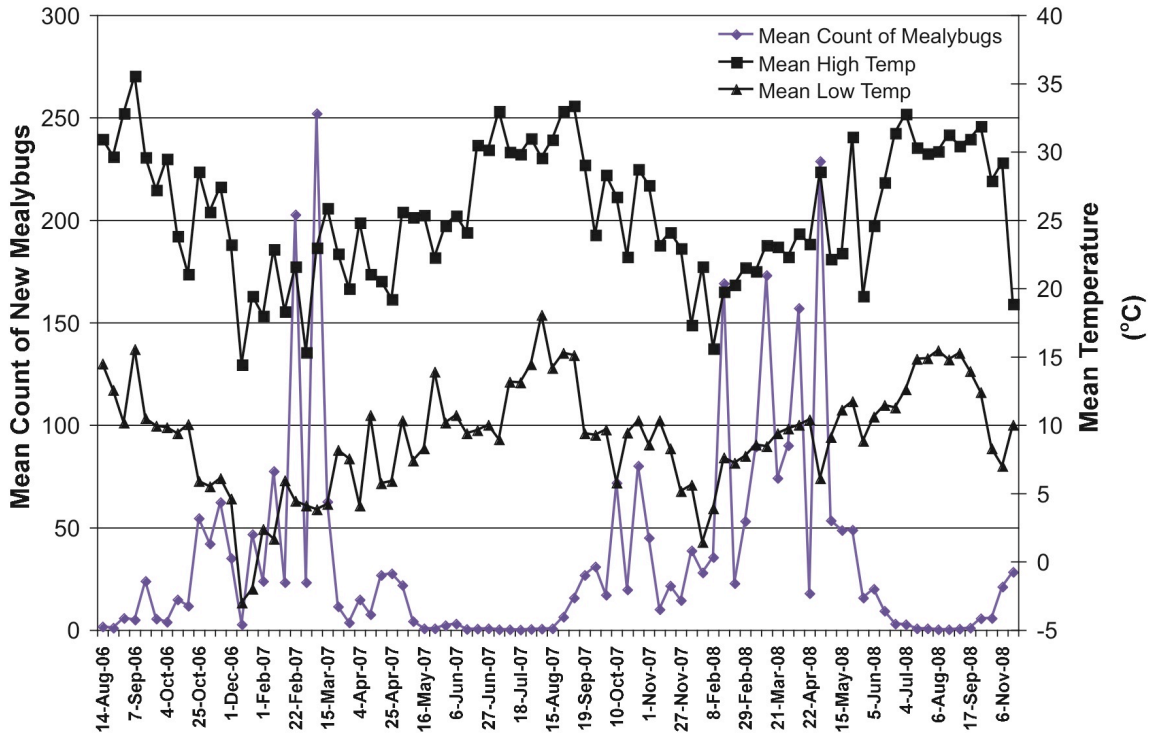


Figure 2.3.

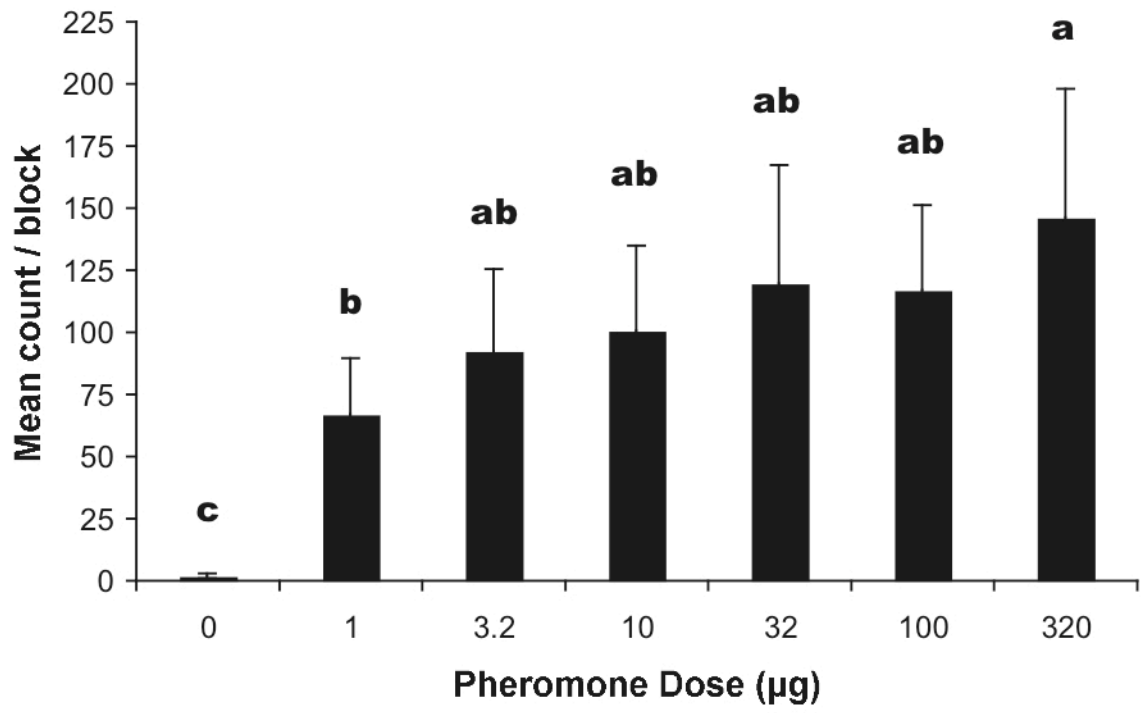


Figure 2.4.

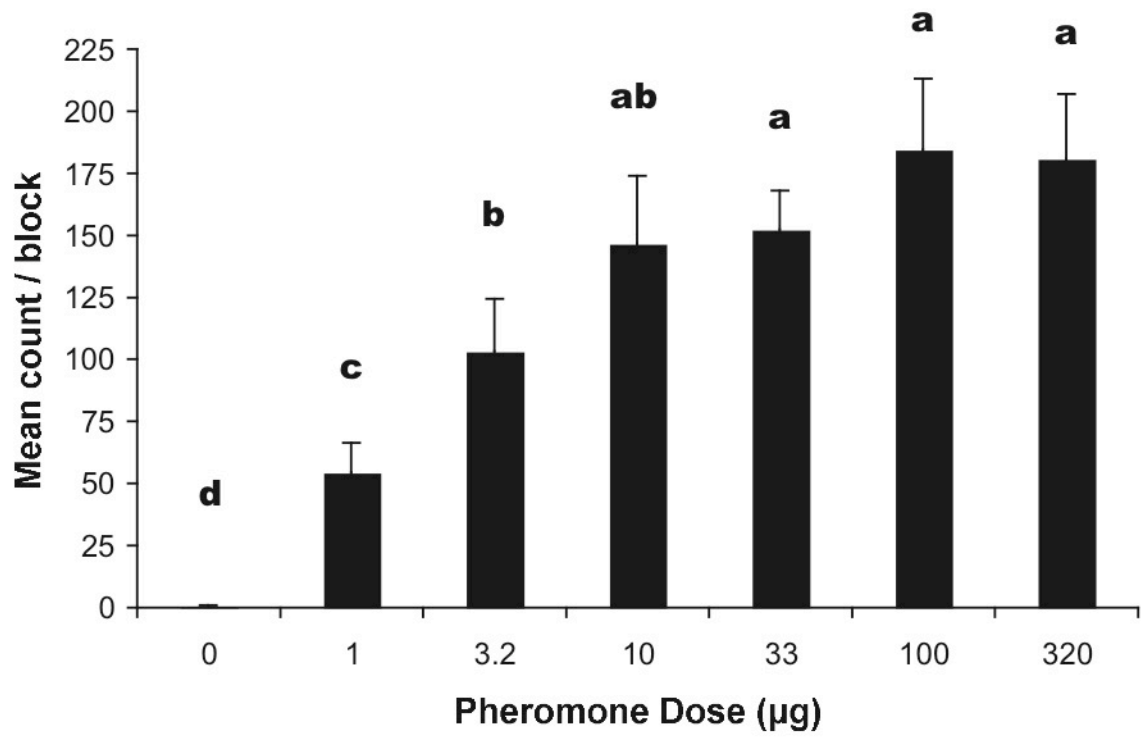


Figure 2.5.

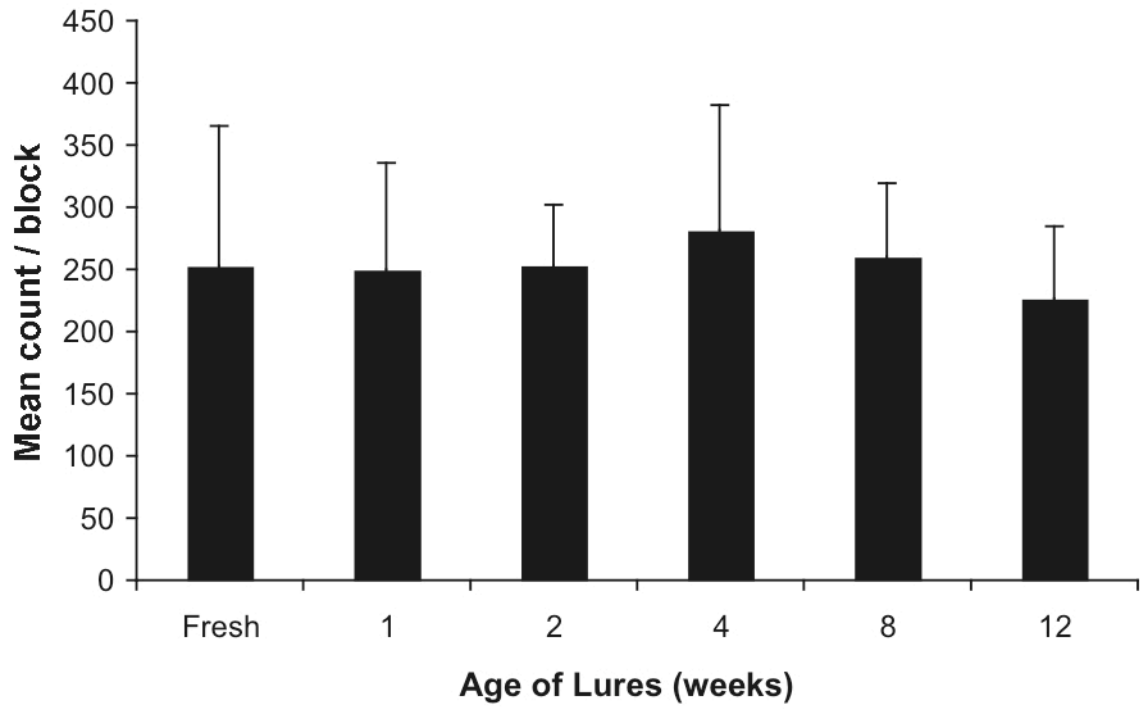
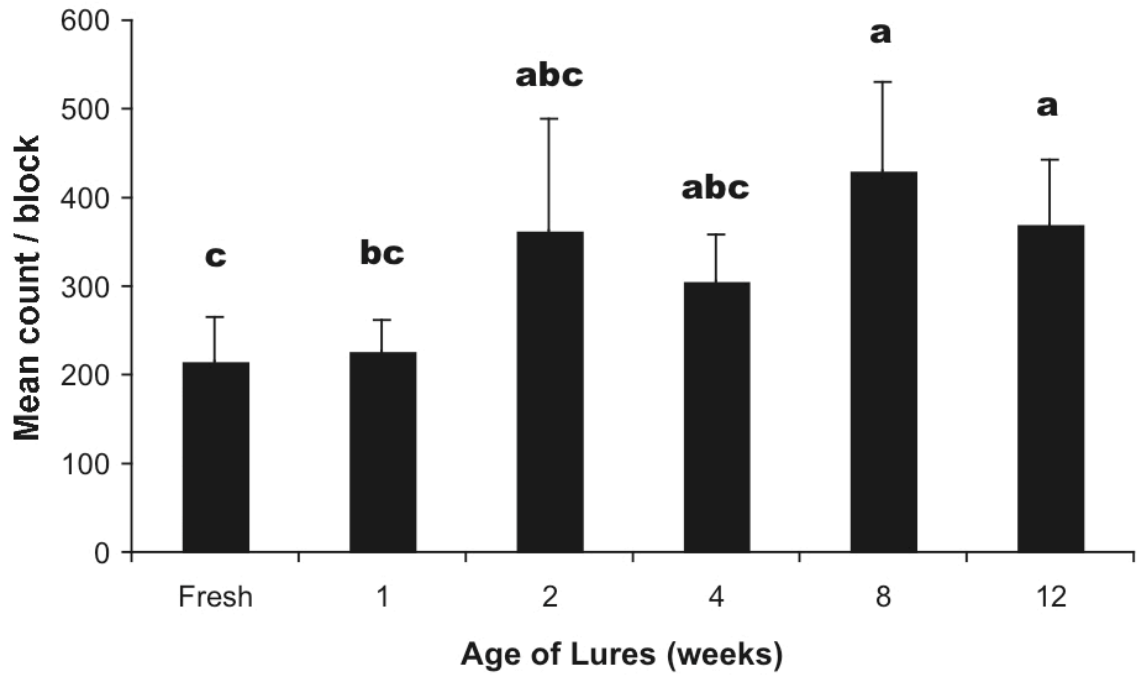


Figure 2.6.



Figures 2.7a-b.

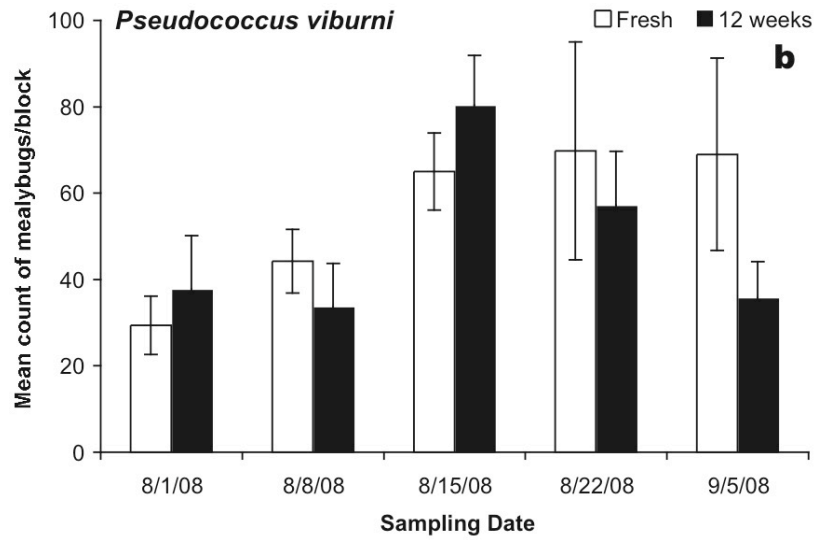
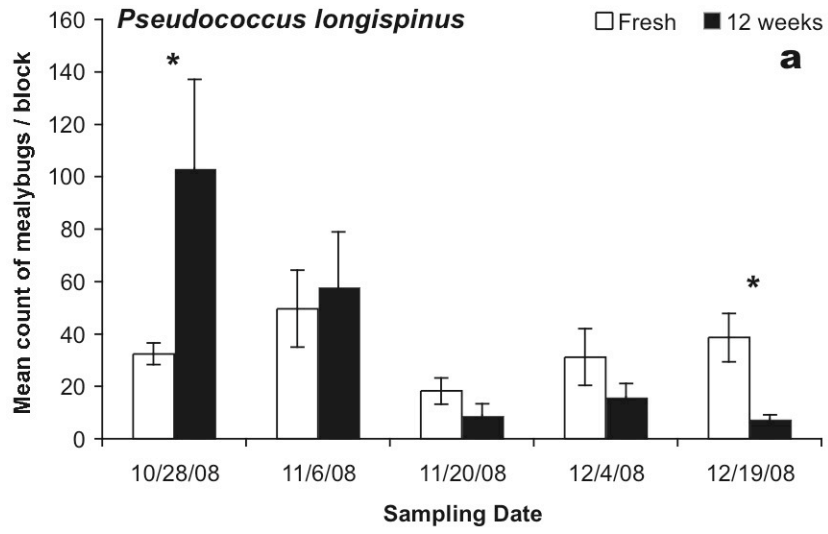


Figure 2.8.

