Title

Implications of Argentine Ant Management for Biological Control of the Asian Citrus Psyllid and Other Ant-Tended Pests in Citrus

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Implications of Argentine Ant Management for Biological Control of the Asian Citrus Psyllid and Other Ant-Tended Pests in Citrus

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

Doctor of Philosophy

in

Entomology

by

Kelsey Ann McCalla

December 2019

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- Dr. Mark Hoddle, Co-Chairperson
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ABSTRACT OF THE DISSERTATION

Implications of Argentine Ant Management for Biological Control of the Asian Citrus Psyllid and Other Ant-Tended Pests in Citrus

by

Kelsey Ann McCalla

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2019
Dr. Mark Hoddle and Dr. Richard Redak, Co-Chairpersons

The establishment of the Asian citrus psyllid-huanglongbing (*Diaphorina citri* Kuwayama [Hemiptera: Liviidae]) pest-disease complex has severely diminished the profitability of the citrus trade worldwide. California's citrus industry is valued at $7.1 billion annually and supports some 21,700 full-time positions. Economic forecasts predict that the spread of huanglongbing into major production areas from the residential areas within which it is currently restricted would have devastating financial consequences for the state. My doctoral research investigates the use of integrated, sustainable strategies for improving biological control of *D. citri*. A major impediment to this pest management approach is the invasive Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), a notorious tramp ant species that is ubiquitous across southern California. *Linepithema humile* establishes trophobiotic mutualisms with honeydew-producing hemipterans in the citrus agroecosystem, many of which are invasive, economically damaging pests. Although ant-hemipteran pest partnerships are well-documented for many systems, little is known about the long-term consequences of the relationship between *L. humile* and *D. citri*.

I aimed to bridge this knowledge gap by first, determining the host-killing capacity of the *D. citri* parasitoid *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) under field realistic thermal cycles, second, examining the impact of *L. humile* control on infestation and biological control of *D. citri* and other ant-associated citrus pests, and third, developing and evaluating an alginate hydrogel bait transport system as a high efficacy, low-cost treatment option for control of *L. humile* in commercial citrus. Results indicated that *T. radiata* inflicts a significant portion of *D. citri* mortality through host feeding. Consequently, its biocontrol contributions may have previously been undervalued. In addition, *L. humile* management is critical to achieving optimal biological control of populations of *D. citri* and other hemipteran pest populations. Finally, excellent *L. humile* suppression can be achieved with both a traditional liquid-bait-and-dispenser program or applications of bait-loaded alginate hydrogels. The work presented herein provides foundational information on the relationships among *L. humile*, *D. citri*, other ant-associated hemipteran species, and biological control agents. Application of these results could greatly improve the existing integrated pest management framework for control of these pests in California citrus.

Table of Contents

Chapter 1: Introduction.....1

References.....14

Chapter 2: The Influence of Temperature Variation on Life History Parameters and Thermal Performance Curves of *Tamarixia radiata* (Hymenoptera: Eulophidae), a Parasitoid of the Asian Citrus Psyllid (Hemiptera: Liviidae).....22

Abstract.....22

Introduction.....23

Materials and Methods.....27

Results.....35

Discussion.....40

References.....51

Figures and Tables.....61

Chapter 3: A low-toxicity baiting program precipitates collapse of Argentine ant and ant-associated hemipteran pest populations in commercial citrus.....74

Abstract.....74

Introdction.....75

Materials and Methods.....82

Results.....90

Discussion.....100

References.....115

Figures.....124

Chapter 4: A biodegradable alginate hydrogel bait delivery system effectively controls high-density populations of Argentine ant in commercial citrus……........143

Abstract......143

Introduction......144

Materials and Methods......151

Results......157

Discussion......159

References......168

Figures and Tables......173

Chapter 5: Conclusion......178

References......168

List of Figures

Figure 2.1 Relationship between temperature (^{o}C; x-axis) and development rate (days^{-1}; y-axis) of *Tamarixia radiata* as described by eight thermal performance functions. Solid lines represent model predictions for constant temperatures and dashed lines for fluctuating temperatures. Experimentally measured values are represented by circles (constant) and triangles (fluctuating). The 35^{o}C data point in both the constant and fluctuating dataset has been omitted from the linear model due to outlier status. The 12^{o}C data point (fluctuating) has been omitted from both the linear and nonlinear models to facilitate direct comparison between constant and fluctuating datasets…………………67

Figure 2.2 Average longevity (days) of adult *Tamarixia radiata* maintained under various constant (circle) and fluctuating (triangle) average daily temperatures (^{o}C)……69

Figure 2.3 Comparison of parasitism rates of *Diaphorina citri* by *Tamarixia radiata* in fluctuating regime cohorts across average daily temperatures (^{o}C). Means followed by the same letter are not significantly different at α = 0.05……………………………………70

Figure 2.4 Developmental mortality of *Tamarixia radiata* in fluctuating regime cohorts compared across average daily temperatures (^{o}C). Means followed by the same letter are not significantly different at α = 0.05…………………...………………………………71

Figure 2.5 Average total mortality rate (A) and average mortality rate excluding parasitism (B) of *Diaphorina citri* nymphs reared under fluctuating temperature regimes for control cohorts (white) and *Tamarixia radiata*-exposed cohorts (black). Grey bars represent total mortality in *T. radiata*-exposed cohorts corrected for baseline mortality with the Schneider-Orelli formula. Asterisks indicate a significant difference in *D. citri* mortality between control and parasitoid-exposed cohorts at α = 0.05…….………72

Figure 3.1 Mean visual estimates (± SEM) of *Linepithema humile* activity in treated and control trees across the 18-month experimental duration. The dashed line separates pre- and post- treatment sampling dates. Mean differences between treated and untreated trees across all analyzed months (Feb 2017 – Jan 2018) were statistically significant at α = 0.05 (estimated marginal means)……………………………………………………..……130

Figure 3.2 Relationship between *Linepithema humile* and four hemipteran pest species (i.e., *Aonidiella aurantii*, *Coccus hesperidum, Diaphorina citri,* and *Planococcus citri*) infesting twigs, flush, and fruit in control trees. The proportion of pest colony species tended by *L. humile* (A), absolute attendance (ants per colony; B) and relative attendance (ants per individual per colony; C) are shown. Means (± SEM) labeled with the same lowercase letter are not significantly different at α = 0.05 (estimated marginal means).132

Figure 3.3 Mean (± SEM) cumulative infestation of combined niches in insect days (A) and colony size of hemipteran pests in insect days (B) in ant-treated and control trees across the final year of study (Feb 2017 – Jan 2018). Mean differences between treated and untreated trees across all analyzed months were statistically significant at α = 0.05 (estimated marginal means)…………………………………………………………133

Figure 3.4 Mean (± SEM) pooled hemipteran pest infestation rate of twigs, flush, and fruit prior to (Aug 2016) and following (Aug 2017) liquid bait deployment in treated and control trees. Asterisks represent statistically significant differences between pre- and post-baseline estimates between treated and control trees and "NS" represents non-significant differences at α = 0.05 (estimated marginal means)……………………….…134

Figure 3.5 Mean (± SEM) infestation of twigs (A), flush (B), and fruit (C) by hemipteran pest species pooled (i.e., *Aonidiella aurantii*, *Coccus hesperidum*, *Diaphorina citri*, and *Planococcus citri*) in ant-treated and control trees across sampling date. The dashed line separates pre- and post- treatment sampling dates. Asterisks represent statistically significant differences between treated and control trees in concurrent sampling months and "NS" represents non-significant differences at α = 0.05 (estimated marginal means). "N/A" denotes that infestation rates could not be compared across treatment due to the absence of variance in one or both groups…………………………………………...136

Figure 3.6 Mean (± SEM) infestation of niches by individual hemipteran pest species (i.e., *Aonidiella aurantii*, *Coccus hesperidum*, *Diaphorina citri*, and *Planococcus citri*) in ant treated and control trees across sampling date. The dashed line separates pre- and post- treatment sampling dates. Asterisks represent statistically significant differences between treated and control trees in concurrent sampling months and "NS" represents non-significant differences at α = 0.05 (estimated marginal means). "N/A" denotes that infestation rates could not be compared across treatment due to the absence of variance in one or both groups……………………………………………………………...……139