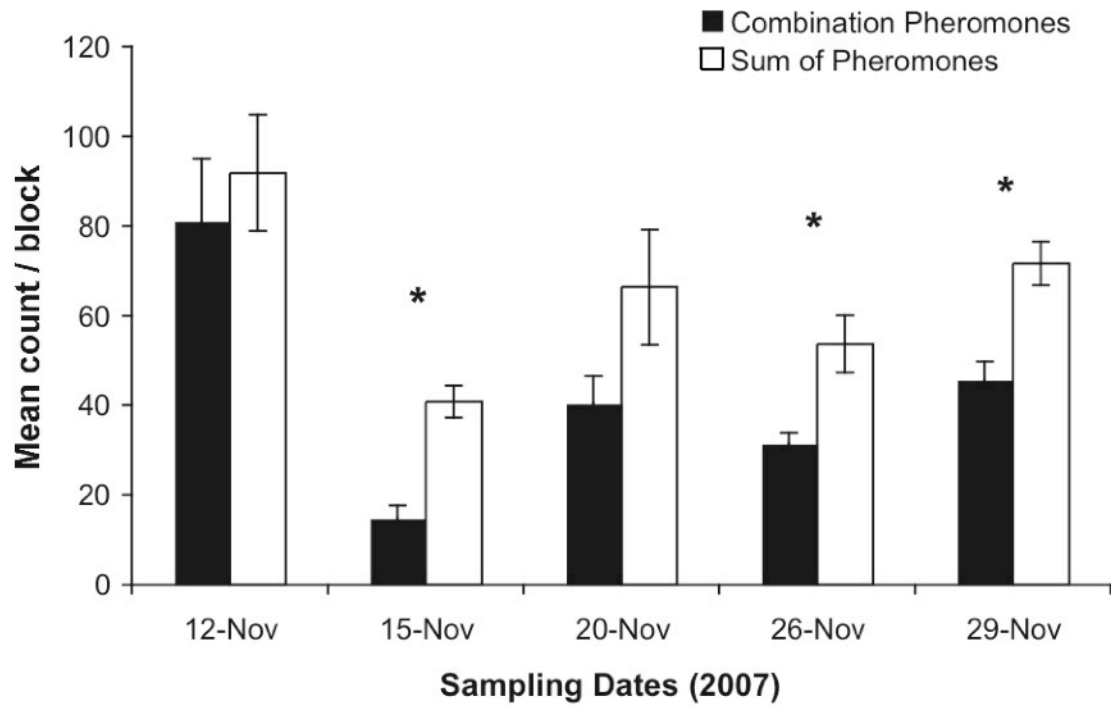
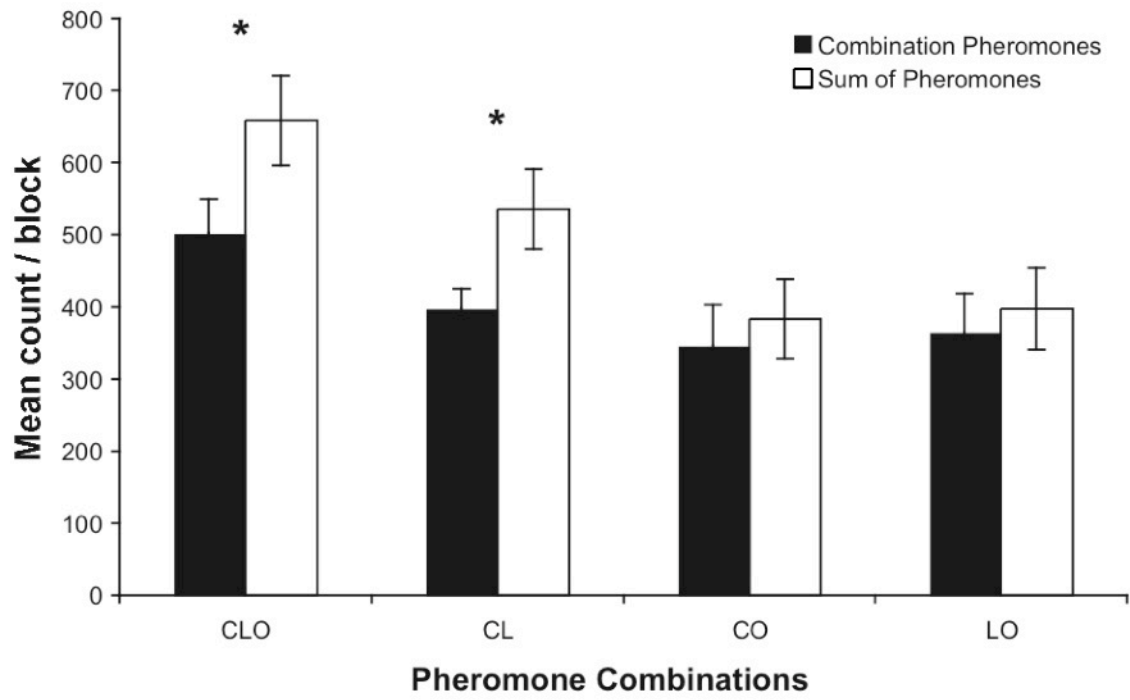


Figure 2.9.



Figures 2.10a-f.

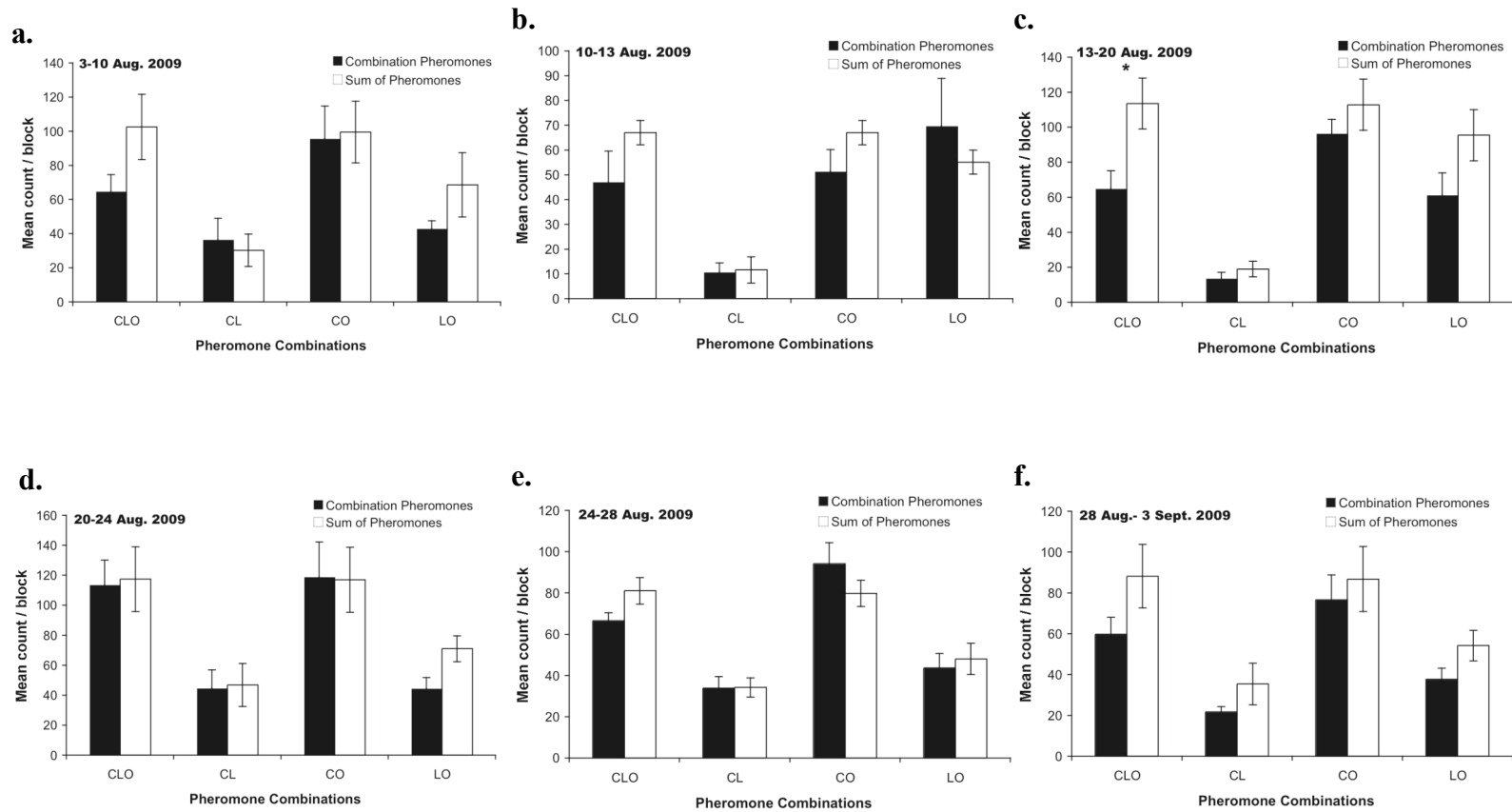
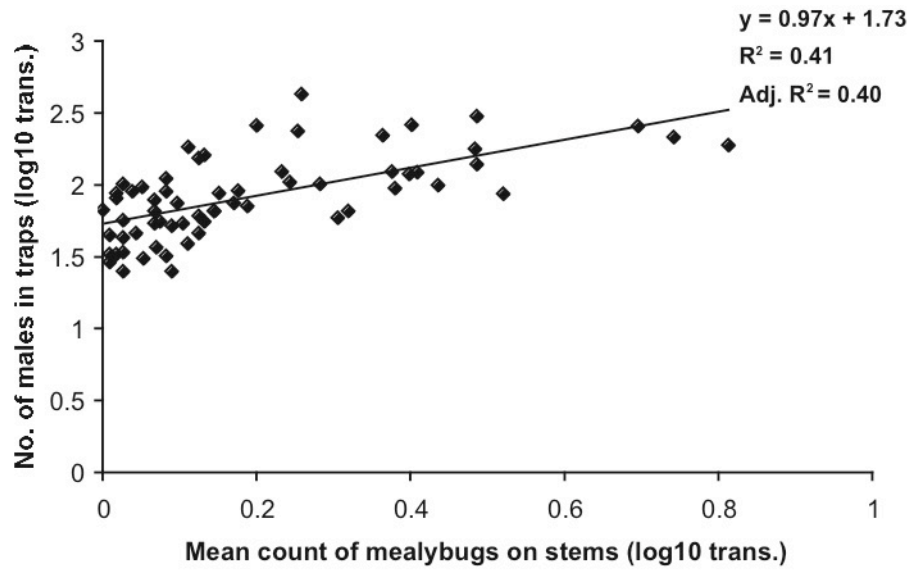


Figure 2.11.



Chapter 3: Reproductive biology of three cosmopolitan mealybug (*Hemiptera: Pseudococcidae*) species

Abstract

Female *Pseudococcus longispinus* (Targioni Tozzetti), *Pseudococcus viburni* (Signoret), and *Planococcus ficus* Signoret were capable of mating multiple times on the same day and on sequential days (range 1 – 8 times). Female reproductive output was unaffected by multiple copulations. Male *P. longispinus*, *P. viburni*, and *P. ficus* also mated multiple times during their lifetimes (maximum of 9, 11, and 19 times, respectively). Male *P. ficus* had the highest mean number of copulations (9.6 ± 0.6), followed by *P. longispinus* and *P. viburni*. Over half of the *P. ficus* males survived their first day of copulations and remated the next day when presented with unmated females. *Pseudococcus viburni* males also readily mated with unmated females on the day subsequent to their first copulations. Median times between copulations were short for males of all species (< 2 minutes). Constant exposure to pheromone had no detectable effect on the activity levels of male *P. ficus* and *P. longispinus*, whereas *P. viburni* males exposed to pheromone emerged significantly earlier from their cocoons than control males without pheromone exposure. Constant exposure to pheromone had no effect on the longevity of males of any species compared to controls.

Introduction

The majority of mealybug species reproduce sexually by an unusual lecanoid genetic system. Males and females are diploid (typically $2n = 10$) but males are functionally haplodiploid in their transmission genetics (Brown and Nur 1964), because the haploid set of paternal chromosomes is heterochromatic, and is eliminated at spermatogenesis so that sperm carry only maternal chromosomes. Thus, males only transmit the chromosomes inherited from their mothers to their offspring (McKenzie 1967, Nur 1980, Varndell and Godfray 1996, Ross et al. 2010). There are no sex chromosomes in the more derived mealybug species; instead, in embryos destined to become males the chromosomes from the sperm that fertilized the egg condense into a heterochromatic mass, whereas this condensation does not occur in embryos that become females (McKenzie 1967, Buglia et al. 2009).

There are also a number of mealybug species that reproduce parthenogenetically. Nur (1971) reviewed the mechanisms of parthenogenetic reproduction in several species. For *Antonina bambusae* Khalid & Shafee, *Phenacoccus solani* Ferris, and several *Trionymus* spp., reproduction is thelytokous with females producing only female offspring that develop from unfertilized eggs, and with diploidy being restored later in the insects' development. In contrast, *Antonina graminis* (Maskell) and *Dysmicoccus brevipes* (Cockerell) develop from diploid eggs, a product of meiosis when homologous chromosomes do not pair (Nur 1971).

A more controversial area of mealybug reproductive biology has been with species that are possibly facultatively parthenogenetic. This phenomenon is known to

occur in some Hymenoptera and Hemiptera that typically reproduce sexually, but in which unmated females are capable of producing some viable offspring by thelytoky (Normark 2003). Similarly, there have been reports that some mealybug species may be able to reproduce both sexually and asexually, including *Dysmicoccus brevipes* (Cockerell) (Beardsley 1965), *Ferrisia virgata* (Cockerell) (Padi 1997), *Planococcoides njalensis* (Laing) (Padi 1997), *P. citri* (Myers 1932, Padi 1997), *Planococcus vovae* (Nasonov) (Francardi and Covassi 1992), and the vine mealybug *Planococcus ficus* Signoret (K. Daane pers. comm.).

A number of other studies have demonstrated that many mealybug species clearly are incapable of reproducing parthenogenetically. For example, James (1937) and Gray (1954) isolated virgin *P. citri* females on potato sprouts and did not observe reproduction. Male mealybugs were observed attempting to enter the chambers with virgin females, thus demonstrating that females were emitting pheromone and were unmated (Gray 1954). James (1937) also determined that females of longtailed mealybugs, *Pseudococcus longispinus* (Targioni Tozzetti), and grape mealybugs, *Pseudococcus maritimus* (Ehrhorn), must mate to reproduce. Grimes and Cone (1985) later confirmed James' results with *P. maritimus*, finding that some female *P. maritimus* produced ovisacs, but these were devoid of eggs. Another very recent study by da Silva et al. (2010) also determined that *P. citri*, citrophilous mealybug (*Pseudococcus calceolariae* (Maskell)), and obscure mealybug (*Pseudococcus viburni* (Signoret)) were obligately sexual in their reproduction. Females of these three species were allowed to mate with males 65 days after their isolation from males, with greater than 70% of these females

then producing an ovisac (da Silva et al. 2010). During their isolation from males, no ovisacs or eggs were produced.

One of the important reproductive characteristics of mealybugs is the high fecundity of females. Because each female produces hundreds of eggs or crawlers, populations can increase dramatically in one generation. For example, mated female *P. viburni* produced an average of 395 eggs (Nur 1962). In general, fecundity can be quite variable (Table 3.1).

Males have been shown to copulate with more than one female. For example, James (1937) demonstrated that the mean number of copulations for 13 male *P. citri* was 9.1 females (range: 0 – 23 females) whereas 20 male *P. longispinus* mated with an average of 8.2 females (range: 0 – 20 females).

Sex ratios of mealybug offspring have also been determined for many of the economically important species. This information is important for proper interpretation of the counts of male mealybugs caught in pheromone-baited traps (Chapter 2). Sex ratios that are heavily skewed may lead to inaccurate interpretation of trap catches, resulting in inappropriate management decisions. Bartlett (1978) reported that the sex ratio for *P. longispinus* was about 7 females: 1 male and James (1937) reported about 6 females: 1 male. *Pseudococcus maritimus* females were reported to outnumber males three to one (James 1937). In contrast, the sex ratio calculated by James (1937) for *P. citri* was ~1: 1, and Buglia et al. (2009) and Ross et al. (2010) concurred with James' results.

Other researchers have demonstrated that the sex ratio of *P. citri* can be strongly influenced by environmental or other conditions (James 1937, Nelson-Rees 1960, Varndell and Godfray 1996, Buglia et al. 2009, Ross et al. 2010). For example, the sex ratio of *P. citri* could be skewed towards males by delaying copulation of females (James 1937, Nelson-Rees 1960, Buglia et al. 2009). Nelson-Rees (1960) showed that mealybug reproductive output was reduced to an average of 75 offspring by rearing at 30.2°C, 68% of which were males. In contrast, under cooler conditions (20-26°C), 43 % of the 527 offspring produced were males.

The effect of population density on sex ratio of offspring is unclear. In one set of experiments, Varndell and Godfray (1996) showed that crowding during the adult stage resulted in a more female-biased sex ratio in *P. citri*, whereas crowding as juveniles had the reverse effect. In a similar set of experiments, Ross et al. (2010) found that *P. citri* females crowded as adults, regardless of crowding or not as juveniles, produced a more male-biased sex ratio (Ross et al. 2010). One possible explanation for these apparently contradictory results may lie in the fact that Ross et al. (2010) used female *P. citri* that were unrelated, whereas Varndell and Godfray (1996) did not. The former authors hypothesized that more unrelated females (e.g., crowded adult treatment) would produce offspring in a more equal sex ratio under the paradigm of local resource competition. They concluded that global competition for resources might have led to the higher male bias in their study; male offspring might be ‘preferred’ in dense populations of mealybugs, as they require fewer resources (feeding ceases after the second instar) and can disperse away from their mothers and siblings.

Now that sex pheromones for a number of mealybug species have been identified and synthesized (Chapter 1), there is increasing interest in developing practical applications for these pheromones, including mating disruption or other methods of direct control (Walton et al. 2006). Before attempting mating disruption, a sound knowledge of mealybugs' basic reproductive biology is desirable, because this has direct implications on the methods employed (e.g., length of pheromone deployment) in control measures. Male mealybugs eclose to adults with limited energy reserves, and locating mates quickly is imperative before their reserves are exhausted. Releasing synthetic pheromone may alter the success of male mealybugs in locating mates. Yet as evidenced by the summaries above, the literature provides only fragmented information about the reproductive biology of various mealybug species, including some aspects of parthenogenetic reproduction, fecundity, sex ratios, and mating behaviors for a limited number of species. Understanding these basic reproductive parameters may also affect the success of pheromone-based control measures. The goals of my research were to expand this knowledge base, specifically for the agriculturally important species *P. longispinus*, *P. viburni*, and vine mealybug (*Planococcus ficus* Signoret). My specific objectives were to:

1. Describe the sequence of reproductive behaviors of males and females that culminate in copulation;
2. Determine whether females of these species could reproduce both sexually and asexually;

3. Quantify parameters of their reproductive behaviors, such as the number of possible copulations for each sex, the periods of time that males and females were receptive to copulation, duration of copulation, and intervals between copulations;
4. Determine whether reproductive output of females was influenced by the number of times that they mated; and
5. Determine the possible effects of exposure to pheromone on male activity and longevity.

Materials and Methods

Mealybug cultures. Starter cultures of *P. longispinus* were collected from orchids and cycads in San Diego, CA, *Pseudococcus viburni* were field collected from potted azaleas in Riverside, CA, and *P. ficus* came from an established colony at the Kearney Agricultural Center in Parlier, CA (Kent Daane, UC Berkeley). Colonies of each species were maintained in widely separated rearing rooms to prevent cross-contamination of colonies. Crawlers of *P. longispinus*, *P. viburni*, and *P. ficus* were established on green beans by placing several beans on top of the containers holding the colony, and allowing crawlers to infest the beans. The beans were then held as a group in ventilated plastic containers. Crawlers were collected every 2-3 days, and the date of their collection was recorded to produce cohorts of known age for reproductive biology studies. After a period of two weeks, females were easily distinguished from the elongate purple-colored males (late second instars). Sexually immature females of *P. longispinus* and *P. ficus* (2nd and 3rd instars) were removed from the rearing containers and placed as a group in ventilated plastic vials with fresh green beans. *Pseudococcus viburni* females, which performed poorly on green beans, were placed as a group on seed potatoes (var. Dark Red Norland) in groups of 20. Females were held in their respective containers until they sexually matured and were subsequently used in experiments. Male mealybugs of all three species spun their cocoons at the conclusion of the second instar and were isolated into ventilated plastic containers away from females to ensure that both sexes remained unmated.

Sexual or asexual reproduction. Immature female *P. longispinus*, *P. viburni*, and *P. ficus* were removed from the green beans as second or third instars and placed individually onto a piece of seed potato (var. Dark Red Norland) held in plastic containers with ventilation holes covered with fine brass screening. The containers were kept in a rearing room on a 16:8 (L: D) cycle at 25°C and ~50% relative humidity.

Every three to four days, females were inspected to determine if they were alive and whether they had produced an ovisac (*P. viburni* and *P. ficus*) or crawlers (*P. longispinus* is viviparous), as indicators of reproduction. Fifty-eight *P. viburni*, 27 *P. ficus*, and 50 *P. longispinus* females were used in total. Females were monitored until their deaths.

Mating arenas. For studies assessing the number of possible copulations between males and females, for all three species mating arenas were constructed from a piece of yellow squash with the cut-off top of a microcentrifuge tube (0.6 mL, Fisher Scientific, without the snap cap lid) pressed into the vegetable's skin. Once the insects were transferred to this arena, a glass slide was placed over the arena to prevent the insects' escape. The diameter of the arena was 7 mm (Figs. 3.1a-b). The squash was placed into a petri dish with water to minimize desiccation during the observational period.

Number of copulations for females. Preliminary observations of male and female mealybugs showed that there were differences in the attractiveness of females and the receptivity of males, often delaying the start of copulation, if it occurred at all. The simplest way to ensure that copulation began quickly (with *P. longispinus*) was to introduce multiple females and males together or a single female with two males (*P.*

viburni and *P. ficus*) at 08:30 PST into mating arenas. Once the first copulation was initiated, the copulating pair was confined to the mating arena and superfluous males and females were removed. Arenas were checked every 15 minutes until 17:00 PST. Due to the time-intensive nature of these studies, different groups of females were observed over successive days. Table 3.2 summarizes the slight differences among the studies carried out with the three species, including the number of days females were initially mated, female ages, and the number of females observed.

At every quarter hour, males that had completed copulation were removed, and depending on the stock on hand, 2 to 3 unmated males were introduced into the arena. Behaviors of males and females were noted during these observation periods. After one to two hours, males that did not successfully mate with female mealybugs or remained stationary in the arena were replaced with other unmated males. During this second phase of the experiments, number and duration of copulations were noted.

Copulations for females on successive days following an initial copulation. Every morning for three consecutive days after the start of the experiment, two unmated male mealybugs were introduced into an arena with a previously mated female at 08:30 PST. Arenas were observed every 15 minutes until 10:00 PST, and after the behavior was recorded, males were removed. After three days, two males were introduced to the arenas every other morning until females began producing crawlers (*P. longispinus*) or eggs (*P. viburni* and *P. ficus*). Observations were made of the behaviors of both females and males. Behaviors of males were categorized as walking, arrestment (Fig. 3.2a), investigating the female ('assessing') (Fig. 3.2a), attempting copulation (probing) (Fig.

3.2b), and mating. The proportion of males engaging in each behavior was noted, rather than the duration of each behavior. Behaviors of females were most often observed when males were attempting copulation. These behaviors included downward movement of the abdomen away from the male's copulatory stylets and walking around the arena with the male on her dorsum. If copulation did occur on the mornings following the initial day of copulation, it was noted. Males were introduced into the arenas until females began producing crawlers (*P. longispinus*) or eggs (*P. viburni* and *P. ficus*).

Fecundity of females with multiple copulations. Females of all three species were checked daily for signs of reproduction (crawler or ovisac production). Once crawlers or ovisacs appeared, they were counted and removed from the arena. Females then were examined for crawlers and eggs every three to seven days, depending on the number of females producing offspring at one time. Towards the end of reproduction, the number of crawlers and eggs declined, and weekly counts of mealybug offspring were sufficient. Females were removed from degrading squash every 5 to 7 days and placed into arenas composed of fresh squash.

Fecundity data was analyzed using SAS version 9.1.3 (SAS Institute, Cary, North Carolina). The relationships between egg counts (*P. viburni* and *P. ficus*) or crawler counts (*P. longispinus*) and the total number of copulations over time for each female were determined (PROC REG) for each species (three regression lines). Data were square root ($x + 0.5$) transformed for *P. ficus* and *P. longispinus* to satisfy assumptions of variance homogeneity and normality. A lack of fit test for all species demonstrated that

the assumption of a linear relationship was also met between reproductive output and number of copulations.

Male copulatory characteristics. Experiments were conducted to determine whether male mealybugs were capable of multiple copulations. For each species, eight unmated females from the same crawler cohort (same age) were placed into a mating arena. An unmated male mealybug was selected at random from a pool of newly active males and placed into the arena with the females. Due to developmental differences, females from different species were of different calendar ages but were at the same developmental ages when these studies were conducted (Table 3.3). Replication among the three species varied (see Table 3.3).

Some males of *P. viburni* and *P. ficus* survived the first day of mating and were introduced into a new arena the following day with a new cohort of females. As *Pseudococcus viburni* females were challenging to rear (their development was inconsistent once they were isolated from males), there often was not a sufficient quantity of females to mate with male *P. viburni*. Only four male *P. viburni* were evaluated the day following their initial copulations. *Planococcus ficus* males consistently survived the first day of matings and females were readily available, with 14 males evaluated again. *Pseudococcus longispinus* males rarely were alive the following morning (8 individuals) but were not introduced to additional females.

Prior to making observations, the eight females were marked with food coloring with a camel's hair brush. This aided in the identification of individual females while

viewing videotapes and in monitoring the females following recording to check for the production of crawlers or an ovisac.

All reproductive experiments were video taped by placing each arena under a dissecting scope connected to a camera-VHS recording system. Recordings were made for six hours beginning between 08:30 and 09:30 (PST), after which the females and male were removed and isolated into individual chambers, with food provided for the females. Videotapes were used to quantify the duration of each mating event and the intervals between mating events.

Activity and longevity of male mealybugs constantly exposed to pheromone. Male mealybugs were isolated individually into 3.7 ml shell vials at the end of their second instar. They were provided with a circular piece of tissue (Kimwipes[®], Kimberly-Clark, Roswell, GA) slightly smaller than the circumference of the vial to provide a shelter under which to spin a cocoon. Male pupae were checked daily for their developmental status. Once males had developed fully expanded wings while inside their cocoon, they were randomly assigned to a pheromone treatment or a blank control. Treatment containers were wide-mouth mason jars (7.5 cm × 12 cm, 470 ml), with two pieces of paper towel balled inside. Pheromone treatments consisted of gray rubber septa (11 mm; The West Company, Lititz, PA) impregnated with 25 µg of each species' pheromone, with solvent-treated septa used as controls. Mason jars were placed in a fume hood, with treatment and control jars separated by one meter. Fume hood temperatures were 20.8°C for the *P. longispinus* and *P. viburni* experiments and ranged between 22.3 and 22.9°C for the *P. ficus* experiment. Experiments were conducted under natural light. Newly

eclosed males were continually added to either treatment or control jars when vials were checked twice daily (09:00 and 21:00). Replicates were individual males. During the observations, I determined the activities of males including: signs of emergence (away from cocoon), resting (no movement within vial), walking (actively moving within the vial), or death. The length of time between eclosion and emergence from cocoons (activity) and male longevity were recorded.

Differences in activity and longevity between control and pheromone treatments were analyzed by t-tests using SAS version 9.1.3 (SAS Institute, Cary, North Carolina) in PROC TTEST. Male mealybugs were exposed to pheromone (25 µg) or the control for their entire adult life (< 5 days). Replication was as follows: *P. longispinus*: Control (C) = 32 males, Pheromone (P) = 33 males; *P. viburni*: C = 22 males, P = 25 males; and *P. ficus*: C = 39 males, P = 40 males. If the test demonstrated that population variances were homogeneous between the two treatments, the TTEST output labeled 'equal' was used (for pooled variances). Otherwise, for heterogeneous variances between treatments, the TTEST output labeled 'unequal' was used.

Results

General description of mating behaviors. Moreno et al. (1984) described the copulatory behaviors of *P. citri* in detail, and the reproductive behaviors of my three study species were similar. When males and females were confined in mating arenas, female mealybugs walked occasionally but were mainly sessile. In contrast, males walked until they encountered a female, at which point they climbed onto and walked back and forth over the female while occasionally antennating her, followed by vigorous probing with the genitalia around the females' body margin. Males that began probing in areas away from the females' genitalia would gradually move toward the posterior. A receptive female apparently sensed the presence of the male (either actively probing her or simply walking over her back) and raised the tip of her abdomen to allow the male access to her genitalia. Males would then rapidly couple with the receptive females and remain in copula for periods of several minutes (see below). Behavior of males during these observational periods was categorized as walking or arrestment (noncopulatory behavior) and assessing, probing, or mating (copulatory behavior) (Figs. 3.2a-b).

During mating studies between previously mated females and virgin males, females sometimes were unreceptive to copulation attempts by males. However, a male rarely left a female once he had started probing her body margin (probing continued for up to 75 min., pers. obs.). For reasons that are unclear, some males remained inactive despite the fact that females were clearly producing pheromone and attractive, as demonstrated by the active searching and mating attempts exhibited by the other males in the arena. Males never attempted to fly in any instance in the presence of females.

An additional behavioral component observed with *P. longispinus* and *P. viburni* occurred prior to males initiating copulatory attempts (see Fig. 3.2b = probing). Males were observed standing on the posterior half of the female's dorsum and then curving their bodies around the side of the female with their heads beneath her ventral side. This behavior was termed 'assessing' (Fig. 3.2a). *Planococcus ficus* males rarely exhibited this behavior. Upon introducing *P. ficus* males to the mating arena, they immediately approached a female and began copulatory attempts, often initially probing the female's head. In contrast to the behaviors of male *P. citri* as described by Moreno et al. (1984), males of any of these three species did not extensively antennate females before initiating mating attempts.

Sexual or asexual reproduction. Females of *P. longispinus*, *P. viburni*, and *P. ficus* must copulate to reproduce. Interestingly, 54 of the 58 unmated *P. viburni* females (93%) used in the experiment did produce an ovisac, but the ovisacs contained no eggs. Nineteen of the 27 unmated *P. ficus* females also produced ovisacs, two of which did not contain eggs. On average, there were 24.9 ± 5.6 eggs in *P. ficus* ovisacs. These eggs were often obviously deformed, and crawlers never emerged. Females in these experiments also lived a remarkably long time. From when they were collected as crawlers, female *P. longispinus*, *P. viburni*, and *P. ficus* survived for means of 137.7 ± 3.4 days, 105.4 ± 2.7 days, and 93.6 ± 2.9 days, respectively. Unmated *P. viburni* females produced an empty ovisac on average 75.4 ± 2.5 days after they were collected as crawlers whereas half of the *P. ficus* females that produced an ovisac did so between 59 – 69 days after they were collected as crawlers. Female *P. longispinus* that had free

access to males usually began producing crawlers 37 – 41 days after they had been collected as crawlers. Mated female *P. viburni* and *P. ficus* began producing ovisacs 34 – 43 days and 32 – 40 days, respectively, after their collection as crawlers.

Number of copulations for females. Females of all three study species mated multiple times in a single day (Figs. 3.3a-c). *Pseudococcus longispinus* females had the fewest copulations during the first day of mating (mean = 2, range = 1 – 4), whereas *P. viburni* females mated most frequently (mean = 5.3 copulations, range 1 – 8). *Planococcus ficus* females mated an average of three times (range 1 – 8 copulations).

Copulations for females on successive days following the first day of mating. Female mealybugs of all species mated again on days subsequent to their first copulation (Figs. 3.4a-c). Half of the 31 *P. viburni* females mated again on day 1 after the first copulation, and the number of females that mated again on days 2, 3, and 5 declined (2, 1, and 1 female(s) during each morning, respectively). Further matings were not attempted with *P. viburni*, because females began producing ovisacs on day 4. In contrast, female *P. ficus* and *P. longispinus* did not mate again until day 2, and the pattern of remating was different. With *P. longispinus* females, relatively low levels of subsequent mating (1-5 females per day, out of the 29 females) occurred up to 23 days after the first mating. *Pseudococcus longispinus* females exhibited the longest period between mating and reproduction (first crawlers produced at 26 days post mating), hence the extended period for introducing new males. With *P. ficus*, no females mated again on the day following the first copulation, but up to two females (out of 30 total) mated again up to 11 days after the first mating. The first ovisac was produced on day 9, but the majority of *P. ficus*

females did not start producing ovisacs until days 14 – 17. Overall, these results demonstrate that females of all three species can copulate multiple times over a number of days.

The behavior of virgin males towards mated females exhibiting copulatory behavior (e.g., assessing or probing) indicated that many males were attracted to females for several mornings (Figs. 3.5a-d). The changes in the patterns of behavior of males over successive days are shown in Figures 3.5a-d. As days progressed, males exhibited less copulatory behavior and more walking or arrestment behaviors (compare 5a with 5d). There were species differences at nine days (Fig. 3.5d); one hundred percent of the *P. viburni* males were resting in the arenas, whereas on the same day and time, behaviors of *P. ficus* males were more varied, although nearly 80% of *P. ficus* males were either walking or resting (Fig. 3.5d).

Fecundity of females with multiple copulations. There was no relationship between reproductive output (egg or crawler abundance) and total numbers of copulations (Figs. 3.6a-c) (*P. longispinus*: $F = 0.83$, $df = 1, 26$, $P = 0.37$; *P. viburni*: $F = 0.27$, $df = 1, 29$, $P = 0.61$; *P. ficus*: $F = 3.2$, $df = 1, 28$, $P = 0.08$).

Male copulatory characteristics. When presented with an excess of females, male mealybugs of all three species mated multiple times within a single 6-hour observation period (Figs. 3.7a-c, Fig. 3.8). *Planococcus ficus* males had the highest frequency of mating (day 1 mean = 9.6 ± 0.6 , range 6 – 19). *Pseudococcus longispinus* and *P. viburni* males mated an average of 5.8 ± 0.3 (range 3 – 9) and 4.6 ± 0.7 (range 1 – 11) times on day 1, respectively (Fig. 3.8). Durations of copulations for male mealybugs tended to be

longest for the first copulation with median times of 28, 13, and 3 minutes for *P. longispinus*, *P. viburni*, and *P. ficus*, respectively. Median copulation times, overall, were longest for *P. longispinus* followed by *P. viburni* and *P. ficus*. Of the four male *P. viburni* that were recorded on the second day, all mated readily with more females (Fig. 3.8). Fourteen out of 25 male *P. ficus* survived to day two and were allowed to mate again with eight virgin females, and all mated multiple times (Fig. 3.8, Table 3.4). Overall, the mean number of copulations for *P. ficus* was less (mean = 5.6 ± 0.5 , range 3 – 9) than the first day, but two of the fourteen males did mate nine times each (Fig. 3.10a, Table 3.4). Male *P. ficus* mated a mean of 15 times over two days, four more copulations than males that were recorded for 1 day (Table 3.4). Median copulation length was also much longer on the second day for *P. ficus* than the first (Figs. 3.7c and 3.10a).

The median refractory period between matings for males of all species was short (~1-20 minutes), especially for the earlier copulations (Figs. 3.9a-c). *Planococcus ficus* males typically had the shortest periods between copulations (~1-16 minutes). During the second day that *P. ficus* males were mated, the median time between copulations was also short, with less than five minutes elapsing between the first six copulations (Fig. 3.10b).

Activity and longevity of male mealybugs with constant pheromone exposure. Constant exposure to pheromone in sealed jars had no effect on the time taken for male *P.*

longispinus and *P. ficus* to emerge from their cocoons and become active (*P. longispinus*: $t = 0.89$, $df = 63$, $P = 0.38$; *P. ficus*: $t = -0.71$, $df = 77$, $P = 0.48$) (Fig. 3.11). My hypothesis had been that early and constant pheromone exposure would result in males

detecting pheromone and emerging from their cocoons earlier to locate mates. Male *P. viburni* did become active significantly sooner than control males (Fig. 3.11, $t = 3.52$, $df = 25.6$, $P = 0.002$).

There were no differences in longevity between males with or without exposure to pheromone for any of the three species (*P. longispinus*: $t = 0.27$, $df = 63$, $P = 0.79$; *P. viburni*: $t = 1.57$, $df = 45$, $P = 0.12$; *P. ficus*: $t = 1.44$, $df = 77$, $P = 0.15$) (Fig. 3.12). Male *P. ficus* lived the longest of the three species after eclosion (~4.5 days, Fig. 3.12).

Discussion

Sexual or asexual reproduction. My data confirmed earlier findings that females of all three species only reproduce sexually. No viable eggs were produced from unmated females. These results concur with previous work on *P. longispinus* (James 1937) and very recently published work on *P. viburni* (da Silva et al. 2010). Similarly to da Silva et al. (2010), *Pseudococcus viburni* females in this study also produced ovisacs, but eggs were never observed. Also, in the work reported here, more *P. viburni* females formed an ovisac (93%) than in the study of da Silva et al. (2010) (54%).

For *P. ficus*, preliminary studies had raised the possibility that parthenogenetic reproduction might occur (K. Daane, pers. comm.). My results, carried out under carefully controlled conditions to ensure that no males were present, demonstrated that this does not occur. Thus, the viable eggs and offspring seen in the preliminary studies carried out by Daane's group were probably due to the presence of a few undetected males, or the use of females that were already mated by the time they were isolated individually in vials. Overall, the results of the studies presented here, in combination with the fact that females of all three study species produce highly attractive sex pheromones, provide strong evidence that reproduction by parthenogenesis seems highly unlikely in any of these species.

During these studies, I noted that the lifespans of unmated females were surprisingly long, as had been previously observed with virgin *P. citri* females that survived for nearly eight months after maturity (James 1937). Production of an ovisac

with viable eggs clearly had a major cost because females of all species died fairly soon after the egg sac was produced.

An implication that the species investigated here are not facultatively parthenogenetic is that if pheromone-based control (e.g., mating disruption) eliminates males or disrupts mating, females will never reproduce. However, the longevity of females will present an additional problem for mealybug control. If pheromone is used in an effort to control mealybugs, it must be deployed and persist for long periods to ensure that copulation rarely if never occurs. As da Silva et al. (2010) mentioned, females (including *P. viburni*) were attractive to males 65 days after their sexual maturity and were able to deposit viable eggs. Although males were never introduced to aged females in this study, females produce pheromone until they are mated (Rotundo and Tremblay 1980). James (1937) indicated that after mating, only a third of 10-week old *P. citri* females oviposited. This suggests that there may be time limits for successful reproduction.