Figure 3.7 Mean proportion (± SEM) of natural enemies (NEs) present in hemipteran pest species within twigs, fruit, and flush. Natural enemies are pooled (A) or separated by morphotaxa group (B). Means labeled with the same lowercase letter are not significantly different at α = 0.05 (estimated marginal means)……………………………………141

Figure 3.8 Association between syrphid prevalence in pest colonies (i.e., proportion of pest colonies with syrphids present) and densities (i.e., colony size) of *Diaphorina citri* (A) and *Planococcus citri* (B) over the full 18-month sampling period………………142

Figure 4.1 Mean visual (A) and 24-hour baited vial (B) estimates (± SEM) of foraging Argentine ants in hydrogel treated and control trees averaged across all sites. The dashed line separates pre- and post-baseline samplings…………………………..…………176

Figure 4.2 Mean cumulative insect days (± SEM) across all sampling dates (A) and mean total cumulative insect days averaged across all sampling dates (B)…………………..177

List of Tables

Table 2.1. Fluctuating temperature profiles utilized for rearing *Tamarixia radiata*…….61

Table 2.2: Mean development times and adult longevity (days ± SE) of *Tamarixia radiata* under constant and fluctuating temperature regimes. For development time and longevity, means followed by the same letter within the same temperature are not significantly different α = 0.05 (estimated marginal means), *n* denotes number of individuals……………………………………………………………………………..62

Table 2.3: Mathematical models and parameter estimates for eight performance functions describing the relationship between development rate (D_r) and temperature for *T. radiata* reared under constant and fluctuating temperature regimes. See corresponding references for full description of models and parameters…………………………….………….…64

Table 2.4: Comparison of development times, degree-days, theoretical minimum developmental thresholds, and R-squared values for *Tamarixia radiata* populations from China, Brazil, Réunion Island, Taiwan, and California. [a]Outliers excluded from *K*, *T*min and *R*2 calculations. [b]Development times provided for female *T. radiata* only. [c]Values averaged across all citrus varieties investigated in the study. [d]Values estimated from figures……………………………………………………………………………...65

Table 2.5: Comparison of goodness-of-fit, percent deviation, and theoretical developmental thresholds among eight thermal performance functions fitted to the development rate of *Tamarixia radiata* under constant and fluctuating temperature regimes. [a]Beta model achieved convergence at absolute zero…………………………66

Table 3.1 Results of generalized linear mixed models evaluating the effects of treatment and environmental factors on the infestation rate (%) of twigs, flush, and fruit by various hemipteran pest species. Model selection was based on comparison of AICc scores through likelihood ratio tests and the function "*dredge*" in the R package MuMln. Only variables retained in the final model are shown below…………………………………125

Table 3.2 Rate of infestation of niches by various hemipteran pest species in treated and control trees. Parentheses delineate the proportion of total infestation for each pest species within treatment categories. Within each column (control vs. treatment), means (± SEM) followed by the same lowercase letter are not significantly different at α = 0.05 (estimated marginal means)…………………………………………………….……126

Table 3.3 Comparison of mean colony size (number of individuals/colony) and percent parasitism (number of parasitized individuals/[parasitized + susceptible individuals]) of hemipteran pest colonies collected from treated and control trees (Feb 2017 – Jan 2018). Within each column (control vs. treatment), means (± SEM) followed by the same lowercase letter are not significantly different at α = 0.05 (estimated marginal means).128

Table 4.1 Comparison of active ingredient concentrations, product application rates, and treatment costs for various Argentine ant control strategies used in California commercial citrus production……..…………………………………………………………....174

Table 4.2 Comparison of mean Argentine ant activities (± SEM) as estimated visually and with baited vials on hydrogel treated and control citrus trees across all sites following applications………………………………………………………………………..175

Chapter 1: Introduction

The invasive Asian citrus psyllid, *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae) and its vectored disease pose the largest biotic threat to world citriculture (Halbert and Manjunath 2004; Bové 2006; Chen and Stansly 2014). *Diaphorina citri* is the primary vector of *Candidatus* Liberibacter asiaticus (*C*Las), one of the gram-negative alpha-proteobacteria responsible for huanglongbing (HLB; citrus greening). HLB is a lethal, incurable phloem-limited disease of all commercial citrus cultivars and many related species in the family Rutaceae (Bové 2006). The *D. citri*-*C*Las-HLB complex has spread to most citrus-growing regions worldwide, resulting in losses in productive acreage, reduced fruit quality and quantity, and increased nutritional and pest management costs (Gottwald 2010; Khan et al. 2014). The current distribution of HLB forms (i.e., caused by the Liberbacters "asiaticus", "africanus", and "americanus") spans more than 40 countries located primarily within Asia, Africa, and the Americas (Bové 2006; Gottwald 2010). In the United States, *D. citri* is present in ten states (i.e., Alabama, Arizona, California, Florida, Georgia, Hawaii, Louisiana, Mississippi, South Carolina, and Texas) and *C*Las-HLB in seven (i.e., Alabama [2017], Louisiana [2008], South Carolina [2009], Georgia [2009], Texas [2011], and California [2012] and Florida [2005]) (Jepson 2008; USDA 2010; Grafton-Cardwell et al. 2013). HLB management programs typically include monitoring for *C*Las using PCR-based detection methods, quarantine and rouging of trees in areas where *C*Las has been detected (and sometimes replanting with nursery stock produced in insect-proof screen houses), and vector (*D. citri*) management with biological and chemical controls. In Florida where the disease is widespread, growers are additionally attempting to extend the lifespan of infected trees with nutritional programs (Coletta-Filho et al. 2014).

HLB transmission and symptoms. *C*Las is acquired from and transmitted to trees by *D. citri* adults and nymphs through feeding. Following pathogen intake, bacteria enter the insect's alimentary canal and replicate within the salivary glands. During feeding, *C*Las migrates from the salivary glands through the piercing-sucking mouthparts and into the vascular tissue of the plant (Ammar et al. 2011). The rate of *C*Las acquisition generally increases with *C*Las titer in the tree, although some *D. citri* may acquire bacteria from newly infected hosts (Coletta-Filho et al. 2014). Despite highly variable transmission rates of *C*Las by *D. citri* (Inoue et al. 2009; Pelz-Stelinski et al. 2010; Coletta-Filho et al. 2014), field observations suggest *C*Las spreads rapidly through *D. citri* populations and citrus orchards (Hall et al. 2013). This may be due, in part, to the high invasion potential of *D. citri* (i.e., small size, excellent dispersal capability, and high reproductive output) and the positive taxis exhibited by *D. citri* to infected trees (as a result of canopy discoloration [yellowing] and increased plant-defense volatile release) (Mann et al. 2012; Khan et al. 2014; Lewis-Rosenblum et al. 2015; Wu et al. 2015). In addition, *C*Las-infected *D. citri* develop more quickly, have higher fecundity, and tend to disperse more frequently and across longer distances than uninfected conspecifics (Inoue et al. 2009; Martini et al. 2015). These factors facilitate spread of *D. citri*-*C*Las and expression of HLB in orchards.

Following acquisition of *C*Las by trees, bacteria proliferate both locally and within the root system, triggering rapid root turnover and associated early disease symptoms: reduced plant vigor, stunted or slowed growth, foliar loss, and chlorosis of the leaves (Gottwald 2010). The systemic spread of infection increasingly impairs nutrient transport in the phloem, often resulting in deficiencies. Under heavy bacterial titers, extensive root, limb, and foliar dieback compromises tree productivity, manifested as premature fruit abortion (30-100%) and growth of irregularly ripened, deformed, bitter fruit unsuitable for sale, especially in California's fresh fruit market (Gottwald 2010). Consequently, orchards may become unprofitable in as little as two years after initial *C*Las detection, with tree death generally occurring within six to ten years (Roistacher 1996; Bové 2006; Bassanezi and Bassanezi 2008; Gottwald 2010). Although symptoms may not manifest visibly for multiple years, plants can be active sources of inoculum for disease spread throughout this time (Gottwald 2010; Coletta-Filho et al. 2014). This asymptomatic period in combination with variability in the rate of infected tree decline and discontinuous bacterial distribution and titer impedes *C*Las detection (Gottwald 2010). Furthermore, growers may be hesitant to remove infected but still productive trees due to immediate economic losses and lack of education on the risks associated with maintaining diseased trees in orchards (Spann et al. 2011; Hall et al. 2013). Disease progression in citrus groves has an increasingly large impact on spread of infection to nearby trees, reductions in yield, and proportion of marketable product, which collectively have important implications for management strategies.