Number of copulations for females. For all three species investigated, at least some females remated on one or more days following their first copulation. *Pseudococcus viburni* females were most promiscuous, with half of the mated females mating again the morning after the first copulation(s) (Fig. 3.4b). Additional matings for females of the other two species occurred two mornings later at the earliest and were more dispersed across time (Figs. 3.4a, 3.4c). Copulations decreased and stopped altogether as females began to reproduce. The fact that male mealybugs were attracted to previously mated females suggests that any hormonal substances transferred to females to induce them to

become refractory to further mating attempts and begin production of eggs do not take effect immediately. Clearly copulation does trigger physiological changes in females because mated females produced ovisacs much sooner than unmated females

Number of copulations for females on successive days. For all three study species, some females remated on one or more days following their first mating. *Pseudococcus viburni* females were the most promiscuous with half of the mated females remating the morning after the first copulation(s) (Fig. 3.4b). Additional matings for females of the other two species occurred two mornings later at the earliest and were more spread out overall (Figs. 3.4a, 3.4c). Copulations decreased and stopped altogether as females began to reproduce. The fact that male mealybugs were attracted to previously mated females suggested that these females were still releasing traces of pheromone or were contaminated with sufficient amounts of pheromone to elicit responses from males. Even though individual females of all three species apparently remained attractive to males, these females were generally unwilling to mate, and males were rarely successful in copulation.

Fecundity of females mated multiple times. Overall fecundity for the three species was lower than what has been reported in the literature (see Table 3.1). These lower numbers may have been due to suboptimal diet (females in these experiments were maintained on pieces of yellow squash) or to trauma from frequent handling because females were transferred to fresh pieces of squash every 5 – 7 days. In addition to lower reproductive output, there was no relationship between reproductive output and the number of copulations. A larger reproductive output might have been expected from females that

mated multiple times. Females with multiple partners should have more sperm in their reproductive tracts to fertilize eggs. It is not known whether male mealybugs have the capacity to remove another male's sperm as is known to occur in some other insect orders (e.g., Odonata, Simmons 2001) or if females select for sperm quality from amongst the sperm in the spermatheca (e.g., *Drosophila subobscura* Collin, see Chapman 1998). My results suggest that sufficient sperm are transferred during a single copulation to inseminate all of the female's eggs. Nur (1962) showed that for every one egg laid there were 2.7 sperm deposited into the reproductive systems of female *P. viburni*.

Male copulatory characteristics. During the 6 hours that males were held with eight virgin females, males of all three study species mated multiple times. James (1937) had previously documented that male *P. longispinus* mated with an average of 8.2 females, with a maximum of 20 copulations. Similarly, male *P. citri* mated with 9.1 females with one male mating 23 times (James 1937). Due to the small size of the mating arenas, it was not feasible to introduce any more than eight females; thus, an individual male was restricted to mating with a maximum of eight different females. However, it was not unusual for males to mate more than once with some females, as can be seen from the data in Figures 3.6a-c. For example, one *P. ficus* male mated 19 times, so that at least three of the eight females present must have been mated at least 3 times.

Median copulation times were typically short for *P. ficus* (~3 minutes), somewhat longer for *P. viburni* males (~13 minutes), and nearly 30 minutes for *P. longispinus*. Nur (1962) observed a similar duration of copulation for *P. viburni* (10.1 minutes, range: 6 – 18 minutes). It was not clear why there were species level differences in the durations of

copulations. Physiologically, there may be differences in the rates of sperm transfer in these three species. For example, one or two minutes after copulation was initiated between *P. viburni* adults, sperm bundles were already present in the spermatheca and its duct or in the common oviduct and ovarian junction (Nur 1962). *Planococcus ficus* sperm transfers are obviously fast because copulations only lasted for a median of 3 minutes.

However, for the *P. ficus* males that were exposed to a new group of females on mornings subsequent to the initial copulations, the median copulation lengths were much longer, and males mated fewer times than on the first day (maximum of nine). One or both of these may be a result of the depletion of sperm and energy reserves in these males that had already mated multiple times the day previously. To my knowledge, there is no literature documenting the number of copulations over time for male mealybugs.

During the first day of copulations, refractory periods between copulations were brief for all species, usually only a few minutes, indicating that male mealybugs can copulate with multiple females rapidly to maximize their reproductive output. This was not unexpected, given that the sole function of male mealybugs is reproduction (i.e., they do not feed, and so must reproduce quickly and efficiently before they deplete their limited energy reserves and die).

Longevity of males constantly exposed to pheromone. Contrary to our expectations, exposure to pheromone had surprisingly little effect on the initiation of male mealybug activity. Because the lifetimes of males are severely restricted by their limited energy reserves as described above, I had predicted that adult males exposed to pheromone

would leave their cocoons more quickly than unexposed males. Yet, control males and males of *P. longispinus* and *P. ficus* that were exposed to pheromone were all active at about the same time. Exposure to pheromone did appear to have some effect on *Pseudococcus viburni* males, which became active about 9 hours sooner than controls when exposed to pheromone. The lack of effect or relatively small effect of pheromone on the initiation of male activity may be due to the fact that male mealybugs have an obligatory sexual maturation period after eclosing to an adult. For example, when *Pseudococcus viburni* males were dissected immediately after eclosion, their testes were not mature (Nur 1962). Thus, the initiation of male activity was likely controlled by physiological development, rather than the detection of female sex pheromone.

I had also predicted that males exposed to pheromone would exhibit higher overall activity levels as they searched for the females whose putative presence was signaled by the pheromone, and consequently, that they would die sooner than control males. My experiments showed that the presence of pheromone had no discernable effect on the lifespans of males in the three study species. One possible explanation for these results is that constant exposure to pheromone may have resulted in habituation or desensitization, so that males ceased responding, particularly because the dose of pheromone (12.5 micrograms of the active stereoisomer) was relatively high. That is, my field experiments have shown that this dose of pheromone on a rubber septum lure is an effective attractant for male mealybugs for a period of several months. Thus, males may simply have been overwhelmed by the concentrations of pheromone in the closed confines of the jars used in these experiments.

Planococcus ficus males tended to live the longest (ca. 4.5 days), regardless of treatment. This was in accord with the mating experiments described above, in which *P. ficus* males were often alive the day after the first concentrated mating bouts and these experienced males courted and mated vigorously with virgin females. *Pseudococcus viburni* males tended to live longer than *P. longispinus* males, with some *P. viburni* males still alive on mornings following initial copulations.

Implications for pheromone-based control of mealybugs. My results have a number of positive and negative implications for the possible use of pheromones for mealybug control. First, the fact that females of all three species must mate in order to reproduce is a major advantage; the efficacy of pheromone-based mating disruption cannot be compromised by unmated females still producing offspring in areas under treatment. Thus, the efficacy of the strategy will be dependent only on how effectively the pheromone treatment disrupts mate finding. In contrast, the fact that individual males of all three species can mate numerous times in rapid succession, either on the same day or on successive days, is a serious disadvantage for mating disruption, mass trapping, or attract-and-kill strategies, because even a relatively small number of males can mate with essentially all the available females. This disadvantage is compounded further by two other points. First, my data showed that a single copulation is sufficient for females to maximize their lifetime reproductive output. Second, I found that unmated females lived for a remarkably long time, and that the period in which they could mate and successfully produce offspring extended over many weeks. Thus, with sexually mature females continually advertising their presence for many weeks, mating disruption treatments

would have to remain effective for long periods. This in turn might require repeated applications of the pheromone treatment, which would substantially increase control costs.

On the other hand, the fact that females mated several times, both on the same day and on days following the initial mating suggests that previously mated females might act as a sink for male mating efforts. That is, because multiple matings did not increase reproductive output by females in terms of overall population increase, in this context copulation with a previously mated female may be wasted effort, although it may increase an individual's reproductive output if mechanisms such as sperm precedence are operative. This distraction of males by previously mated females would be an advantage for pheromone-based methods of control.

Finally, it was disappointing to find that exposure to pheromone had little apparent effect on the maturation or longevity of male mealybugs. I had hypothesized that exposure to pheromone would shorten male lifetimes through increased male activity and accelerated use of their limited energy reserves, but this proved not to be the case. Thus, pheromone deployed for mating disruption may effectively disrupt mate location by males, but it will have no "bonus" effect by decreasing male longevity.

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Table 3.1. Literature data on fecundity of economically important mealybug species that reproduce sexually, and for which sex pheromones have been identified. The references did not state the number of copulations for each female.

Species	Fecundity (eggs)	Reference
<i>Maconellicoccus hirsutus</i>	150 – 300	Bartlett 1978
<i>Phenacoccus madeirensis</i> ³	288 – 491	Chong et al. 2003
<i>Planococcus calceolariae</i>	400 – 600	Bartlett 1978
<i>Planococcus citri</i> ¹	300 – 500	Bartlett 1978
	38 – 404	James 1937
<i>Planococcus ficus</i> ²	150 – 400, max. 1,000	Flaherty 2008
<i>Pseudococcus comstocki</i>	200 – 300, max 700	Bartlett 1978
<i>Pseudococcus cryptus</i>	200 – 300	Bartlett 1978
<i>Pseudococcus longispinus</i> ¹²	200 – 300 (crawlers)	Bartlett 1978
	92 – 398 (crawlers)	James 1937
<i>Pseudococcus maritimus</i>	7 – 186	Grimes and Cone 1985
	8 – 232	James 1937
<i>Pseudococcus viburni</i> ¹²	≤ 500	Bartlett 1978

¹ Mealybug species discussed in Chapter 2.

² Mealybug species discussed in Chapter 3.

³ Fecundity data was strongly temperature dependent.

Table 3.2. For the mating studies that examined the number of copulations possible for female mealybugs, the initial ratio of females to males was modified to ensure rapid and uniform copulation. Different cohorts of females were observed during different days due to the time- and male-intensive nature of the studies. The calendar ages of females (from collection as crawlers) and the number of females in the studies are shown.

Species	Initial no. of females : males	Number of Days for Initial Copulations	Female Age (days)	No. of Females
<i>Pseudococcus longispinus</i>	3:3	2	28-29	29
<i>Pseudococcus viburni</i>	1:2	3	29-30	31
<i>Planococcus ficus</i>	1:2	3	21-25	30

Table 3.3. For the studies that examined the number of copulations possible for male mealybugs during their first 6-hour mating bout (out of a possible two), calendar ages of the females varied with species, but females were of the same developmental stage. Mean and median female age (days since collection as crawlers) are presented as well as the number of males used for each species.

Species	Female Age (Days)		No. of Males
	Mean	Median	
<i>Pseudococcus longispinus</i>	27.6	27	25
<i>Pseudococcus viburni</i>	30.7	31	23 ¹
<i>Planococcus ficus</i>	23.0	23	25 ²

¹ Four of these males were exposed to 8 additional females the following morning.

² Fourteen of these males were exposed to 8 additional females the following morning.

Table 3.4. Total number of copulations for 14 *Planococcus ficus* males that mated on successive days.

Male #	Copulations per day	
	Day 1	Day 2
1	8	9
2	12	5
3	10	5
4	8	6
5	10	3
6	14	4
7	6	6
8	9	6
9	9	7
10	8	9
11	6	7
12	8	6
13	7	3
14	11	3
Mean (\pm SE)	9 (\pm 0.6)	5.6 (\pm 0.5)

Figure Legends

Figures 3.1a-b. (a) Side view of mating arena showing yellow squash with microcentrifuge top implanted into the skin and covered with a glass slide. (b) Overhead view of mating arena showing the 7 mm diameter opening.

Figures 3.2a-b. (a) A male mealybug that is ‘assessing’ a female at her posterior. Another male is exhibiting arrestment to the right. (b) Two male mealybugs ‘probing’ a female’s posterior in an attempt to copulate with her.

Figures 3.3a-c. The number of female mealybugs and their number of copulations in an 8-hour time interval (initial day). a = *Pseudococcus longispinus*, b = *Pseudococcus viburni*, c = *Planococcus ficus*

Figures 3.4a-c. The number of female mealybugs that were mated on mornings subsequent to the initial copulation trial. a = *Pseudococcus longispinus*, b = *Pseudococcus viburni*, c = *Planococcus ficus*

Figures 3.5a-d. The percentages of male *Pseudococcus longispinus*, *Pseudococcus viburni*, and *Planococcus ficus* exhibiting various behaviors (walking, resting, assessing, probing, and mating) at: a = 1 day, b = 3 days, c = 7 days, d = 9 days after an 8-hour initial copulation period.

Figures 3.6a-c. The number of offspring produced (eggs or crawlers) for females that were mated once or multiple times. a = *Pseudococcus longispinus*, b = *Pseudococcus viburni*, c = *Planococcus ficus*

Figures 3.7a-c. Bars represent the range in time (minutes) male mealybugs spent in copulation for each mating event during the initial 6-hour mating trial. The diamond in

each bar is the median time. a = *Pseudococcus longispinus*, b = *Pseudococcus viburni*, c = *Planococcus ficus*.

Figure 3.8. Mean number of copulations per male during 6 hours for the first and second days that males were exposed to eight virgin females. Only eight *P. longispinus* males were alive on the second morning so this species was not included.

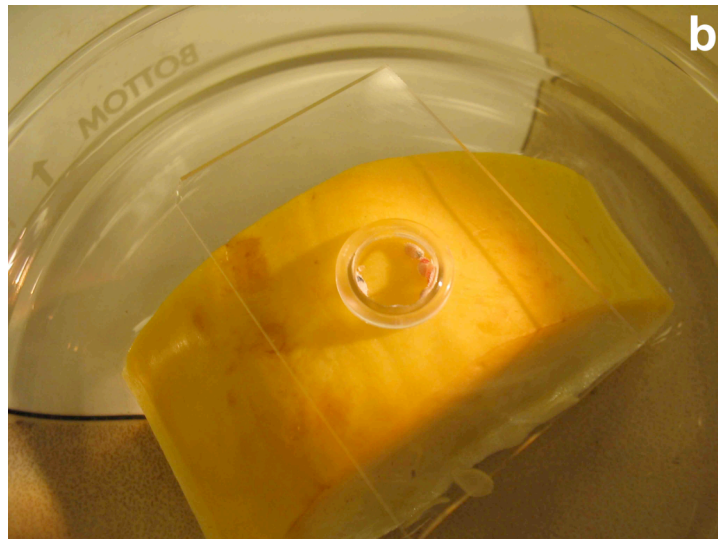
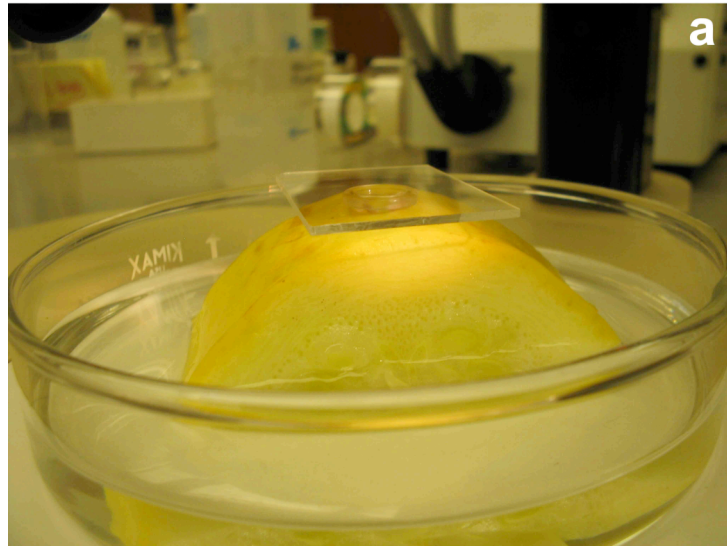
Figures 3.9a-c. Bars represent the range in time (minutes) male mealybugs spent between copulations. The diamond present in each bar is the median time spent between copulations. a = *Pseudococcus longispinus*, b = *Pseudococcus viburni*, c = *Planococcus ficus*.

Figures 3.10a-b. Fourteen *P. ficus* males were observed on a second day mating with a additional set of eight females. (a) Bars represent the range in time (minutes) male mealybugs spent in copulation for each mating event during 6 hours. The diamond present in each bar is the median time spent in copulation for that mating event. (b) Bars represent the range in time (minutes) male mealybugs spent between copulations. The diamond present in each bar is the median time spent between copulations.

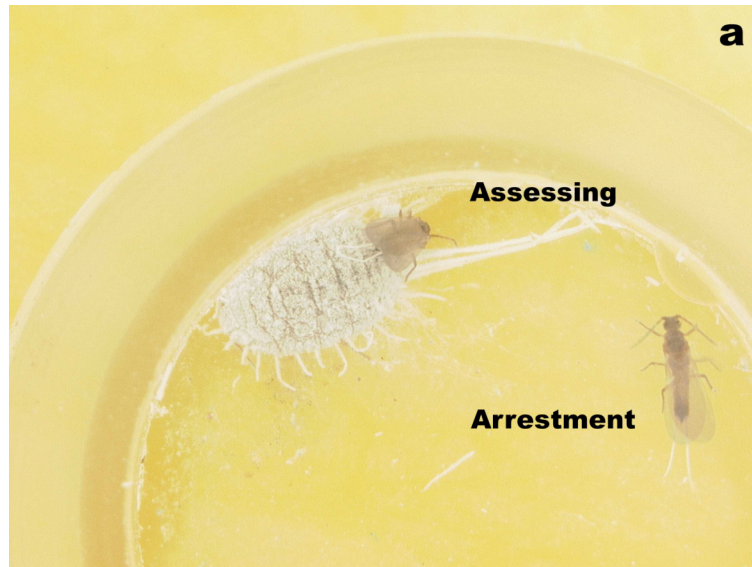
Figure 3.11. Time until males emerged from cocoons in the presence (white bars) or absence (black bars) of pheromone for *P. longispinus*, *P. viburni*, and *P. ficus*. The asterisk denotes a significant difference between treatment and control for each species.

Figure 3.12. Longevity of male *P. longispinus*, *P. viburni*, and *P. ficus* in the presence (white bars) or absence (black bars) of pheromone. There were no significant differences between the treatments or controls for any species.