***Diaphorina citri*-*C*Las in California.** The California citrus industry generates $7.1 billion annually (i.e., $3.4 billion from sale of fresh fruit and $3.7 billion from industries and households supported by citrus) (Babcock 2018). California is the leading producer of fresh fruit and fruit for export (i.e., >85% of whole oranges and tangerines and >90% of whole lemons in the United States), markets with low tolerance for the malformed or astringent fruit produced from *C*Las-infected trees (Bennet 2016; Babcock 2018; USDA 2018). In California, *D. citri* was initially detected in Imperial and San Diego Counties in 2008 (Grafton-Cardwell 2010). Following early detections, the California Department of Food and Agriculture (CDFA) initiated an expensive, insecticide-based (i.e., imidacloprid soil drenches and cyfluthrin foliar sprays) *D. citri* eradication program in urban southern California (Hoddle 2012). Despite considerable management efforts, *D. citri* rapidly spread throughout southern California and subsequently into the central and northern coast, the Sacramento valley, and the San Joaquin Valley, where over 70% of citrus is produced (USDA 2008). Despite the establishment of *D. citri* across southern California, *C*Las has been restricted to residential areas (i.e., >1,100 cases located in Riverside, Los Angeles, Orange, and San Bernardino Counties as of March 2019) (Hoddle and Pandey 2014; Milosavljević et al. 2018; Milosavljević and Hoddle 2019). Urban infestation foci serve as reservoirs for infected trees and psyllids, which can disperse to commercial groves and expedite the establishment of *C*Las and HLB, despite best-efforts of growers maintaining rigorous control programs (Bayles et al. 2017; Gottwald 2010).

Biological control of *D. citri*. Biological control is a critical component of the comprehensive, long-term management strategy for *D. citri* in California. Higher densities of *C*Las infected psyllids and longer feeding periods increase bacterial titers and the number of infestation foci, which in turn increases the rate of HLB symptom onset and decline in tree productivity (Gottwald 2010; Pelz-Stelinski et al. 2010). Consequently, continuous *D. citri* population reduction is critical to minimizing the spread of *C*Las and expression of HLB.

Reductions in vector populations through biological control can increase the efficacy and sustainability of other control strategies (e.g., insecticide programs) by reducing the number of pests that need to be managed. In addition, integrated pest management programs with a biocontrol component can delay the development of insecticide resistance in pest populations and minimize outbreaks of secondary pest populations (Tiwari et al. 2011). In residential areas of California, there are an estimated 1.23 million residences with citrus in Los Angeles County alone and the cost of sustained chemical management is exorbitant (Hoddle 2012). The CDFA's insecticide-based *D. citri* management program cost $158 per residence and total control costs would have totaled nearly $200 million dollars if it had been completed for every targeted residence (Hoddle 2012; Hoddle and Pandey 2014). Because continuous chemical management of *D. citri* is cost-prohibitive in residential areas, a program that incorporates biological control is critical for achieving effective, sustainable control of vector populations (Qureshi and Stansly 2007; Hoddle and Pandey 2014).

The classical biological control program for *D. citri* has focused on establishment of *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) sourced from Punjab, Pakistan (Hoddle and Pandey 2014). *Tamarixia radiata* is a host-specific, idiobiont ectoparasitoid capable of inflicting substantial *D. citri* mortality through parasitism of nymphs (third through fifth instar) and host feeding (Chien et al. 1991; Chen and Stansly

2014; Hoddle and Pandey 2014; Kistner et al. 2016; Milosavljević et al. 2017). The CDFA has released over twelve million *T. radiata* at more than 1,000 sites covering 4,000 square miles of urban southern California (David Morgan CDFA, personal communication 19 November 2018), resulting in widespread establishment (Hoddle et al. 2016) and significant impacts on *D. citri* populations (Kistner et al. 2016). The observed seasonal and regional variation in efficacy of *T. radiata* in California may be partially mediated by temperature, as it is known to strongly affect parasitoid development, longevity, reproductive output, and mortality inflicted on hosts (McFarland and Hoy 2001; Chen and Stansly 2014; Kistner et al. 2016). Estimates of *D. citri* parasitism in the field likely underestimates the biocontrol efficacy of *T. radiata* as host-feeding is known to be a significant mortality factor for *D. citri* reared under constant temperature regimes in laboratory settings (Chien et al. 1991, 1994; Yang and Huang 1991). A better understanding of the impact of realistic daily thermal cycles on the life history (including host-killing capacity) of *T. radiata* is key to accurately assessing the potential of this parasitoid in the field. This topic is investigated in chapter 2. Findings from fluctuating temperature studies may better inform methods for *T. radiata* mass rearing, storage, and release and provide realistic expectations for performance in the field (Chen and Stansly 2014), ultimately improving our ability to effectively utilize *T. radiata* for suppressing populations of *D. citri*.

Efficacy of *T. radiata*. Several reasons have been proposed for the seasonally and regionally variable rates of parasitism reported for *T. radiata*: intensive broad-spectrum insecticide use (Hall and Nguyen 2010), low genetic variability of released parasitoids

(Roush et al. 1990; Barr et al. 2009; Chen and Stansly 2014), and intraguild predation by generalist predators, and mutualisms with ants (Michaud 2004; Qureshi and Stansly 2009; Navarette et al. 2013). Commonly used insecticide sprays and residues are known to cause 80 – 100% mortality of *T. radiata* 24 – 72 hours following exposure (Hall and Nguyen 2010). However, widespread *T. radiata* mortality from insecticide use is unlikely in California, where parasitoid releases have been focused primarily in chemically unmanaged urban areas. In addition, the population of *T. radiata* released and established in southern California is genetically diverse, as it was seeded by thousands of Pakistan-collected individuals accumulated over multiple collecting trips. Furthermore, in the California mass rearing program, 17 female isocage lines are maintained and regularly reintroduced into breeding stock in order to limit the effects of genetic drift (Hoddle et al. 2014). Little is known about intraguild interactions for *D. citri* in California, but they are suspected to occur (Tena et al. 2013; Kistner and Hoddle 2016). In Florida, generalist predators such as coccinellids have been reported to cause high mortality of *D. citri* parasitized by *T. radiata* and are thought to be a key contributing factor to low parasitism estimates in the field (Michaud 2004; Qureshi and Stansly 2009). Ant disruption of biological control of hemipteran pests in agricultural settings is well-documented (Delabie 2001; Ness and Bronstein 2004; Styrsky and Eubanks 2007; Helms 2013; Yao 2014). The positive, negative, or neutral impact of ant activity on a biological control program relies upon a complex mix of factors such access to external sugar resources, ant tending intensity, ant aggression level, and the presence or absence of ant-mitigation adaptations in natural enemies (e.g., short searching and oviposition time, avoidance behavior patterns, or visual or chemical camouflage, etc.) (Völkl 1997; Yoo and Holway 2011; Helms 2013; Sime and Daane 2014). A superior understanding of the interactions between ants, *D. citri* and ant-associated hemipteran pests, and natural enemies such as *T. radiata* may better inform biological control programs which target or utilize these species. This was the topic of Chapter 3.