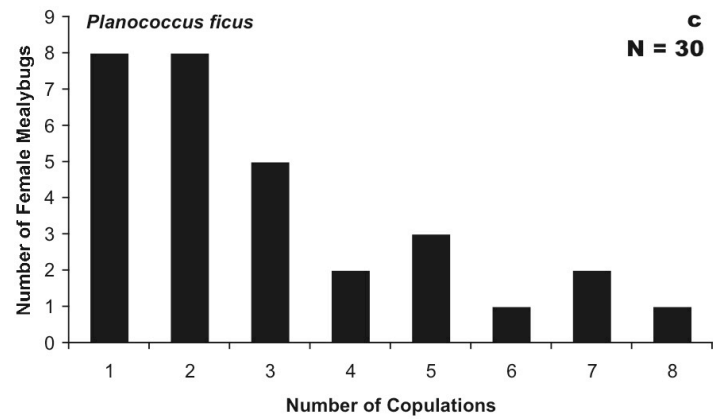
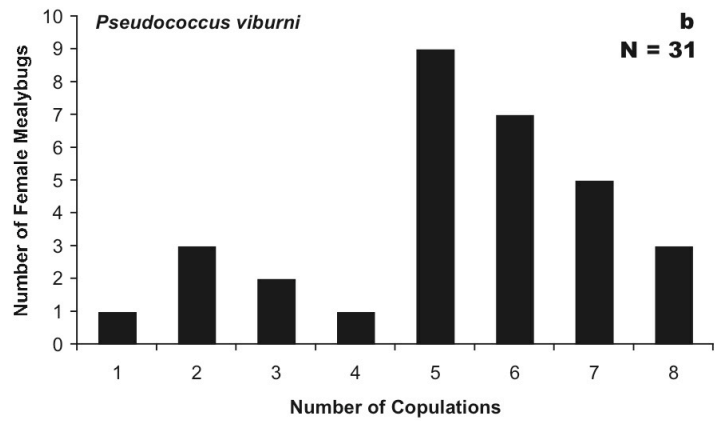
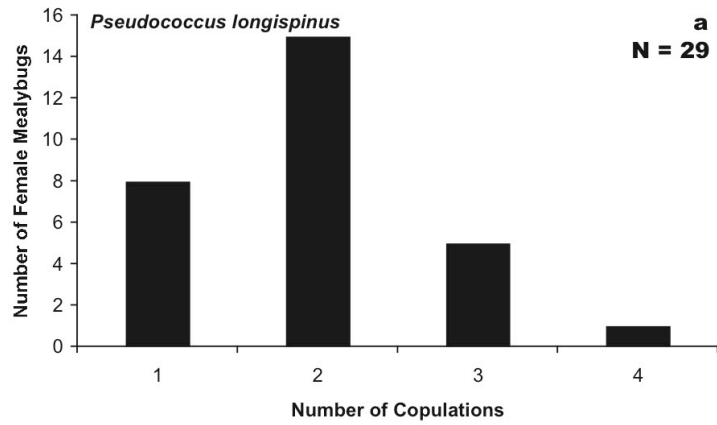
Figures 3.1a-b.



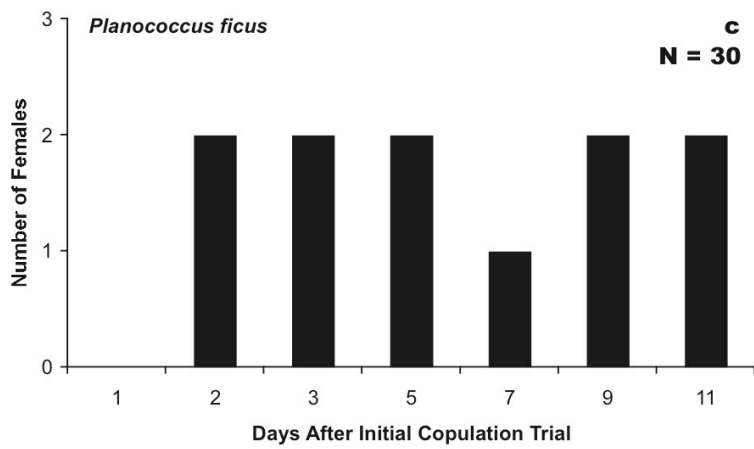
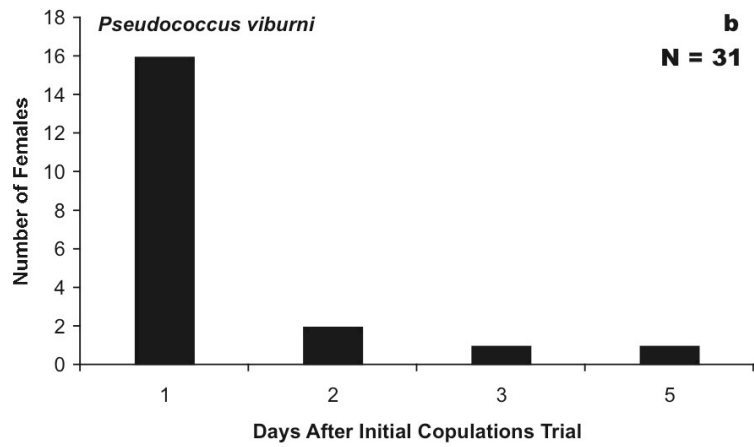
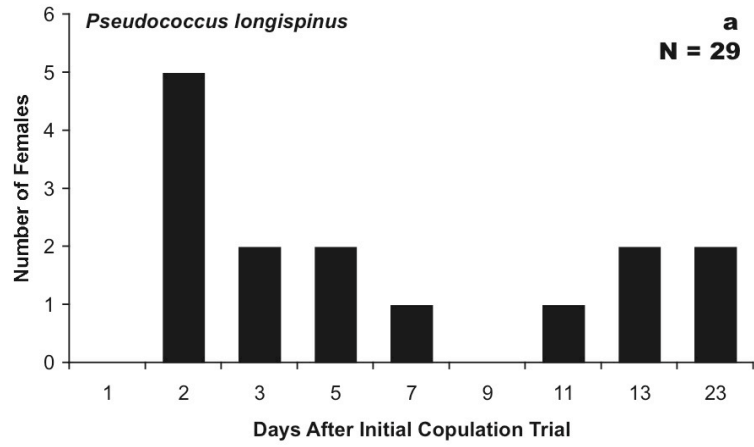
Figures 3.2a-b.



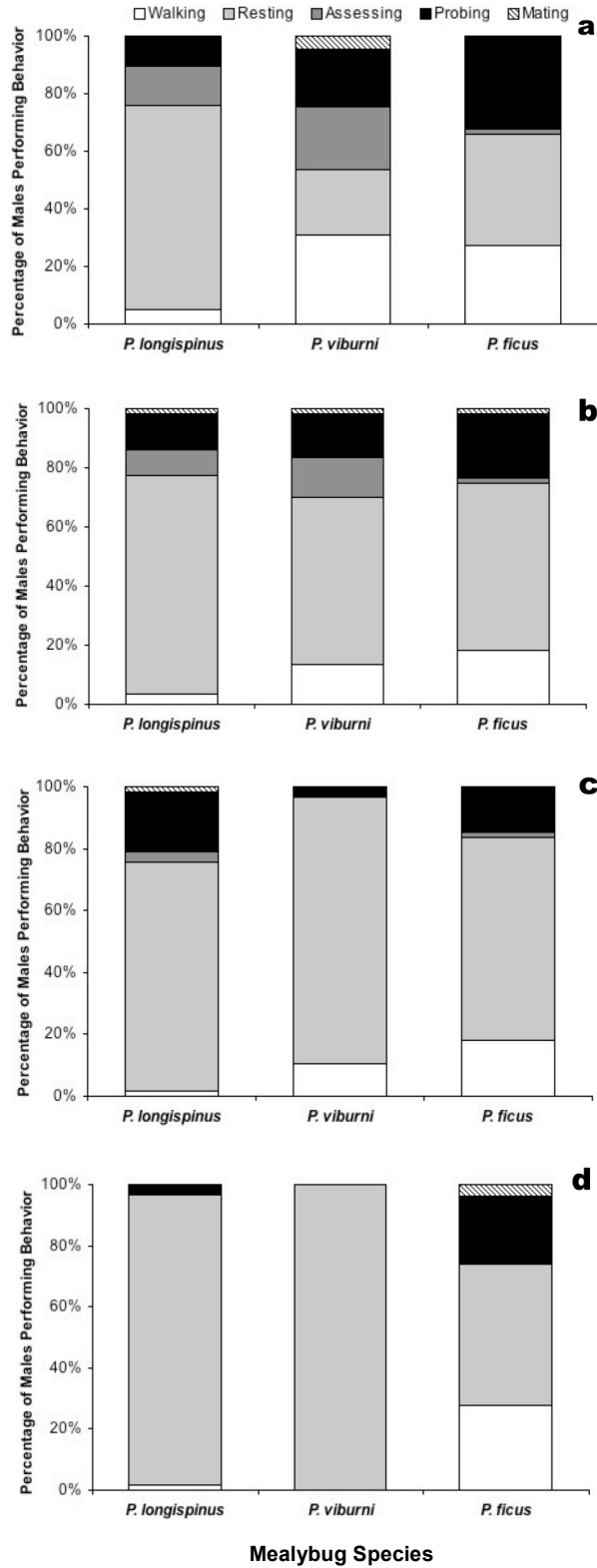
Figures 3.3a-c.



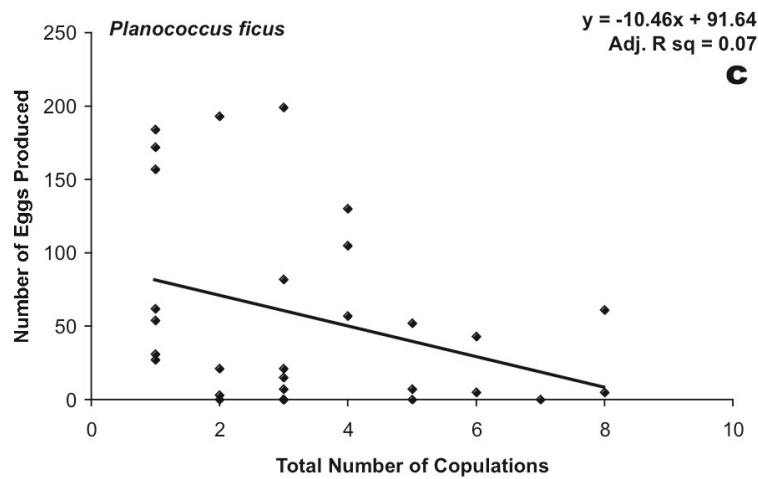
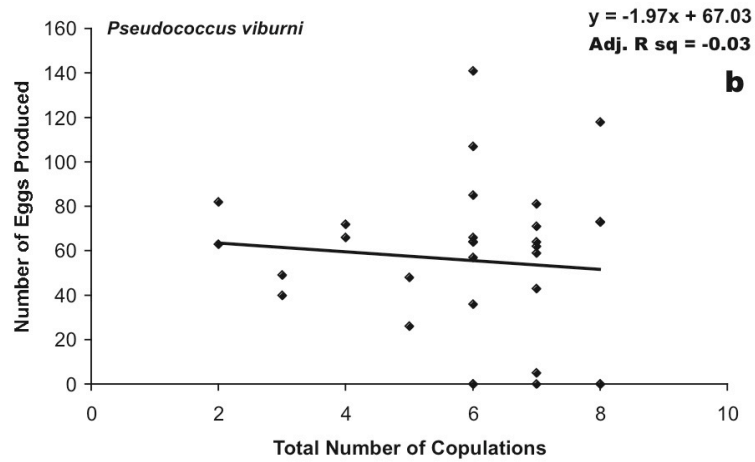
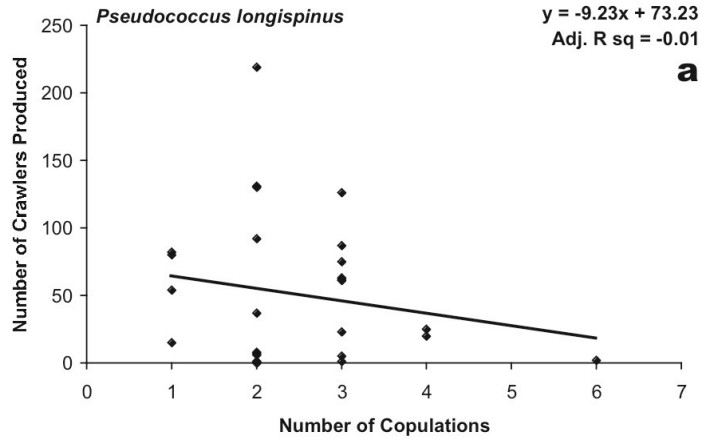
Figures 3.4a-c.



Figures 3.5a-d.



Figures 3.6a-c.



Figures 3.7a-c.

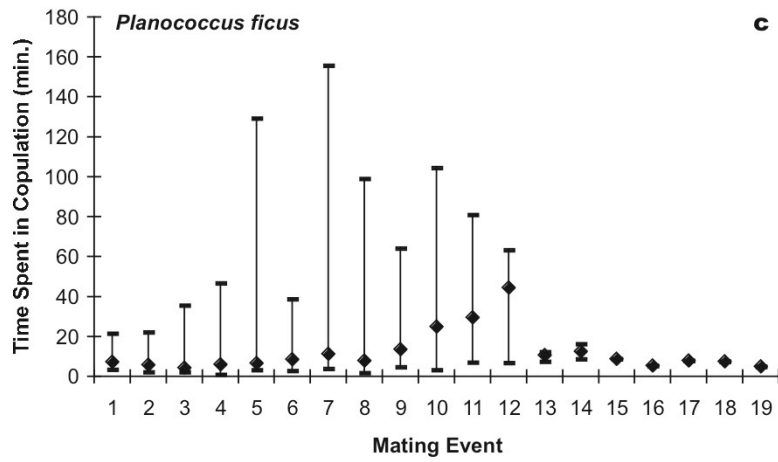
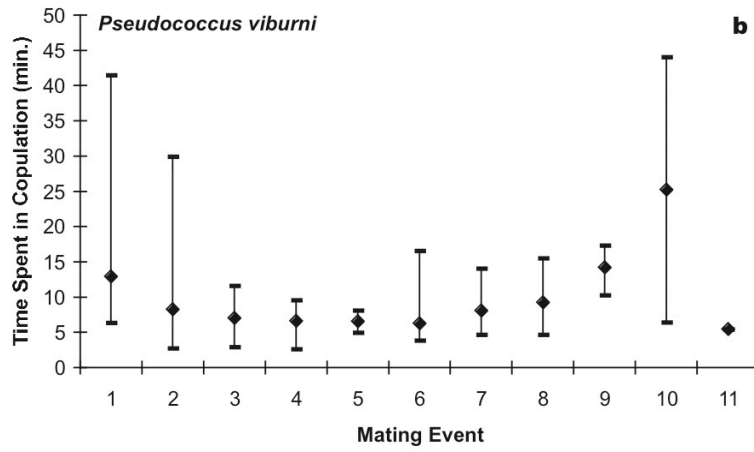
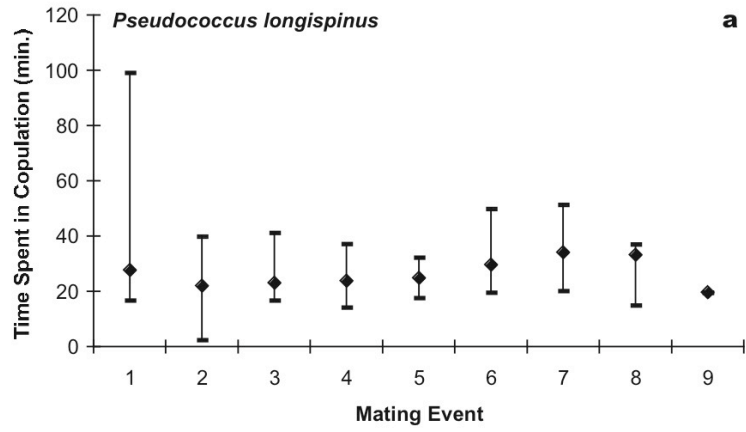
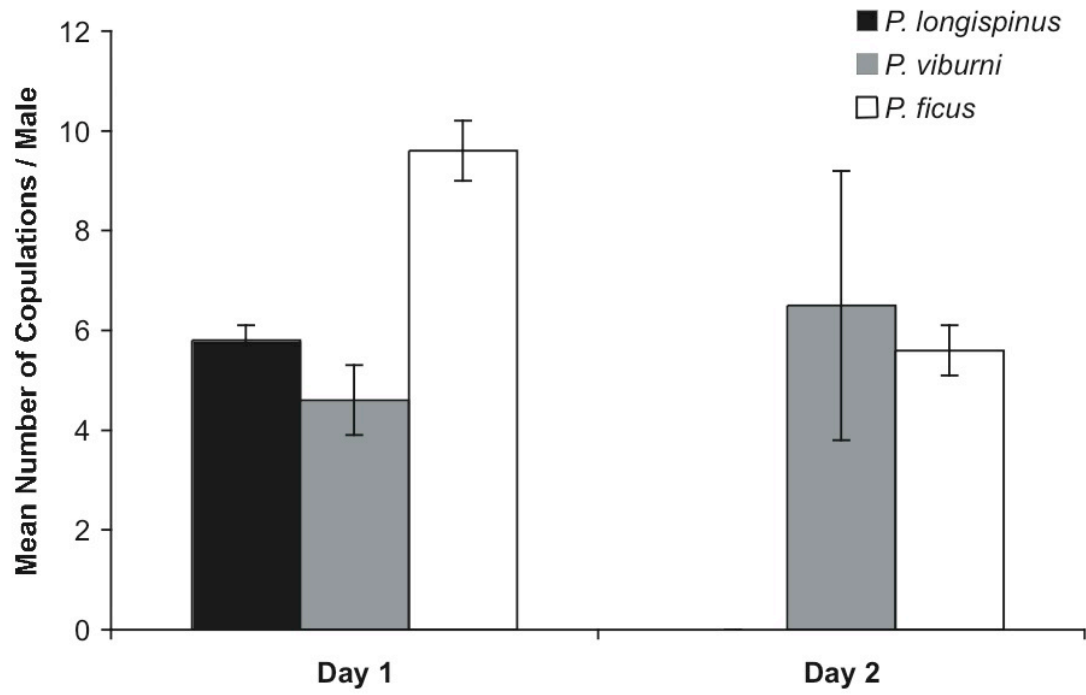
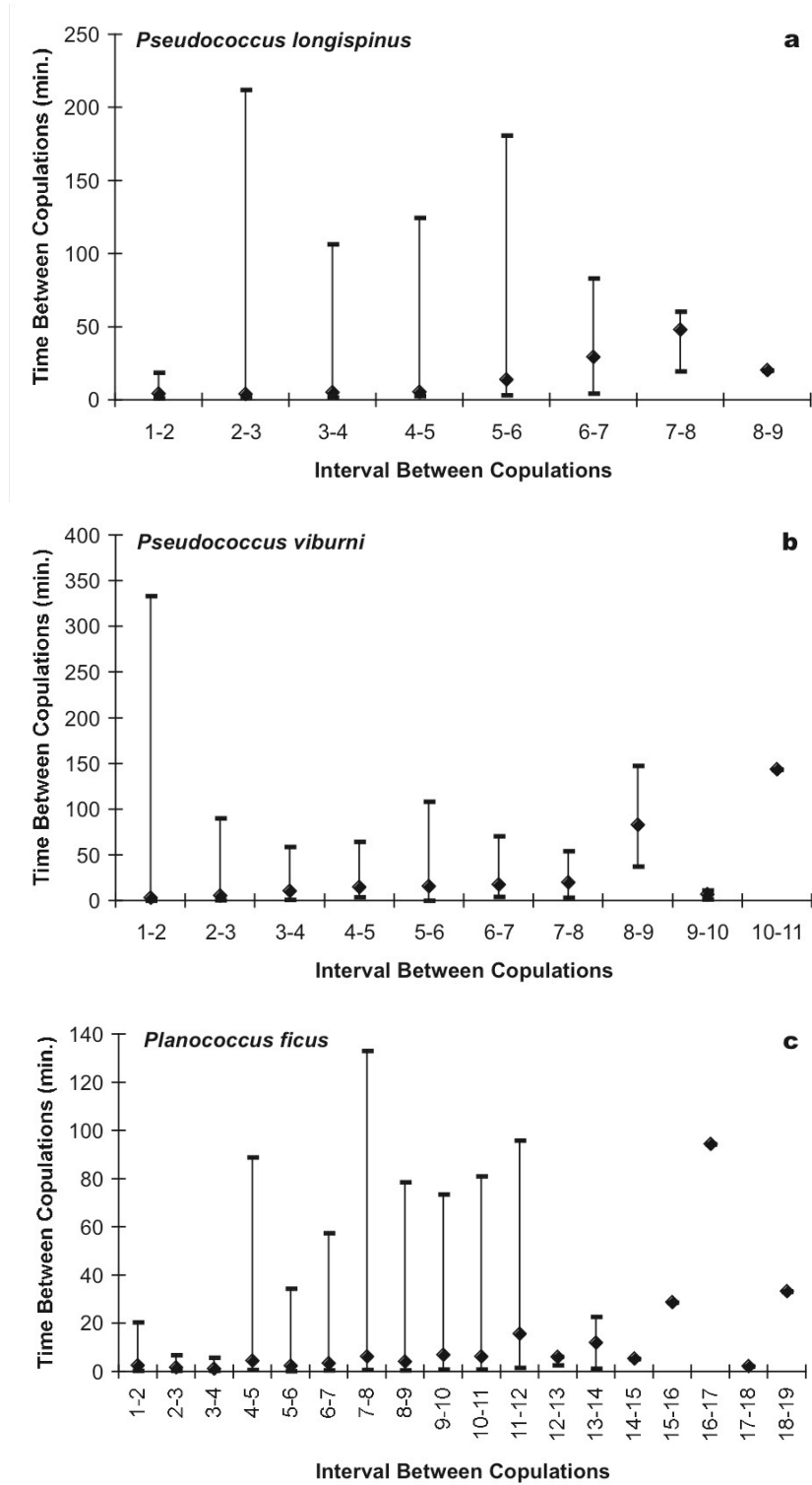