Implications of *L. humile–D. citri* mutualisms for biological control. Complex, multi-species interactions involving ants are likely a major contributing factor to the low estimates of *D. citri* parasitism observed in the field. A key player in southern California citrus is the Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), a highly invasive and widely distributed tramp ant species which holds notoriety as a major pest of urban, agricultural, and natural systems. Originating from the Paraná River drainage and surrounding regions of Argentina, Uruguay, Paraguay, and Brazil, the global distribution of *L. humile* has, over the last two decades, spread to six continents, including 15 countries and several oceanic islands (Vega and Rust 2001; Silverman and Brightwell 2008). It flourishes in disturbed natural and managed environments, particularly those that are at low altitude, are irrigated or have water runoff year-round, and have warm, mild winters (Cook 1953; Holway et al. 2002; Silverman and Brightwell 2008). *Linepithema humile* is well-established throughout costal and southern California as a massive cooperative supercolony (i.e., conspecifics have low-to-no intraspecific aggression due to highly related cuticular hydrocarbon profiles [Tsutsui et al. 2000]). This supercolony is part of a larger global megacolony which is estimated to expand over

11,000 km^2 and contain hundreds of trillions of individuals (Horton 1918; Vogel et al. 2009; Van Wilgenburg et al. 2010).

Although *L. humile* is commonly branded as a nuisance household invader, its damage extends well beyond the urban landscape (Vega and Rust 2001; Silverman and Brightwell 2008). *Linepithema humile* is an efficient invader and fierce competitor that is consistently better at exploiting resources than native ant species. *Linepithema humile* workers are quick to locate food resources, rapidly recruit high numbers of nestmates, effectively defend resources, and forage for long periods of time (Holway 1999). The monopolization of shared food resources and numerical dominance in combination with cooperative fighting strategies (Buczkowsi and Bennett 2008), potent chemical defenses, and aggressive behavior (Zee and Holway 2006) allows *L. humile* to outcompete and displace other ant species in as little as several hours after arrival (De Kock 1990). Consequently, *L. humile* invasion in natural and managed areas is often characterized by a reduction in the abundance and diversity of other arthropods (particularly native ant species), fundamentally altering community structure and function and disrupting critical ecosystem services (e.g., pollination, seed dispersal, biological control, etc.) (Bond and Slingsby 1984; Ward 1987; Cole et al. 1992; Human and Gordon 1997; Folgarait 1998; Styrsky and Eubanks 2007; Lach 2007; Helms 2013; Hanna et al. 2015; LeVan and Holway 2015).

Central to the invasional success of *L. humile* (and subsequent displacement of native ant and other arthropod species) is its ability to secure high-energy carbohydrate resources in the form of sugary honeydew (Human and Gordon 1996; Holway 1999;

Helms 2013). *Linepithema humile* readily form mutualisms with a wide variety of sap-feeding, honeydew-producing hemipteran pest species in the citrus agroecosystem. Workers aggressively defend their partners from natural enemies, often considerably reducing the efficacy of biological control agents (Vega and Rust 2001; Silverman and Brightwell 2008; Powell and Silverman 2010; Helms 2013). In addition to protection from natural enemies, *L. humile* provide hemipteran mutualists "sanitation services" (honeydew removal limits the growth of pathogenic fungi and honeydew drowning) (Gullan 1997), actively disperse pests to new areas (Flanders 1951; Way 1963), and can increase pest development rate, fecundity, and longevity by stimulating an increased rate of phloem ingestion and assimilation (Yoo and Holway 2011). Consequently, ants may cause population explosions of tended hemipteran mutualists, providing an ever-increasing supply of honeydew and creating positive feedback cycles which allow *L. humile* and tended hemipteran pests to invade new areas together (Davidson 1998; Silverman and Brightwell 2008; Rowles and Silverman 2009; Helms 2013). Increased densities of hemipteran pests in agricultural systems can result in the spread of vectored plant diseases and direct feeding damage (e.g., leaf curling, foliar dieback, stunted growth, etc.), potentially resulting in economically significant losses in yield or acreage (Godfrey et al. 2002; Styrsky and Eubanks 2007). Consequently, ant control is may be considered a critical constituent of integrated pest management programs targeting ant-tended hemipteran pests.

Although the impact of ant-hemipteran pest interactions on biocontrol and population growth is well-documented in the literature, most studies focus on ant interactions with aphids, mealybugs, and membracids (Delabie and Fernandez 2003), while mutualisms with psyllids (and *D. citri* in particular) remain poorly studied by comparison. However, a few studies have investigated the relationship between ants and *D. citri*. Navarette et al. (2013) reported that exclusion of invasive ant species from citrus canopies led to increased parasitism of *D. citri* by *T. radiata* in Florida. A survey of urban citrus gardens in southern California revealed that ~60% of *D. citri* colonies are tended by *L. humile*, with the rate of attendance and parasitism negatively associated with the density of neighboring hemipteran competitors (Tena et al. 2013). There is often heavy competition among hemipteran species for ant attendance in the field. Some species of honeydew-producing hemipterans increase the quantity of honeydew produced or shift honeydew sugar composition to promote ant attendance (McGlynn 1999; Fischer and Shingleton 2001; Lester and Tavite 2004; Abbott 2005; Wetterer 2005; Strysky and Eubanks 2007; Green et al. 2011). Ants favor tending hemipteran colonies that are spatiotemporally stable and produce copious quantities of high-quality honeydew. Consequently, the intensity of attendance is typically a direct reflection of mutualist attractiveness (Fischer et al. 2001; Helms 2013). Considering the competitive interactions among *D. citri* and other honeydew-producing hemipteran pests present in citrus may provide an explanation for differences in the response of hemipteran species to ant control (Chapter 3).

That *L. humile* disrupts biocontrol and induces population increases of citrus-infesting, sap-feeding hemipteran pests is a well-accepted paradigm. However, comparatively fewer studies have examined the impact of *L. humile* control on infestation of citrus by multiple hemipteran pest species simultaneously (DeBach 1951; Moreno et al. 1987; Yoo et al. 2013; Calabuig et al. 2014), and none to date have examined the impact on populations of *D. citri*. In addition, all of these studies utilized sticky or insecticide barriers to exclude ants from honeydew-producing hemipterans in tree canopies. However, barriers are ineffective treatments for control of *L. humile* in realistic commercial scenarios (McCalla et al. in press). Treatments that create a zone of ant suppression which extends beyond the citrus canopy may provide a superior refuge for natural enemies, potentially leading to improved biological control. Thus, chapter 3 investigated the efficacy of a low-toxicity liquid baiting program for biocontrol-mediated suppression of *L. humile*, *D. citri*, and other honeydew-producing hemipteran pests.

To reap the biocontrol benefits of ant control reported in chapter 3, targeted, efficacious, low labor input treatment options for management of *L. humile* in citrus are needed. Commercially available treatments are limited to bait-and-dispenser systems, which are effective but expensive, and barrier sprays of broad-spectrum insecticide, which have poor efficacy and often cause mortality of the natural enemies needed to provide biocontrol of hemipteran pests (McCalla et al. in press). In addition, use of chlorpyrifos, the only registered toxicant for use in the barrier-spray format in California was banned by the state in early 2019 and use will be phased out by 2021 (California Department of Pesticide Regulation 2019 a,b). Novel ant control treatments are greatly needed. Chapter 4 investigates the efficacy of a broadcastable, biodegradable alginate hydrogel bait transportation system for suppressing *L. humile* populations in commercial citrus. Controlling infestations of sugar-feeding ants in vulnerable cropping systems (e.g., citrus, wine grapes, nuts) may help minimize economic losses sustained through ant damage and outbreaks of hemipteran pest mutualists such as *D. citri*.

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Chapter 2: The Influence of Temperature Variation on Life History Parameters and Thermal Performance Curves of *Tamarixia radiata* (Hymenoptera: Eulophidae), a Parasitoid of the Asian Citrus Psyllid (Hemiptera: Liviidae)

Abstract

This study examined the effects of seven constant and fluctuating temperature profiles with corresponding averages of 12 to 38°C on the life history of the Punjab, Pakistan-sourced *Tamarixia radiata* (Waterston) released in California for biological control of *Diaphorina citri* Kuwayama. One linear and seven nonlinear regression functions were fit to egg-to-adult development rate data to characterize thermal performance curves. Temperature fluctuations significantly affected both development and longevity of *T. radiata*. Estimates of degree-days predicted by the linear model were 30% higher for the fluctuating regime than the constant regime. Nonlinear model estimations of theoretical minimum and maximum developmental thresholds were lower for the fluctuating regime when compared to the constant regime. These predictions align with experimental observations. Parasitoids reared under fluctuating profiles at low average temperatures developed faster (15°C) and survived longer (15 – 20°C) when compared to those reared under constant regimes with corresponding means. In contrast, high average fluctuating temperatures produced parasitoids with an extended developmental period (35°C) and reduced longevity (30 – 35°C). A meta-analysis of published *T. radiata* development datasets, together with the results of this study, indicated convergence in degree-days and theoretical minimum developmental thresholds among geographically distinct parasitoid populations. These findings demonstrate the significant effects of temperature on *T. radiata* life history and have important implications for optimization of mass-rearing and

release efforts, improvement of predictions from climate modeling, and comparison of *T. radiata* population performance across climatic gradients and geographic regions.