Figure 3.8.



Figures 3.9a-c.



Figures 3.10a-b.

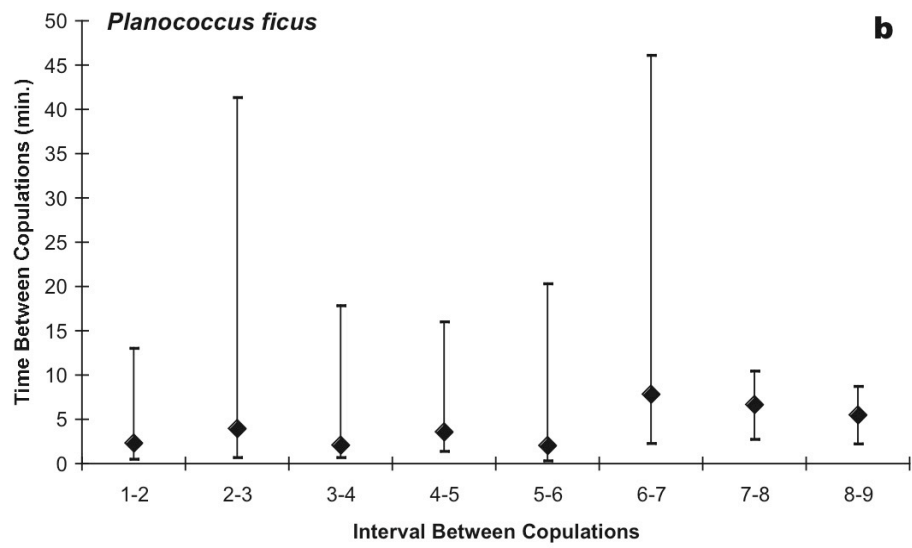
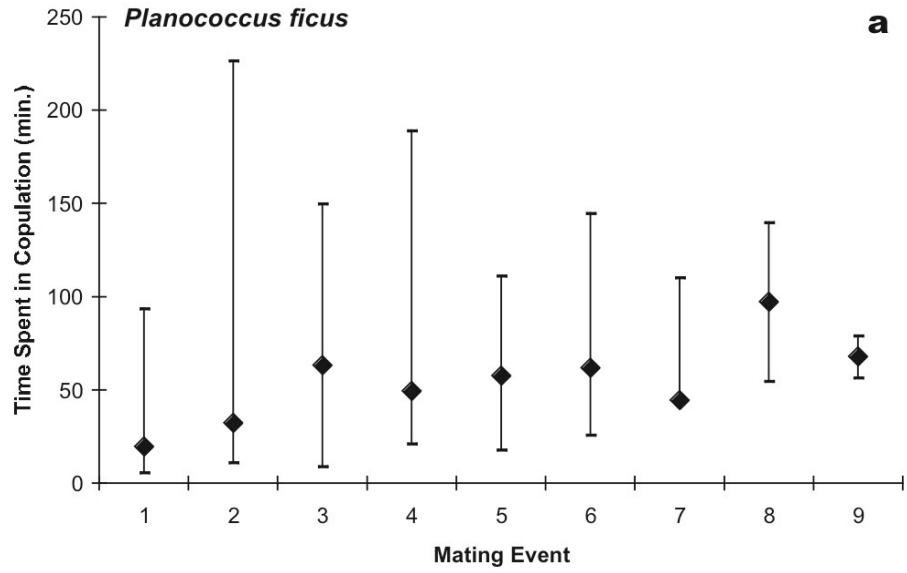


Figure 3.11.

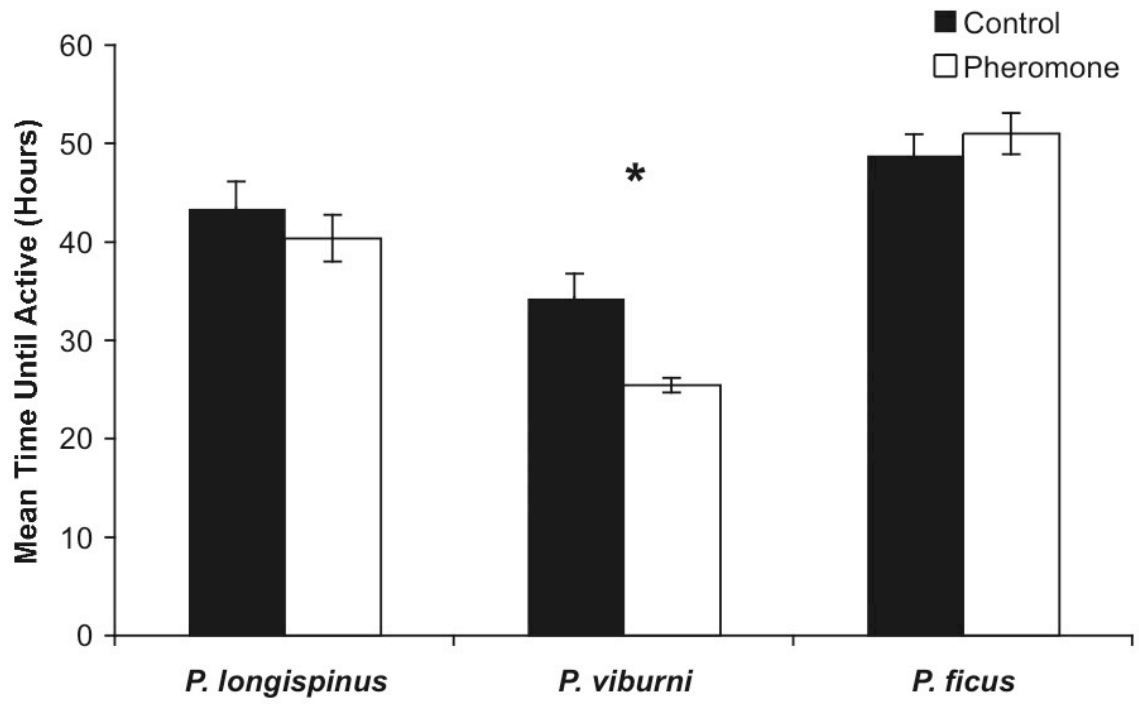
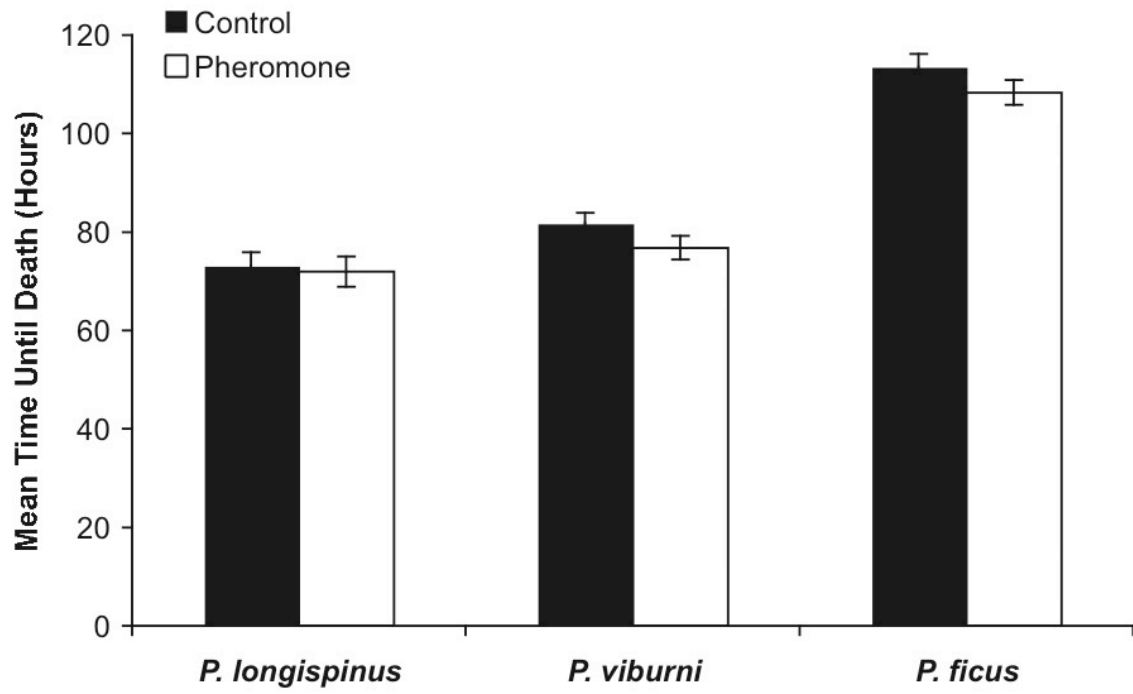


Figure 3.12.



Chapter 4: Probable site of sex pheromone emission in female vine and obscure mealybugs (Hemiptera: Pseudococcidae)

Abstract

Pseudococcus viburni (Signoret) and *Planococcus ficus* Signoret females were cut into body sections that were then exposed to male mealybugs. Males of both species were most attracted to the section with the hind legs. Males did not discriminate between extracts of the anterior body section or the posterior section with the hind legs, suggesting that most of the female's waxy body becomes contaminated with pheromone. Significantly more males chose extracts of body sections with the third pair of legs than sections with the second pair of legs. Males also exhibited copulatory behaviors towards filter paper discs treated with extracts of the hind legs. In sum, these experiments suggest that the sex pheromone of females is produced by glands on or close to the hind legs.

Introduction

Sexually mature adult female mealybugs in biparental species produce a sex pheromone to attract males (Millar et al. 2005). Gravitz and Willson (1968) first demonstrated that female mealybugs produce sex pheromones using *Planococcus citri* (Risso) as a model species, and the first mealybug sex pheromone was identified and synthesized in 1980 for *Pseudococcus comstocki* (Kuwana) (Negishi et al. 1980), followed shortly by the identification and synthesis of pheromone of *P. citri* (Bierl-Leonhardt et al. 1981). Despite knowing that these insects produce sex pheromones for more than 40 years, and the fact that a number of pheromones have been identified and synthesized (listed in Chapter 1), the site of pheromone production remains unknown. The sites of pheromone emission for other members of the Sternorrhyncha, such as diaspidid scales and aphids, were located in the early 1970s. For example, sex pheromone is released from pseudosensoria on the swollen hind tibiae of adult female aphids (Pettersson 1970, Marsh 1975). In contrast, female scales have pygidial glands connected to the pygidium via ducts, so that the pygidium, and more specifically the anal opening, is the site of pheromone emission in these species (Moreno 1972). Once a female scale has been mated, the cells that produce the pheromone break down and pheromone production ceases (Moreno and Fargerlund 1975).

Adult female mealybugs are covered with hundreds of pores (McKenzie 1967, Williams 1985, Cox and Pearce 1985) (Fig. 4.1), most of which produce various types of wax for different purposes (Williams 1985, Cox and Pearce 1985). These pores are distinct sclerotized structures that act as molds to produce structurally different forms of

wax in different areas of the body (Cox and Pearce 1985). For example, wax produced by trilocular and quinquelocular pores protects all mealybug instars from contamination by honeydew and defensive exudates whereas tubular ducts and multilocular disc pores produce wax that forms the female's ovisac and the male's cocoon (Cox and Pearce 1985, Williams 1985). Discoidal pores are associated with the eyes of female mealybugs, but their function is unknown (McKenzie 1967, Williams 1985).

Adult females of most mealybug species also have another type of pore located on the coxae, femur, or tibiae of the hind legs, visible as minute, thin patches of translucent cuticle (McKenzie 1967, Williams 1985, Watson and Kubiriba 2005). It has been suggested that these pores, named translucent pores, are the sites of pheromone emission in mealybugs, because they are present only on the adult female (Williams 1985, Watson and Kubiriba 2005). The location of these pores on the hind legs of mealybugs also would be analogous to the location of the pheromone-emitting pores of aphids. However, female *Phenacoccus herreni* Cox & Williams do not possess morphologically recognizable translucent pores anywhere on the hind legs, although, male mealybugs are attracted to females, suggesting that this species does indeed have a female-produced sex pheromone (Williams 1985).

Obligately parthenogenetic mealybug species (Nur 1971) such as *Phenacoccus solani* Ferris, *Dysmicoccus brevipes* (Cockerell), and most mealybugs in the genus *Trionymus* also possess translucent pores on the third pair of legs (McKenzie 1967, USDA 2007). Unlike *Planococcus ficus* Signoret, which has translucent pores on the coxa, femur, and tibia, *P. solani* and most *Trionymus* species possess pores on a single

leg segment, usually the coxa or the tibia only (McKenzie 1967, USDA 2007).

Dysmicoccus brevipes is known to have a sexual race in Brazil (Beardsley 1965) and so the presence of pores on the legs possibly may have an explanation for this species at least.

It would be useful to unequivocally determine the site of pheromone production in mealybugs for two reasons. First, if the pheromone-producing glands could be reliably located, it might simplify the identification of pheromones for additional species. That is, dissection and extraction of the specific pheromone-producing tissues would provide much cleaner extracts than, for example, whole body extracts. Second, mealybug pheromones are highly irregular terpenoid structures, which must be synthesized by unusual biosynthetic pathways or enzymes. Location of the site of the pheromone-producing tissues would allow detailed studies of these enzymes and pathways, including the genes that are ultimately responsible for their production. Thus, my goal was to locate the sites of pheromone production in two species for which pheromones have been identified, *P. ficus* and *Pseudococcus viburni* (Signoret). My specific objectives were to:

1. Determine the body region of female *P. viburni* and *P. ficus* to which males were attracted;
2. Determine whether male *P. ficus* were attracted to solvent-extracted sections of bodies of females;
3. Determine whether pheromone could be located and identified in extracts of female body parts (*P. ficus*) by coupled gas chromatography-electroantennogram analyses, using antennae of male mealybugs as detectors; and

4. Examine the legs of immature and adult *P. ficus* using scanning electron microscopy, with the aim of locating possible pheromone pores.

Materials and Methods

Bioassays of body sections of females. Female *P. viburni* and *P. ficus* were separated as late second or third instars from males as described in Chapter 3 and placed on fresh squash segments. Upon reaching maturity, individual females of both species were selected at random and were briefly exposed to 10 randomly selected males (from a pool of newly active males) in mating arenas (Chapter 3). Only females that were clearly attractive to males were used in the subsequent bioassays, and females were separated from males before copulation occurred. This allowed pre-selection of only those females that were actively producing pheromone.

Attractive females were chilled and divided into different body parts under a dissecting microscope. A glass slide was placed over a petri dish containing crushed dry ice, and a female was placed on the slide with its legs directed upward. Females froze within seconds, and once a female had completely frozen, the carcass was divided with a scalpel into different body sections, including anterior and posterior segments with and without the third pair of legs. Great care was taken to avoid cross contamination of dissected body parts. Scalpels were rinsed with acetone and air-dried between each cut. Probes were constructed from glass micropipette tubes with insect pins (size 1) inserted into one end. Each probe was assigned for use in one body region only (e.g., holding down the anterior while incisions were made or moving the anterior to the arena). Probes were rinsed with acetone between dissections. Scalpels and forceps were rinsed three times at the conclusion of the dissections and baked overnight in a drying oven.