Introduction

Climate is the primary driver of many important biological and ecological processes in insects (Atkinson 1994; Damos and Savopoulou-Soultani 2012). Among abiotic factors, temperature exerts the strongest effects (Taylor 1981; Hallman and Denlinger 1998), influencing biochemical reactions, physiology, behavior (Denlinger and Yocum 1998; Colinet et al. 2015), phenology and population dynamics (Porter et al. 1991; Kang et al. 2009; Nelson et al. 2013), and the structure and functioning of communities and ecosystems (Nooten et al. 2014; Bjorkman and Niemela 2015). Many of these temperature-influenced phenomena, such as life history and phenology, play a fundamental role in the management of insect pests by informing the timing and implementation of control actions (Horn 1998; Roy et al. 2002; Terblanche et al. 2015).

The majority of empirical mathematical functions used to model insect development rates are temperature-driven (Damos and Savopoulou-Soultani 2012; Shi et al. 2015; Mirhosseini et al. 2017; Quinn 2017). The most widely utilized models are linear (Quinn 2017), which provide estimates of degree-days (i.e., accumulation of thermal energy necessary for completion of development in a specific life stage) and, through extrapolation of the regression line, the theoretical minimum temperature threshold below which development cannot occur (Campbell et al. 1974; Damos and Savopoulou-Soultani 2012). However, these simple linear models cannot fit the curvilinear portion of responses typically observed at the higher end of thermal clines (Davidson 1944; Colinet et al. 2015; Mirhosseini et al. 2017). Nonlinear models more accurately describe development rate across broader thermal gradients, allow estimation of the optimum developmental temperature and theoretical minimum and maximum developmental thresholds, and often incorporate biologically meaningful parameters (Shi et al. 2015; Mirhosseini et al. 2017; Ratkowsky and Reddy 2017). In conjunction with other life history data, thermal performance curves are highly relevant for pest management as they are used to calibrate climate-based software (e.g., CLIMEX) which forecast the growth, geographic distribution, spread, synchrony, and stability of pest and natural enemy populations across varying spatiotemporal scales (Roitberg et al. 2001; Regniere et al. 2012). These predictions may help improve biological control programs by facilitating the selection and utilization of climatically adapted biocontrol agents, optimizing release strategies (e.g., timing releases when targets are concentrated in vulnerable life stages), and promoting synergy among natural enemies (such as through geographic partitioning) (Horn 1998; Hart et al. 2002; Roy et al. 2002; Damos and Savopoulou-Soultani 2012; Terblanche et al. 2015). Further, temperature-driven developmental biology data can assist with the development of protocols for optimized mass-rearing, storage, release, and monitoring of biocontrol agents, potentially reducing costs and improving product quality (Leopold 1998; Mirhosseini et al. 2017).

Biological control has played an important role in management programs for the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), vector of the phloem-limited bacterium *Candidatus* Liberibacter asiaticus (*C*Las) which causes the lethal citrus disease huanglongbing (HLB; Bove 2006; Gottwald 2010; Grafton-Cardwell et al. 2013). Most major citrus-growing regions have been invaded by the *D. citri*-*C*Las pest complex, resulting in greatly increased pest management costs, yield quantity and quality reductions, and loss of productive acreage (Halbert and Manjunath 2004; Yang et al. 2006; Bassanezi et al. 2011; Durborow 2012; Grafton-Cardwell et al. 2013; Hall et al. 2013; Khan et al. 2014; Spreen et al. 2014). California's citrus industry is valued at $7.1 billion annually, generating $3.4 billion in sales of fresh fruit and an additional $3.7 billion through dependent industries and induced economic activities (i.e., income spent by households supported by the citrus industry) (Babcock 2018). As the leading producer of fresh-market citrus in the USA (Babcock 2018; USDA 2018), the profitability of California's citrus industry is particularly susceptible to *C*Las-associated economic losses. Although *D. citri* is widely established in southern California, *D. citri*-*C*Las is currently restricted to residential areas (Hoddle and Pandey 2014; Milosavljević et al. 2018; Milosavljević and Hoddle 2019). Spillover of *C*Las-infected *D. citri* from urban infestation foci into major growing regions could expedite establishment of *C*Las and HLB expression in commercial production areas (Bayles et al. 2017).

Because sustained chemical management of *D. citri* infestations in California's vast urban citrus landscape is cost-prohibitive, biological control is the primary means of suppressing *D. citri* populations and delaying *C*Las spread to adjacent production zones (Hoddle and Pandey 2014; Milosavljević et al. 2017). The classical biocontrol program for *D. citri* has centered on establishment of *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) sourced from Punjab, Pakistan (Hoddle and Pandey 2014).

Tamarixia radiata is a host-specific, idiobiont ectoparasitoid capable of inflicting substantial *D. citri* mortality through parasitism of nymphs (third through fifth instar) and host feeding (Chien et al. 1991; Chen and Stansly 2014; Hoddle and Pandey 2014; Kistner et al. 2016; Milosavljević et al. 2017). The California Department of Food and Agriculture (CDFA) has released over twelve million *T. radiata* at more than 1,000 sites covering 4,000 square miles of urban southern California (David Morgan CDFA, personal communication 19 November 2018), resulting in widespread establishment (Hoddle et al. 2016) and significant impacts on *D. citri* populations (Kistner et al. 2016). The observed seasonal and regional variation in efficacy of *T. radiata* in California may be partially mediated by temperature, as it is known to strongly affect host and parasitoid development, longevity, and fecundity (McFarland and Hoy 2001; Chen and Stansly 2014; Kistner et al. 2016).

Temperature-driven development rate models for *T. radiata* have previously been constructed from data collected under constant temperatures (Quilici and Fauvergue 1990; Chien et al. 1993; Gomez-Torres et al. 2012, 2014; Li et al. 2018). However, under natural field conditions, ambient temperatures follow a thermal cycle that exposes insects to temperatures above and below daily temperature means. The asymmetric nature of thermal performance curves means the warming phase of a cycle has a stronger influence on the physiological and biochemical responses of insects (e.g., metabolism) than the cooling phase (Jensen 1906; Colinet et al. 2015). Because constant temperature profiles fail to account for such thermal complexities independent of average conditions, they may produce unrealistic estimates of biological performance (Ruel and Ayres 1999;

Lyons et al. 2013; Warren and Anderson 2013; Spanoudis et al. 2015; Wu et al. 2015). Thus, laboratory studies examining the development rate of *T. radiata* across a range of temperatures should account for the effects of thermal variance to more accurately model conditions experienced in the field (Colinet et al. 2015).

Understanding the effects of temperature on *T. radiata* life history could facilitate improvements in mass-rearing methods, inform the timing of inoculative and augmentative parasitoid releases, assist prediction of parasitoid establishment, spread, and efficacy across climatic gradients in citrus-growing regions, and enable comparisons among parasitoid populations sourced from different geographic areas for biological control programs (e.g., Pakistan vs. China). Thus, this study investigated the effects of constant and fluctuating temperatures (12 to 38°C) on the developmental biology (as modeled by eight thermal performance functions) and host-killing capacity of *T. radiata* sourced from Punjab, Pakistan. In addition, a meta-analysis of published work (10 studies) examining the effects of constant temperature on geographically distinct *T. radiata* populations was conducted.