Two sets of bioassays were carried out with the dissected body sections, comparing the attraction of males to:

1. The posterior portion of the body including the third pair of legs versus the anterior portion with the first two pair of legs.
2. The posterior portion of the body (without any legs) versus the anterior body section with all three pairs of legs.

Freezing the insects was imperative for precise sectioning of the females' bodies. Their soft bodies otherwise exuded fluid as incisions were made. Incisions for study one were made between the second and third pairs of legs (Fig. 4.1, green line). The incisions for study two were made between the second and third abdominal segments (Fig. 4.1, purple line).

While the body sections were still frozen, they were transferred with forceps to opposite sides of a glass petri dish arena (Pyrex[®], 100 mm x 10 mm) with sections separated by 80 mm. The 10 males previously used to determine that the female was attractive and producing pheromone were introduced into the center of the arena between 10:00 and 12:00 hr (PST) and observed every 15 min. for 1 hr. Due to the time intensive nature of screening males and sectioning females, the first experiment was replicated over several days (n = 9 total for *P. viburni* and n = 3 total for *P. ficus*). Thirteen replicates of the second experiment were carried out with *P. viburni* only. The low numbers of active males also limited the number of replicates that could be carried out each day.

Data from the two experiments were analyzed in SAS version 9.1.3 (SAS Institute, Cary, North Carolina). The sum of males at each body section and the remaining males not at either section were calculated over each observational period (e.g., study one, 4 observational periods). A chi-square analysis (PROC FREQ) determined statistical differences among the three components (i.e., males visiting the posterior, anterior, or neither). An additional chi-square analysis was conducted for the two body sections only. The nonparametric Wilcoxon signed-rank test for paired data was used to analyze differences between the proportions of males that had visited each fragment at each time period. The signed rank and probability were calculated in PROC UNIVARIATE in SAS. For study one, both *P. viburni* and *P. ficus* were examined, and these analyses include both species.

Bioassays of extracts of female body parts. In a third experiment, extracts were made from body parts of *P. ficus* females only. Females were not pre-selected for attractiveness by first exposing them to males. It became apparent from other observational work with *P. ficus* (see Chapter 2) that virgin females were almost always attractive to males when the sexes were first placed together. Under this assumption, males that had not been exposed to odors from females previously were randomly selected for use in bioassays.

Frozen females were dissected as described above and the body parts were assayed as follows. First, the attraction of males to the posterior section of a female with the third pair of legs was compared to attraction to the anterior section including the first

two pair of legs (experiment three). Second, the attractiveness of three pairs of hind legs and three pairs of middle legs was compared (experiment four).

The body sections were then individually extracted in ~20 μ l of hexane in a conical vial insert. Approximately one quarter of each extract was spotted onto a filter paper disc (13 mm diam) and discs were placed in pairs in glass petri dish arenas (Pyrex[®], 100 mm x 10 mm) with edges 45 mm apart. Fifteen to twenty males were introduced into the center of each arena between 10:00 and 12:00 hr PST, and the numbers of males on each disc were counted every 3 – 5 min. for 30 min.

In the fourth experiment that examined the attractiveness of the second and third pairs of legs, smaller glass petri dish arenas were used (Pyrex[®], 60 x 15 mm) along with smaller filter paper discs (7 mm diam.). Three *P. ficus* females were simultaneously frozen and the third pair of legs was removed from each by holding the female down and gently pulling the legs off with fine forceps. *Planococcus ficus* females have translucent pores on three leg segments: the coxa, femur, and tibia. Most of the leg was removed as described above, leaving, at most, a small portion of the coxa attached to the carcass. Legs were held frozen on the glass slide until all hind legs had been removed and then were transferred to vial inserts containing 10 μ l of hexane. The procedure was repeated with clean forceps to remove the second pair of legs from the same three females. While the legs were being removed, female mealybugs were held down with an insect pin (size 1). This pin was used only once and discarded.

Four μ l of each extract were applied to filter paper discs separated by 20 mm in the petri dish arena. Twenty randomly selected males were introduced in the center of

the arena and the numbers of males on each treatment disc were counted every 5 min. for 1 hr starting between 10:00 and 12:00 PST. The experiment was replicated 13 times over several mornings.

The sum of males at each disc treated with an extract of female sections and the remaining males not at either fragment were calculated over each observational period (e.g., study three, 9 observational periods). A chi-square analysis (PROC FREQ) determined statistical differences among the three components (i.e., males visiting the disc treated with posterior extract, anterior extract, or neither). An additional chi-square analysis was conducted for the two extracts only. The nonparametric Wilcoxon signed-rank test for paired data was used to analyze differences between the proportions of males that had visited each treatment disc for each time period. The signed rank and probability were calculated in PROC UNIVARIATE in SAS.

Gas chromatography – electroantennogram analyses of extracts of body parts of females. Individual extracts were made of the third pair of legs of nine virgin female *P. ficus* as described in the previous section. Three composite extracts were also prepared from 10 pairs of hind legs in 5 μ l of hexane. Composite whole body extracts of females with the middle or hind pairs of legs removed also were prepared by extracting 3 females with \sim 30 μ l of hexane (n = 3). One microliter of each extract then was analyzed by coupled gas chromatography-electroantennogram detection (GC-EAD) using equipment and methods previously described (Figadère et al. 2007). Briefly, the head of a randomly selected male mealybug was excised and placed on the saline-filled glass capillary EAD electrodes, with the head mounted on one electrode and the distal ends of the two

antennae touching the saline on the tip of the other electrode. A Hewlett Packard 5890 Series II GC fitted with a DB-5 column (30 m × 0.25 mm internal diam, 0.25 µm film; J&W Scientific, Folsom CA) was used for all analyses. Injections were made in splitless mode, and the column effluent was split equally between the flame ionization detector and the heated outlet port leading to the electroantennogram detector. The temperature program was 40 °C for 1 min., then 10 °C per min. to 275 °C.

Scanning electron microscopy of mealybug legs. Immature and adult female *P. ficus* were selected at random and killed in a small volume of 70% ethanol. After 30 min., the alcohol was decanted, and the insects were soaked sequentially in increasingly concentrated ethanol (10% per half hour soak) to 100% ethanol. The insects were soaked in 100% ethanol for a further hour then placed in sufficient hexamethyldisilazane (HMDS) to cover their bodies, refreshed once after 30 min.. The dried insects were left in the fume hood to dry overnight, then rinsed with pentane to remove some of their wax coating, and transferred to conductive double-sided tape for scanning electron microscopy with a Hitachi TM – 1000 instrument.

Results

Bioassays of body sections of females. For each time period in experiment one, there were significant differences between the number of males visiting the anterior body section of a female with the first two pairs of legs compared to the posterior section with the third pair of legs (Table 4.1). A significantly higher proportion of males were attracted to the posterior during each observational period (Table 4.2, Figure 4.2a-b). Conversely, when the female bodies were sectioned differently so that the hind legs remained with the anterior body section, significantly more males visited the anterior sections at each time period (experiment 2, Table 4.3). The mean proportion was initially significantly higher at time periods one and two for males that were attracted to the anterior (Table 4.4). There were no differences in attraction at time periods three and four.

Bioassays of extracts of female body parts. In experiment three, there were no differences in the frequency of males visiting treatment discs of either anterior or posterior extracts during any of the nine observational periods (Table 4.5). As might be expected, there also were no differences in the mean proportion of males attracted to either treatment (Table 4.6.)

The numbers of males attracted to extracts of the third pair of legs were all significantly different from the numbers of males attracted to extracts of the second pair of legs (Table 4.7). A higher mean proportion of males visited the extracts of the third pair of legs at every observational period (Table 4.8).

Gas chromatography – electroantennogram detection analyses of extracts of females.

None of the extracts had concentrations of pheromone that were detectable by either the flame-ionization or the electroantennogram detector. This suggested that compounds were present at subnanogram levels in the extracts.

Scanning electron microscopy of mealybug legs. Electron microscopy showed pores on the coxae of all three adult females that were examined (Fig. 4.3a), whereas none of the four immature *P. ficus* females examined had these pores on their coxae (Fig. 4.3b).

Thus, the pores seem to be specifically associated with the adult female life stage.

Discussion

Bioassays of body sections of female mealybugs. Experiments one and two demonstrated that males were preferentially attracted to female body sections that included the third pair of legs. Experiment four provided additional evidence that the site of pheromone emission was probably on the third pair of legs, with males being strongly attracted to the hind leg extracts, and exhibiting copulatory behaviors when on the treated filter paper discs, including high rates of turning on the discs and probing the discs with their genitalia.

During bioassays, the majority of males did not orient to a body section or treatment disc and were often walking or resting in areas away from treatments (see Tables 1, 3, 5, 7). Overall, the number of males attracted to treatments was higher in experiment four than in the previous bioassays, probably due in part to the fact that smaller arenas were used in this experiment (e.g., in one observational period of experiment four, initially 18 out of 20 males displayed interest in extracts of the third pair of legs). Gravitz and Willson (1968) had noted that male *P. citri* did not display interest (e.g., recognition and investigation) in sexually mature females until they were within a short distance (5 mm) of them. The enhanced responses seen in experiment four may also have been a result of using aliquots of extracts from three pairs of hind legs, which may have contained more pheromone than the amount released from intact legs or body parts. As might be expected, Gravitz and Willson (1968) had previously shown that male *P. citri* responses increased with an increase in the dose of extract of females that was used as a test treatment. Analogous results correlating increasing dose to increased

responses of males have been demonstrated in field bioassays with *P. ficus* (Millar et al. 2002), *P. longispinus*, and *P. viburni* (Chapter 2).

Pheromone pores located on the hind legs might explain some of the copulatory behavior of male and female mealybugs. Females lift their posterior abdominal segments and expose their hind legs when antennated or investigated by males. This behavior presumably results in greater release of pheromone into the headspace around the female. In addition, as males approached and began assessing females, their investigation typically focused on the female's posterior and the vicinity of the third pair of legs. If a male began assessing the anterior of a female, he modified his position and subsequently assessed the posterior. At this point, the subtle signals that trigger females to raise their abdomens in response to investigation by males are unknown, nor do we know all the signals that males use to orient efficiently to the female's posterior, although the pheromone is clearly involved.

Gas chromatography – electroantennogram analyses of extracts of females. It was disappointing that none of the extracts prepared from body sections or legs of females resulted in any detectable GC response or antennal responses by males, despite the fact that the extracts were prepared in exactly the same way as those that elicited behavioral responses from males in bioassays. This clearly indicates the very small amount of pheromone produced by individual females because the GC can detect subnanogram amounts of the pheromone, and the antennae of male mealybugs may be even more sensitive than the FID detector in the GC.

Scanning electron microscopy of female mealybug legs. Finding the pores on the hind coxae of adult females and the absence of such pores on coxae of immature females provided corroborating evidence that these pores might be the sites of pheromone emission. However, Watson and Kubiriba (2005) described the pores as being “minute, thin patches of cuticle”, whereas close examination of my SEM images showed that these pores appear to have some depth (Fig. 4.3a). Obtaining conclusive evidence that these pores are indeed the sites of pheromone emission may be difficult because of the microscopic size of the pores, which effectively precludes dissection of pores, and because of the small amount of pheromone produced per female. Nevertheless, collectively the evidence to date suggests that the pheromone is produced from the hind legs, and these appendages are a good starting point for further studies on the biosynthesis of the pheromones, including identification of the enzymes that produce their highly unusual terpenoid structures and the genes that code for those proteins.

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Table 4.1. The number of male *Pseudococcus viburni* and *Planococcus ficus* attracted to either the anterior, posterior (with the third pair of legs), or neither female body section at each time period (every 15 min. for 1 hr) (experiment one). Chi-square analysis was conducted including all three components (anterior, posterior, or neither) as well as anterior versus posterior only.

Time	Number of Males at Female Body Sections			Three Components		Anterior vs Posterior Only	
	Anterior	Posterior	None	χ^2 , df=2	<i>P</i>	χ^2 , df=1	<i>P</i>
1	4	22	94	113.4	< 0.0001	12.46	0.0004
2	4	26	90	99.8	< 0.0001	16.13	< 0.0001
3	1	17	102	147.4	< 0.0001	14.22	0.0002
4	3	18	99	133.4	< 0.0001	10.71	0.001

Table 4.2. For each time period in experiment one, the number of replicates, the mean difference between proportions of males visiting the posterior (with the third pair of legs) and the anterior, followed by the signed rank (T) and its probability. Differences were calculated: proportion at posterior – proportion at anterior.

Time	n	Mean Difference	T	P \geq T
1	12	0.15	18	0.008
2	12	0.18	28	0.011
3	12	0.13	18	0.008
4	12	0.125	16.5	0.023

Table 4.3. The number of male *Pseudococcus viburni* attracted to either the anterior with all three pairs of legs, posterior with no legs, or neither female body section at each observational period (every 15 min. for 1 hr) (experiment two). Chi-square analysis was conducted including all three components (anterior, posterior, or neither) as well as anterior versus posterior only.

Time	Number of Males at Female Body Sections			Three Components		Anterior vs Posterior Only	
	Anterior	Posterior	None	χ^2 , df=2	<i>P</i>	χ^2 , df = 1	<i>P</i>
1	16	1	113	170.6	< 0.0001	13.24	0.0003
2	17	2	111	161.1	< 0.0001	11.84	0.0006
3	13	2	115	179.2	< 0.0001	8.07	0.0045
4	13	1	116	184.4	< 0.0001	10.29	0.001

Table 4.4. For each time period in experiment two, the number of replicates, the mean difference between proportions of males visiting the anterior (with the third pair of legs) and the posterior, followed by the signed rank (T) and its probability. Differences were calculated: proportion at anterior – proportion at posterior.

Time	n	Mean Difference	T	P \geq T
1	13	0.12	19	0.027
2	13	0.12	12.5	0.047
3	13	0.08	18	0.09
4	13	0.09	14.5	0.055

Table 4.5. The number of male *Planococcus ficus* attracted to extracts of the anterior body section with the first pairs of legs, the posterior with the hind legs, or neither body section at each time period (every 3 – 5 min. for 30 min.) (experiment three). Chi-square analysis was conducted including all three components (anterior, posterior, or neither) as well as anterior versus posterior extracts only.

Time	Number of Males at Extracts of Females			Three Components		Anterior vs Posterior Only	
	Anterior	Posterior	None	χ^2 , df=2	P	χ^2 , df = 1	P
1	20	22	66	37.6	< 0.0001	0.095	0.76
2	22	24	62	28.2	< 0.0001	0.087	0.77
3	31	29	48	6.06	0.048	0.067	0.80
4	20	27	61	26.7	< 0.0001	1.04	0.31
5	24	51	33	10.5	0.005	1.42	0.23
6	21	36	51	12.5	0.002	3.95	0.05
7	26	31	51	9.72	0.008	0.44	0.51
8	23	33	52	12.06	0.002	1.79	0.18
9	29	29	50	8.17	0.017	0	1

Table 4.6. For each time period in experiment three, the number of replicates, the mean difference between proportions of males visiting the discs treated with extracts of either the anterior with the first two pairs of legs or the posterior with the hind legs, followed by the signed rank (T) and its probability. Differences were calculated: proportion at posterior extracts – proportion at anterior extracts.

Time	n	Mean Difference	T	P \geq T
1	6	0.024	0	1
2	6	-0.025	-1.5	0.84
3	6	-0.02	0	1
4	6	0.080	2.5	0.69
5	6	0.042	1	0.91
6	6	0.086	4	0.47
7	6	0.042	1.5	0.84
8	6	0.059	2.5	0.69
9	6	-0.024	-1.5	0.84

Table 4.7. The number of male *Planococcus ficus* attracted to extracts of the third pair of legs of females, the middle pair of legs of females, or neither extract at each time period (every 5 min. for 60 min.) (experiment four). Chi-square analysis was conducted including all three components (third, middle, or neither) as well as third versus middle leg extracts only.

Time	Number of Males at Leg Extracts			Three Components		Third vs Middle Only	
	Third	Middle	Neither	χ^2 , df=2	P	χ^2 , df=1	P
1	134	18	108	85.5	< 0.0001	88.5	< 0.0001
2	134	15	111	91.9	< 0.0001	95.0	< 0.0001
3	113	6	141	117.1	< 0.0001	96.2	< 0.0001
4	119	8	133	108.2	< 0.0001	97.0	< 0.0001
5	108	6	146	121.0	< 0.0001	91.3	< 0.0001
6	107	10	143	109.2	< 0.0001	80.4	< 0.0001
7	109	9	142	110.7	< 0.0001	84.7	< 0.0001
8	103	9	148	116.1	< 0.0001	78.9	< 0.0001
9	88	11	161	129.8	< 0.0001	59.9	< 0.0001
10	87	11	162	131.5	< 0.0001	58.9	< 0.0001
11	94	13	153	114.0	< 0.0001	61.3	< 0.0001
12	83	11	166	138.8	< 0.0001	55.1	< 0.0001

Table 4.8. For each time period in experiment four, the number of replicates, the mean difference between proportions of males visiting discs treated with extracts of either the third pair or second pair of legs, followed by the signed rank (T) and its probability. Differences were calculated as proportion at the extract of third pair of legs – proportion at the extract of the second pair of legs.

Time	n	Mean Difference	T	P \geq T
1	13	0.45	44	0.0007
2	13	0.46	38	0.001
3	13	0.41	33	0.001
4	13	0.43	38	0.001
5	13	0.39	44.5	0.0005
6	13	0.37	33	0.001
7	13	0.38	44.5	0.0005
8	13	0.36	45.5	0.0002
9	13	0.30	39	0.0005
10	13	0.29	39	0.0005
11	13	0.31	39	0.0005
12	13	0.28	39	0.0005

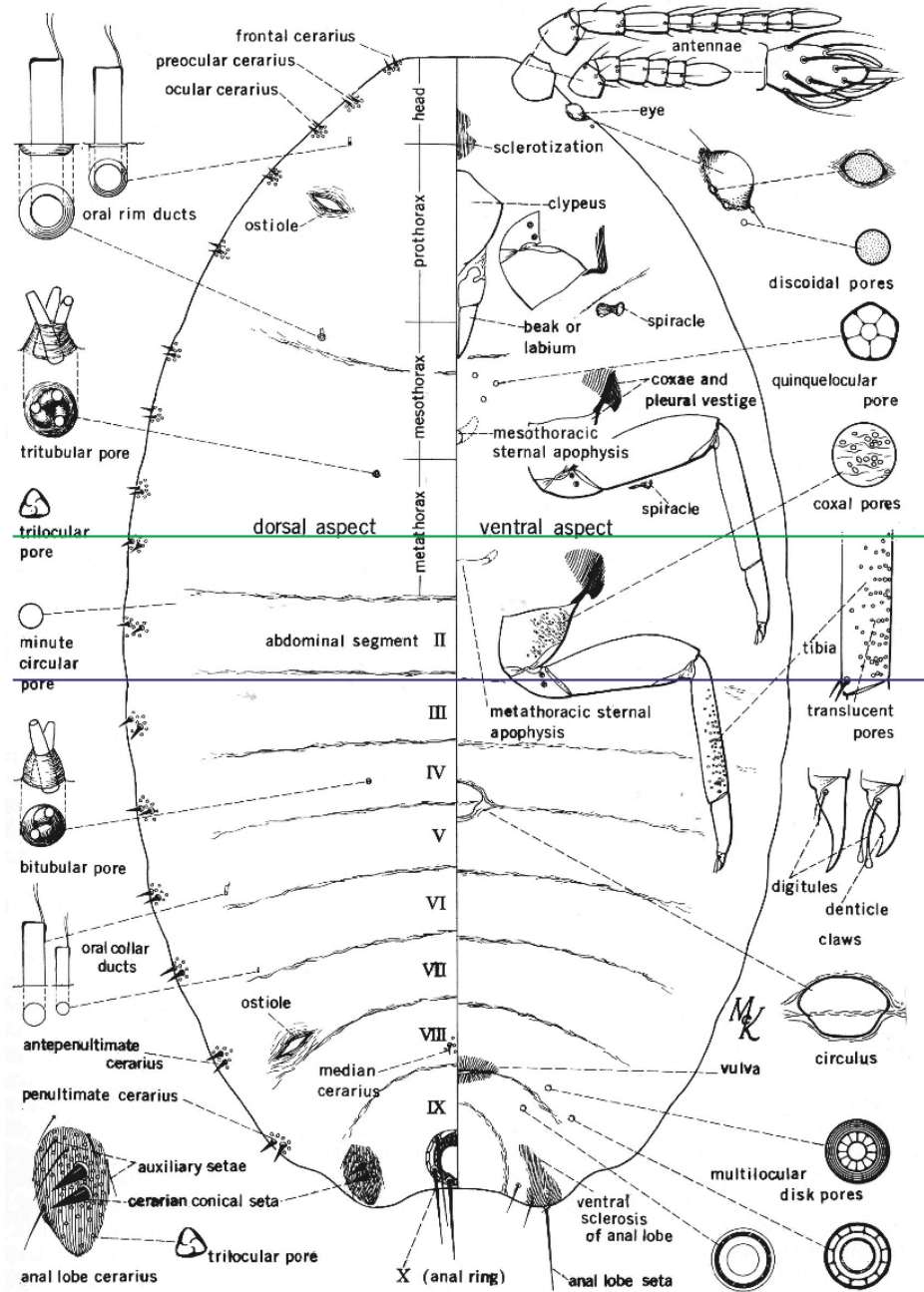
Figure legends.

Figure 4.1. Generalized morphology of adult female mealybugs, with a dorsal view on the left of the longitudinal line and a ventral view on the right. The green line shows where the females were cut into sections for experiments 1 and 4 (see methods). The purple line indicates where females were sectioned for experiment 2 (see methods). Note the relative location of the translucent pores on the mealybug's hind leg.

Figures 4.2a-b. (a) *Pseudococcus viburni* (Signoret) males exhibiting copulatory behavior to the posterior body section with the hind pair of legs of a female. The anterior section is lying to the right. (b) Male *P. viburni* attempting copulation with the posterior body section of a female.

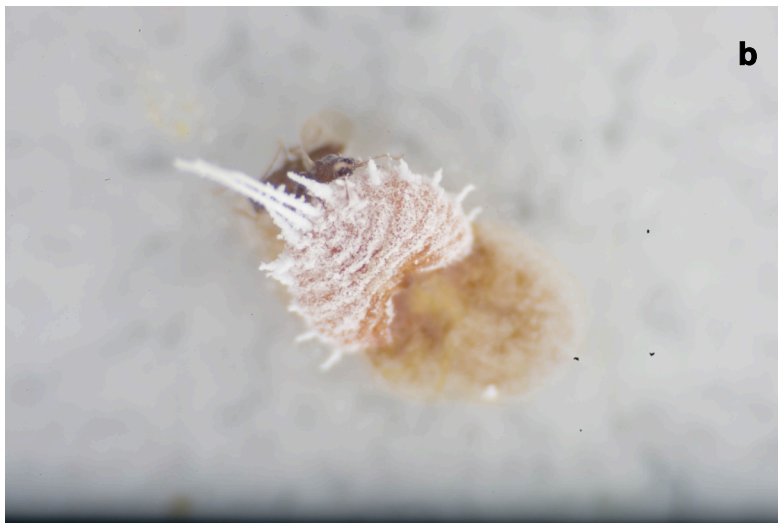
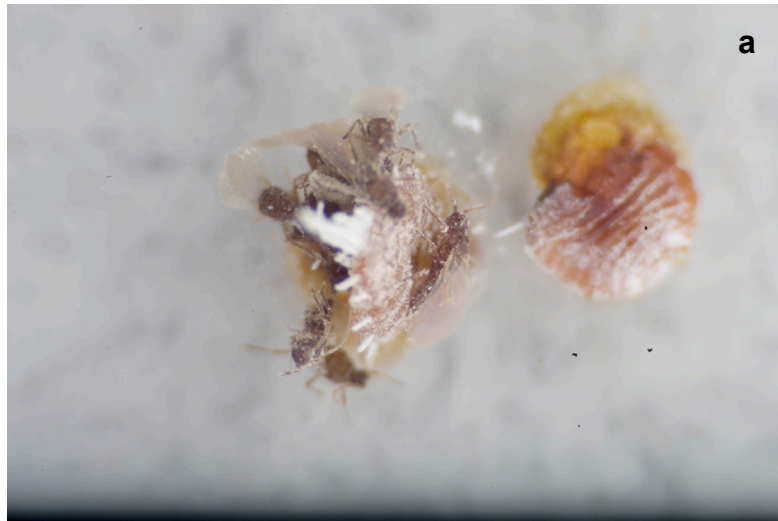
Figures 4.3a-b. (a) Electron micrograph of the hind coxa of an adult female *Planococcus ficus* Signoret at 1200x. Translucent pores are apparent as small openings on the surface. (b) Corresponding vantage point of the hind coxa of an immature *P. ficus* female at 1500x, with no translucent pores visible.

Figure 4.1.

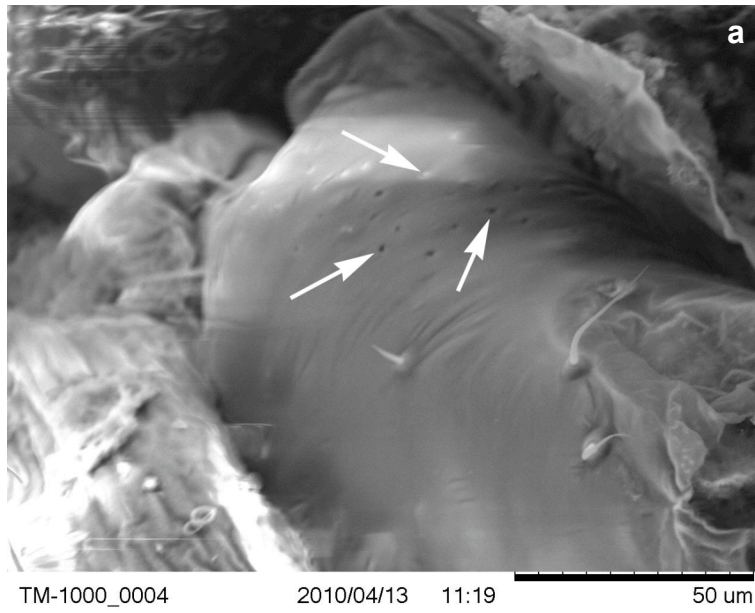


Source: Howard McKenzie, *Mealybugs of California: With Taxonomy, Biology, and Control of North American Species*. Copyright (c) 1967 by the Regents of the University of California. Published by the University of California Press

Figures 4.2a-b.



Figures 4.3a-b.



Chapter five: Concluding remarks

Mealybugs (Hemiptera: Pseudococcidae) are polyphagous insects that feed on plants in hundreds of plant families (McKenzie 1967, USDA 2007). Economic losses as a result of direct feeding damage and indirect damage caused by growth of sooty mold on honeydew and the transmission of pathogens can reach millions of dollars (Chong et al. 2003, Ranjan 2006). Mealybugs are key pests in California greenhouse production (Laflin et al. 2004), especially on rose crops (Casey et al. 2007) and field ornamental crops (see Chapter 2). Despite nursery managers best efforts, mealybugs persist on plant tissue because they conceal themselves among roots, bark crevices, developing meristematic tissue, and other cryptic locations (McKenzie 1967) where insecticide sprays cannot reach them. Many contact insecticides also are ineffective against mealybugs because the mealybugs' waxy covering repels polar chemicals (Walton et al. 2004).

Mealybugs, especially *Planococcus ficus* Signoret, also have become a major problem in vineyards in California (Millar et al. 2002, Godfray et al. 2002), particularly because infestations may be difficult to locate until populations are large. Timely and effective management is currently a critical component in vineyards because *P. ficus* and several other mealybug species are known to transmit grapevine leafroll virus (Golino et al. 2008). Traditionally, growers used tedious and time-consuming visual inspection of plant material to locate infestations, but since the identification of the sex pheromone of *P. ficus* about 10 years ago (Millar et al. 2002), detection with pheromone-baited traps

has become widespread, and this is now the recommended method for monitoring *P. ficus* in vineyards (Flaherty 2008).

Much of my work focused on using mealybug sex pheromones in commercial nurseries producing ornamental plants and flowers, which is a multibillion-dollar industry in California (Census of Agriculture 2007). I demonstrated that pheromone-baited traps were useful for tracking seasonal variation of *Pseudococcus longispinus* (Targioni Tozzetti) populations over multiple growing seasons. Male *P. longispinus* and *Pseudococcus viburni* (Signoret) were very sensitive to the racemic pheromone. Lures loaded with 25 micrograms of the pheromones of *P. longispinus* and *P. viburni* had effective field lifetimes of at least 3 months if not considerably longer. I also showed that the pheromones of *Planococcus citri* (Risso), *P. longispinus*, and *P. viburni* could be combined into a single lure that effectively attracted all three species simultaneously. Conventional mealybug management strategies (typically insecticide applications) are identical in nurseries regardless of the mealybug species. Therefore, combining the pheromones of the most important species increases the efficiency and decreases the cost of the monitoring process (i.e., fewer traps required) while still informing the grower about infestation levels. The apparent slight inhibition of the responses of *P. longispinus* males by the pheromone of *P. citri* was not sufficient to affect the desired goal of using the combined lures to monitor the overall mealybug population. Finally, I showed that the counts of male mealybugs in traps was correlated with the density of mealybugs on plant material such that it should be possible for nursery managers to sample mealybugs quickly and efficiently with pheromone-baited traps, rather than having to use time-

consuming visual sampling methods. All these factors are favorable for the development and commercialization of pheromone products for these species.

The mealybug sex pheromones that have been identified generally are complex molecules that are relatively difficult to synthesize on a large scale (see references in Chapter 1). Nevertheless, because male mealybugs are so exquisitely sensitive to the pheromone, with lures containing only a few micrograms remaining active for at least several months under field conditions, widespread use of pheromone-baited traps for monitoring mealybugs is economically feasible. For example, 1 gram of racemic pheromone is sufficient to prepare ~50,000 lures or more (20 µg/lure). At the time of writing, pheromone lures for *P. ficus* have been widely available for several years and are widely used. Pheromone lures for *P. longispinus*, *P. maritimus*, and *P. viburni* have been used by field researchers for several years in various countries (Bell et al. 2005, Zaviezo et al. 2007), and commercial lures for the former two species became available from Suterra LLC (Bend, OR) in 2010.

There are a number of other agronomically important mealybug species (e.g., *Phenacoccus herreni* Cox & Williams) with males that are attracted to females (Williams 1985), though no sex pheromone has yet been identified. This species is a pest of South American cassava causing damage to growing tips of the host plant (Ben-Dov 1994). Cassava is the fourth most important source of carbohydrates for humans in the tropics (Bento et al. 2000, Calatayud et al. 2003). Classical biological control programs were established in Brazil in 1994 to control *P. herreni* (Bento et al. 2000). Identification of *P.*

herreni sex pheromone might further assist in its management (e.g., determine locations of new infestations).

Overall, there do not appear to be any insurmountable barriers to the adoption of pheromone-based methods for monitoring economically important, sexually reproducing mealybug species. An educational program will be required to instruct new users on how to successfully deploy and read traps. Male mealybugs are small and distinguishing between them and other small insects (e.g., sciarids, cecidomyiids, parasitoids) can be initially challenging. Training sessions, or at the least a training publication, will be important in assuring traps are counted accurately. Another aspect of the use of pheromone traps that needs to be examined is the effective range of traps, and/or the number of traps required per hectare for reliable monitoring. Walton et al. (2004) recommended one *P. ficus* pheromone trap per hectare in vineyards and showed that males could move 50 meters or more upwind. My research did not address this question in the production nurseries and to some extent, accurate monitoring with pheromone traps may be challenging because of the irregular nature of ornamental crop plantings (e.g., greenhouses, various sizes of plots, heterogeneity of crop species). Despite the large size of the nurseries, I had no opportunity to evaluate the effective range of pheromone traps under the real life conditions of California nurseries.

In addition to pheromone-based monitoring, efforts towards pheromone-based control of *P. ficus* in vineyards in California are ongoing (Walton et al. 2006; K. Daane, pers. comm.), and a commercial mating disruption product has been available from Suterra LLC (Bend, OR) for several years. Remarkably, despite their economic

importance, little is known about the detailed reproductive biology of mealybugs, despite the implications and consequences for effective mealybug management. Understanding their reproductive biology is particularly important to the effective development and use of pheromone-based monitoring and control measures (e.g., mating disruption, lure and kill). Thus, my third chapter examined a number of aspects of mealybug reproduction. This included assessments of whether *P. longispinus*, *P. viburni*, and *P. ficus* were obligately sexual, or whether they were also capable of parthenogenetic reproduction. I also conducted detailed studies of their reproductive behaviors, examining factors such as the number of times that males and females could copulate in a single day and over multiple days, and the refractory periods between copulations. I found that all three species are obligately sexual; females never produced viable offspring if they were not mated, although some did produce egg sacs. This finding is of particular importance for pheromone-based mating disruption, the effectiveness of which would be seriously comprised if unmated females were indeed capable of reproduction.

In more detailed studies, I found that both males and females were able to copulate repeatedly on the same day and often over multiple days. Furthermore, females could realize their full reproductive output with a single mating, and females that remained unmated for a number of weeks were still fully capable of reproduction. Again, these facts have major implications for attempting pheromone-based control of mealybugs (i.e., in order to effectively minimize mating, pheromone coverage must be continuous and complete throughout the period when adults are present).

In the last section of chapter 3, I tested whether male activity and longevity were affected by constant exposure to pheromone (but see *P. viburni*, Fig. 3.11). I found very little evidence to suggest that the activity of adult male mealybugs was increased by exposure to pheromone or that male longevity was decreased. This was contrary to my predictions. Because adult males do not feed and thus have very limited time window in which to locate females, I had expected that males exposed to pheromone would be more active and search more vigorously for females, using up their limited energy reserves more quickly and shortening their longevity. Thus, if pheromone were used for mating disruption, there would be no additional advantageous effect from males being removed more quickly from the system.

Pheromone-based control may have some potential to be an effective means to minimize the damage caused by mealybugs in vineyards and production nurseries by minimizing reproduction. These cropping systems are advantageous for pheromone-based control methods such as mating disruption because these crops are of relatively high value and they are grown on relatively limited acreages. Mating disruption in glass houses may be particularly effective because of the confinement and opportunities for exclusion of most other pests. However, for most mealybug species, methods such as mating disruption are not economically feasible because the pheromones are too difficult to produce in the quantities needed for control purposes. In an exception to this general rule, the pheromone of *P. ficus* can be readily made in any desired quantity, and pheromone-based control in vineyards for *P. ficus* has shown some promise, particularly when initial infestations of *P. ficus* are low.

One aspect of the practical application of mealybug pheromones that deserves more attention is the possible effect of the pheromones on natural enemies of mealybugs. For example, incidental research has shown kairomonal properties of mealybug sex pheromones, particularly for *P. viburni* and its parasitoid *Pseudaphycus maculipennis* (Mercet) (Bell et al. 2005). I also have observed parasitoids (*Leptomastix* spp. and *Anagyrus* spp.) stuck in traps baited with *P. citri* pheromone. It will be important to determine if other such interactions exist and the degree to which natural enemies are attracted to mealybug pheromones so that pheromone traps do not become inadvertent sinks for natural enemies. The effects of pheromone used for mating disruption on parasitoid populations also have not been examined yet.

In chapter 4, I attempted to address another gap in our knowledge of mealybug reproductive biology, specifically the tissues from which the female-produced sex pheromones are produced and emitted. Testing the attraction of male mealybugs to various body parts of females suggested that the pheromones of *P. ficus* and *P. viburni* are produced somewhere on the third pair of legs, which have translucent pores that are found only on this set of legs. In aphids, pores on the third pair of tibiae have been shown to be the site of pheromone emission (Pettersson 1970), but I was not able to conclusively verify that pores also found on the hind legs are the sites of pheromone emission in mealybugs. In fact, female mealybugs apparently produce such small quantities of pheromone that I was not able to detect the pheromone in extracts of the hind legs or whole bodies in coupled gas chromatography-electroantennogram analyses. Nevertheless, the combined evidence in favor of pheromone production being associated

with these tissues is strong and will provide the basic knowledge required to initiate studies of the biosynthesis of the irregular terpenoid structures of the pheromones.

I conclude that utilizing mealybug sex pheromones to monitor for mealybugs in production nurseries has shown great promise. Small amounts of pheromone are required for attraction, and the long field longevity of lures will minimize the cost of implementing monitoring programs in nurseries. Multiple species' pheromones can be combined, further simplifying the use of this tactic in the field. Reproductive biology studies of the species to date have indicated that pheromone-based control may be possible, provided that pheromone coverage in the field is continuous and that production of multi-kilogram quantities of the pheromones is feasible.

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