Methods and Materials

Source of experimental insects. *Tamarixia radiata* adults used in experimental trials were obtained from 17 Punjab, Pakistan-sourced bi-parental isocage lines maintained at the University of California Riverside Insectary and Quarantine Facility (UCR IQF) (Hoddle and Hoddle 2013; Hoddle and Pandey 2014). *Diaphorina citri* offered to *T. radiata* for colony propagation and experimental trials were obtained from southern

California-collected, *C*Las-free colonies maintained at UCR IQF (Hoddle and Pandey 2014; Bistline-East et al. 2015; Bistline-East and Hoddle 2015).

Stock colony production. *Diaphorina citri* colonies were maintained on 1- to 2-yr-old *Citrus volkameriana* Tenore and Pasquale (Sapindales: Rutaceae) that were pruned to produce the new foliar growth (i.e., flush) required for oviposition. Flushing plants were transferred from greenhouses maintained at 27 ± 1°C, 50 ± 20% RH, and a 14:10 (L:D) h photoperiod at UCR Agricultural Operations (UCR AgOps) to a climate-controlled rearing room in UCR IQF held at 27 ± 1°C, 40 ± 20% RH, and a 14:10 (L:D) h photoperiod. Individual plants in 0.5 l pots were placed in rectangular clear acrylic and fine mesh cages (15 × 15 × 30 cm WxDxH) (methods in Bistline-East et al. 2015). Caged *C. volkameriana* were inoculated with adult *D. citri*, and the resultant nymphs were allowed to develop to fourth and fifth instar (10 – 14 d post oviposition), stages preferred by *T. radiata* for parasitization (Hall et al. 2008). *Tamarixia radiata* that emerged from parasitized *D. citri* were transferred as male-female pairs to honey-provisioned 2-ml O-ring microcentrifuge vials (Micrewtube brand, Simport Scientific Inc., Vancouver, Canada) to ensure mating and held in cold storage cabinets maintained at 16 ± 1°C, 40 ± 20% RH, and a photoperiod of 14:10 (L:D) h until use in experimental trials.

Experimental plant preparation. *Diaphorina citri* nymphs exposed to female *T. radiata* for parasitization in experimental trials were hand-transferred from stock colonies to 6- to 12-mo-old *C. volkameriana* seedlings with suitable foliar growth for feeding. Seedlings were planted in 114-ml plastic growing cones (Ray Leach cone-tainers, SC7

Stubby, Stuewe and Sons Inc., Portland, OR) and maintained in UCR AgOps greenhouses with *D. citri* stock colony plants.

Experimental procedure. In a UCR IQF preparation laboratory maintained at 25 ± 1°C, 40 ± 20% RH, and a photoperiod of 14:10 (L:D) h, 20 *C. volkameriana* seedlings in cone-tainers each inoculated with 10 fifth instar *D. citri* were contained within clear, ventilated 148-ml vial "arenas" (Thornton Plastic Co., Salt Lake City, UT) (methods in Bistline-East et al. 2015). Enclosed cone-tainers were secured in plastic holding bins (4.92 liters; Greenbrier International Inc., Chesapeake, VA) and *D. citri* nymphs were allowed to settle on plants (4–6 h) prior to introduction of *T. radiata* into experimental arenas.

One mated female *T. radiata* was released from a honey-provisioned 2-ml O-ring microcentrifuge vial into each of the 10 arenas containing *D. citri*. Cone-tainers were then promptly transferred into environmental chambers (model I-30BLL; Percival Scientific Inc., Perry, IN) running one of fourteen constant or fluctuating experimental temperature profiles (see "Temperature Regimes" for further detail). *Tamarixia radiata* were allowed to oviposit for 6 h before removal. This procedure was repeated at least once for each temperature regime to provide sufficient *T. radiata* development data for model fitting and statistical analyses.

Across all temperature regimes, cone-tainers were inspected daily for *T. radiata* emergence to measure development rate. *Tamarixia radiata* were collected upon emergence, sexed, and transferred individually into labeled 2-ml microcentrifuge vials provisioned with honey. Adult *T. radiata* were maintained under their respective experimental temperature regimes and monitored daily for mortality to determine parasitoid longevity.

For fluctuating temperature regimes only, an additional 10 cone-tainers with *D. citri* only were paired with the 10 *T. radiata*-exposed cone-tainers. These control replicates measured baseline mortality rates for *D. citri* in the absence of *T. radiata* across all fluctuating temperature regimes. Daily monitoring of parasitoid-exposed and control cone-tainers included an assessment of *D. citri* nymph mortality, parasitism status, and emergence of adult *T. radiata* and *D. citri*. Daily monitoring of cone-tainers ceased following eclosion or death of all *D. citri*.

Temperature regimes. *Tamarixia radiata* life history parameters were examined across seven average temperatures, 12, 15, 20, 25, 30, 35, and 38 ± 0.5°C, 50 ± 20% RH, and a photoperiod of 14:10 (L:D) h that were held constant or fluctuated over time (**Table 2.1**). To produce fluctuating regimes, climate-controlled cabinets were programmed with ramping temperature increments oscillating over a 24-h cycle with a mean temperature equivalent to the constant counterpart. Incremental steps were based on the hourly temperature profiles of days with target average temperatures from 2 yr of field-recorded data (January 2013 to December 2015; Indio, CA #2 weather station [CIMIS 2018]). Detailed methods for fluctuating temperature profile selection can be found in Milosavljević et al. (2019). Target environmental conditions were verified by HOBO Pro V2 Temperature/RH loggers programmed to record at 15-min intervals (Onset Computer Corp., Bourne, MA).

Statistical methods. Following initial descriptive analyses, means comparisons were carried out using the statistical software package R (version 3.4.4, R Development Core Team [2018]). Generalized linear models (GLMs) were used to examine the main effect of average temperature, thermal variation (i.e., constant or fluctuating regime), gender, and their interactions on *T. radiata* development time (i.e., total number of days required to develop from egg to adult; Poisson distribution) and adult longevity (i.e., total number of days survived as adults; negative binomial distribution ["*glm.nb*" function in MASS package, R]). Because temperature held a nonlinear relationship with both development time and longevity, it was treated as a categorical rather than continuous variable. Although it is recognized that dichotomizing a continuous variable generally results in information loss, dividing a continuous variable up into ordered categories can be justified when the variable is highly nonnormally distributed or when its relationship to a response variable is highly nonlinear (Streiner 2002; Pasta 2009). The estimated marginal means function ("*emmeans*" in emmeans package, R) was used to compare development time and longevity between constant and fluctuating regimes across various temperatures (**Table 2.2**). All comparisons were made at the 0.05 level of significance.

For all fluctuating regime cohorts, mean total mortality of *D. citri* nymphs and mortality excluding parasitism were compared between treatments (i.e., *T. radiata*-exposed and control) and across temperatures using GLMs with a Poisson distribution. *Diaphorina citri* mortality estimates (in the *T. radiata*-exposure treatment) were additionally corrected for baseline *D. citri* mortality (control treatment) using the Schneider-Orelli formula (Schneider-Orelli 1947) following analysis. For fluctuating regime cohorts exposed to *T. radiata*, the effect of temperature on rate of *D. citri* parasitism and developmental mortality of parasitoids (i.e., death prior to emergence) were modeled using GLMs with a negative binomial distribution. All GLMs were followed by multiple comparison tests with emmeans at the 0.05 level of significance.

Mathematical models and evaluation. The performance of eight regression functions (i.e., ordinary linear, Lactin-2, Weibull, Brière-2, Beta, LRF, Ratkowsky, and Performance-2) for predicting development rate (D_r=1/D_t, where D_t is mean duration of egg-to-adult development in days) of *T. radiata* reared under constant and fluctuating thermal profiles (15 – 35°C; **Table 2.3**) was assessed. Models were evaluated on the basis of multiple criteria: model goodness-of-fit, deviation of predicted values from estimated values, estimates of theoretical developmental thresholds, and inclusion of biologically interpretable parameters. To facilitate direct comparison of models, temperatures at which development did not occur in one or both regimes (i.e., 12 and 38°C) were excluded from analyses.

Linear regression was used to calculate the theoretical minimum developmental threshold ($T_{min} = -a/b$, where a is the development rate when T = 0°C and b is the slope), thermal constant or degree-days necessary for completion of development (K= 1/b), and coefficient of determination, a measure of model goodness-of-fit ($R^2 = 1 - \frac{RSS}{TSS}$; where RSS and TSS are the residual and total sum of squares, respectively) (**Table 2.4**). To ensure accurate calculation of these measures, data were plotted in the statistical software SAS (version 9.4, SAS Institute Inc., Cary, NC) using PROC SGPLOT and fit with a linear regression using PROC REG. Temperatures at which development rate deviated from rectilinearity were omitted from analysis (Damos and Savopoulou-Soultani 2012; Milosavljević et al. 2019). Observations were considered outliers if the absolute value of their externally studentized residual (provided by the output term "*rstudent*") was greater than three.

Nonlinear regression models were computed using the PROC NLIN procedure in SAS for temperatures 15–35°C and graphed in Excel. Unless given explicitly as model parameters, theoretical thermal tolerance limits were estimated mathematically using graphic software (i.e., Excel and SAS) (Zahiri et al. 2010; Milosavljević et al. 2019). The optimum development point (T_{opt}) was estimated from the development curve peak (where D_r = max), while the theoretical minimum and maximum developmental thresholds (T_{min} and T_{max}) were measured at the sites of intersection between the curve and temperature axis (where $D_r = 0$) (**Table 2.5**).

All nonlinear models examined had four parameters and, therefore, the same d.f. (**Table 2.3**). This eliminated the need for multiple, complex comparison criteria (e.g., AIC, AIC_c, BIC, etc. [Akaike 1974; Schwarz 1978; Hurvich and Tsai 1989]) and selection-associated bias (Ratkowsky and Reddy 2017; Milosavljević et al. 2019). Though R^2 and its adjusted derivative (R^2_{adj}) are commonly used to describe the fit of nonlinear models to developmental data, this usage is inappropriate. Important mathematical assumptions of the linear model-based R^2 equation are violated by nonlinear models, producing erroneous estimations (see further explanation in Spiess and Neumeyer 2010, who conclude that R^2 usage for nonlinear models is inappropriate and recommend its removal from scientific literature for this purpose). Thus, nonlinear model goodness-of-fit was assessed solely with *RSS* (Shi et al. 2015; Ratkowsky and Reddy 2017; Milosavljević et al. 2019) (**Table 2.5**):

$$RSS = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2$$

here, *n* denotes the sample size and y_i and $\hat{y}_i$ denote the observed and expected development rate at the *i*-th temperature, respectively. A lower value of *RSS* indicates superior model fit.

The relative accuracy of model-predicted development rates was assessed using the percent deviation formula (Liu et al. 1995; Quinn 2017; Sánchez-Ramos et al. 2018) (**Table 2.5**):

$$d = \left(\frac{PDT}{RDT} - 1\right) \times 100$$

here, *PDT* and *RDT* are the sum of predicted and recorded development rates, respectively. The percentage of their deviation is represented as *d*, with overestimations expressed as positive values and underestimations as negative values. It is important to note that this calculation provides an assessment of model precision solely at experimentally measured temperatures.

Meta-analysis of *Tamarixia radiata* development. A literature search was conducted to locate published studies examining the temperature-dependent development of *T. radiata* to compare with results presented in this study. Relevant papers were retrieved manually using keyword searches (i.e., combinations of "*Tamarixia radiata*" or "*Tetrastichus radiatus*" with "development rate", "growth", "performance curve", or "life history", and "temperature" or "thermal cline") in Google Scholar, Web of Science, and the University of Florida

Huanglongbing Bibliographical Database (Vanaclocha and Stansly 2009). In addition to the present study, ten papers containing constant temperature regime-derived and combined gender development data for *T. radiata* sourced from China, Brazil, Réunion Island, and Taiwan were compiled (**Table 2.4**). For the subset of four papers (Quilici and Fauvergue 1990; Chien et al. 1993; Gómez-Torres et al. 2014; Li et al. 2018) that provided development time across multiple temperatures, the reciprocal (i.e., D_r=1/D_t, development rate) was taken. These data were plotted in SAS with PROC SGPLOT and fit to a linear regression with PROC REG. Following identification and removal of outliers (i.e., observations with externally studentized residuals larger than |3|, as indicated by rstudent), T_{min}, degree-days, and R^2 were estimated (for methods, see previous section "Mathematical Models and Evaluation") and compared across published datasets. Gómez-Torres et al. (2012) was not included in this analysis, as development time was presented separately by gender. Furthermore, this study was superseded by Gómez-Torres et al. (2014) (included in the analysis) which examined the same source population of *T. radiata* (São Paulo, Brazil) across a broader range of temperatures.

Results

Effect of temperature and regime type on *T. radiata* development time. *Tamarixia radiata* completed egg-to-adult development under experimental temperature profiles from 15 to 35°C in the constant regime and 12 to 35°C in the fluctuating regime. Average temperature (χ^2 = 1410.1, d.f. = 4, $P < 0.001$), regime type (i.e., constant or fluctuating) (χ^2 = 63.15, d.f. = 1, $P < 0.001$), and their interaction (χ^2 = 73.0, d.f. = 4, $P < 0.001$) significantly affected mean development time. Gender was also a significant model factor (χ^2 = 4.88, d.f. =

1, $P = 0.027$) but its interaction with temperature ($\chi^2 = 0.76$, d.f. = 4, $P = 0.943$) and regime were not ($\chi^2 = 0.30$, d.f. = 1, $P = 0.586$). Egg-to-adult development time decreased with increasing temperature to a minimum of 8.14 ± 0.06 d at 35°C constant and 9.28 ± 0.23 d at 30°C fluctuating (**Table 2.2**). *Tamarixia radiata* reared under the fluctuating regime developed significantly faster at 15°C ($Z = 8.39$, $P < 0.001$) and slower at 35°C ($Z = -3.21$, $P = 0.043$) than at the corresponding constant temperatures. No significant differences in development time between constant and fluctuating regimes were detected at 20°C ($Z = -0.3$, $P = 1.0$), 25°C ($Z = -0.54$, $P = 1.0$), and 30°C ($Z = -1.12$, $P = 0.983$).

Development rate model comparison. The linear model provided a good fit for *T. radiata* development rate in both constant and fluctuating regimes, with an R^2 of 0.99 and 0.98, respectively (**Table 2.4**). The regression line predicted a T_{min} of 9.9°C for the constant regime and 6.0°C for the fluctuating regime. Estimates of the thermal requirement for development completion were lower for the constant regime than the fluctuating counterpart, with 166.7 and 217.4 degree-days, respectively.

Table 2.5 summarizes nonlinear model (i.e., Lactin-2, Weibull, Brière-2, Performance-2, Beta, LRF, and Ratkowsky) goodness-of-fit and theoretical developmental thresholds and **Fig. 2.1** displays thermal performance curves. In general, all models fit the observed data well, producing low values of *RSS* and percent deviation. Mathematically, the best fitting models were Ratkowsky, Beta, and LRF while Weibull, Lactin-2, and Performance-2 provided poorer fits. Estimations of T_{opt} were similar among models, ranging from 33.3 to 33.4°C for constant regimes and 29.4 to 31.8°C for fluctuating regimes. Considerable divergence in model predictions were observed for values of T_{min}, which ranged from 5.3 to 11.5°C (constant) and -273 to 7.3°C (fluctuating), and T_{max}, which ranged from 42.1 to 52.5°C (constant) and 35.2 to 40.5°C (fluctuating) (**Table 2.5**).

Meta-analysis of *T. radiata* development. Among the ten papers evaluated, *T. radiata* development was reported to occur at temperatures between 15 and 35°C. The temperatures most frequently examined were 20, 25, and 30°C, with average development times of 16.98 ± 1.26, 11.01 ± 0.27, and 9.06 ± 0.76 d, respectively. *Tamarixia radiata* from Brazil (unknown origin), Réunion (Pakistan), Taiwan (parasitoids sourced from populations established on Réunion that were originally collected from Pakistan), and California (Pakistan) were estimated to have similar developmental parameters, with a T_{min} ranging from 7.6 to 11.8°C and requiring 137.0 to 178.6 degree-days to complete development (**Table 2.4**). These values diverged considerably from the T_{min} of 0.8 and 333.3 degree-days calculated for the China-sourced *T. radiata.*

Effect of temperature and regime type on *T. radiata* adult longevity. Mean longevity of adult *T. radiata* was significantly affected by average temperature ($\chi^2 = 253.93$, d.f. = 4, $P < 0.001$), thermal variation ($\chi^2 = 223.03$, d.f. = 1, $P < 0.001$), and their interaction ($\chi^2 = 281.44$, d.f.= 4, $P < 0.001$). The main effect of gender was not significant ($\chi^2 = 1.47$, d.f. = 1, $P = 0.226$) but significant interactions with temperature ($\chi^2 = 16.8$, d.f. = 4, $P = 0.002$) and regime ($\chi^2 = 4.32$, d.f. = 1, $P = 0.038$) were detected. Maximum longevity of 79.36 ± 3.35 d was observed at 15°C fluctuating and 32.45 ± 1.74 d at 20°C constant (**Table 2.2**). Above these temperatures, longevity steadily decreased with rising temperature, reaching minimums of 1.17 ± 0.17 (fluctuating) and 6.99 ± 0.46 d (constant) at 35°C. Despite the similarity in overall trend (**Fig. 2.2**), average lifespan duration was significantly different between regimes across all temperatures (15°C: Z = -15.88, $P < 0.001$; 20°C: Z = -9.25, $P < 0.001$; 30°C: Z =

3.26, $P = 0.038$; 35°C: Z = 4.14, $P = 0.001$) except 25°C (Z = -0.78, $P = 0.999$) (**Table 2.2**). The magnitude of this effect was greatest at temperature cline margins. In comparison with constant regimes, fluctuating regimes produced *T. radiata* with a lifespan approximately four times longer at 15°C, two times longer at 20°C, and six times shorter at 35°C.

Effect of fluctuating temperature regimes on *D. citri* parasitism rates. Parasitism of *D. citri* by *T. radiata* occurred under all fluctuating experimental temperatures except 38°C. Temperature significantly affected parasitism rate ($\chi^2 = 83.63$, d.f. = 5, $P < 0.001$). Parasitism was significantly higher at intermediate temperatures 20 ^{o}C (58.0 ± 5.93%), 25 ^{o}C (70.0 ± 6.15%), and 30^{o}C (51.0 ± 6.40%) than at cline margins 12^{o}C (20^{o}C: Z = -5.2, $P < 0.001$; 25^{o}C: Z = -5.79, $P < 0.001$; 30^{o}C: Z = -4.8, $P < 0.001$) and 35^{o}C (20^{o}C: Z = -4.53, $P < 0.001$; 25^{o}C: Z = 5.23, $P < 0.001$; 30^{o}C: Z = 4.06, $P < 0.001$), where parasitism was 9.0 ± 4.82% and 14.44 ± 6.26%, respectively (**Fig. 2.3**).

Developmental mortality of *T. radiata* in fluctuating temperature regimes. Temperature significantly affected the mean developmental mortality rate of developing *T. radiata* reared under fluctuating regimes ($\chi^2 = 90.48$, d.f. = 5, $P < 0.001$). Mortality was similar among intermediate temperatures (less than 25% at 15 to 30^{o}C) but significantly higher at 12^{o}C (15^{o}C: Z = -3.42, $P = 0.008$; 20^{o}C: Z = -5.11, $P < 0.001$; 25^{o}C: Z = -5.29, $P < 0.001$; 30^{o}C: Z = -4.33, $P < 0.001$) and 35^{o}C (15^{o}C: Z = 3.19, $P = 0.018$; 20^{o}C: Z = 4.86, $P < 0.001$; 25^{o}C: Z = 5.04, $P < 0.001$; 30^{o}C: Z = 4.09, $P < 0.001$), where 30.56 ± 19.44% and 73.0 ± 16.70% of *T. radiata* failed to emerge from *D. citri* hosts, respectively (**Fig. 2.4**).

Effect of fluctuating temperature regimes and *T. radiata* exposure on mortality of *D. citri* nymphs. *Tamarixia radiata*-exposure treatment ($\chi^2 = 298.48$, d.f. = 1, $P < 0.001$), average temperature ($\chi^2 = 79.5$, d.f. = 5, $P < 0.001$), and the interaction between treatment and temperature (χ^2 = 113.58, d.f. = 5, *P* < 0.001) were significant factors affecting mean total developmental mortality of *D. citri*. Across all temperatures, total mortality rate was significantly higher in *T. radiata*-exposed cohorts than in controls lacking parasitoid exposure (12^{o}C: Z = -15.21, *P* < 0.001; 15^{o}C: Z = -17.12, *P* < 0.001; 20^{o}C: Z = -21.08, *P* < 0.001; 30^{o}C: Z = -20.21, *P* < 0.001; 35^{o}C: Z = -15.31, *P* < 0.001; **Fig. 2.5)**. Control cohort total mortality was 7 – 10% from 15 to 30°C, increasing slightly at temperature cline margins of 12°C (13.0 ± 3.67%) and 35°C (19.0 ± 5.67%). By contrast, corrected total mortality of *T. radiata*-exposed cohorts was over 55% across all temperatures, reaching 92.0 ± 3.27%, 91.0 ± 4.58%, and 85.0 ± 6.87% at 20, 25, and 30°C, respectively.

As with mean total *D. citri* nymph mortality, mortality excluding parasitism was significantly influenced by *T. radiata*-exposure treatment (χ^2 = 213.61, d.f. = 1, *P* < 0.001), temperature (χ^2 = 79.5, d.f. = 5, *P* < 0.001), and the interaction between treatment and temperature (χ^2 = 32.07, d.f. = 5, *P* < 0.001; **Fig. 2.5**). Non-parasitism related *D. citri* mortality was significantly higher in the *T. radiata*-exposure treatment than the control treatment across all experimental temperatures (12^{o}C: Z = -13.21, *P* < 0.001; 15^{o}C: Z = -11.87, *P* < 0.001; 20^{o}C: Z = -10.76, *P* < 0.001; 25^{o}C: Z = -5.65, *P* < 0.001; 30^{o}C: Z = -11.64, *P* < 0.001; 35^{o}C: Z = -12.04, *P* < 0.001). At 25°C, *T. radiata*-exposed cohorts had the lowest rate of non-parasitism related mortality (21.0 ± 4.58%) but the highest rate of parasitism (70.0 ± 6.15%) (**Fig. 2.3**). Non-parasitism related mortality peaked at temperature cline margins of 12 and 35°C (48.0 ± 6.63% and 53.33 ± 7.99%, respectively), where parasitism rate was observed to be the lowest (9.0 ± 4.82% and 14.44 ± 6.26%, respectively).

Discussion

Establishment of the *D. citri*-*C*Las pest complex in major citrus-growing regions could threaten the sustained profitability of California's multi-billion-dollar citrus industry (Babcock 2018). southern California's extensive residential landscape is a liability for *C*Las spread, as there are millions of backyard citrus trees that could harbor infected *D. citri* (Gottwald 2010; Hoddle and Pandey 2014). In these high-risk urban areas where pesticide use is minimal, *T. radiata* is critical for suppressing vector populations and limiting dispersal into commercial production zones (Milosavljević et al. 2017).

Consequently, a thorough understanding of the effects of temperature on the developmental biology of *T. radiata* sourced from Pakistan and used for biocontrol in California is necessary for interpreting parasitoid impacts in different citrus producing regions with varying climates (e.g., hot desert interior regions and cool coastal zones) and for the optimization of mass-production programs. Thus, this study aimed to provide a comprehensive overview of the effects of constant and fluctuating temperatures on life history parameters of *T. radiata*.

Average daily temperature and regime type significantly affected *T. radiata* development time and longevity, with the greatest mean differences between control and fluctuating profiles observed near the lower and upper extremes of experimental temperature clines. At cooler temperatures, parasitoids reared under fluctuating profiles developed more rapidly (15°C) and survived longer (15 and 20°C) than those reared under constant temperatures with equivalent means (**Table 2.2**). The reverse was true at high temperatures, with fluctuating profiles producing parasitoids with a longer development period (35°C) and shorter lifespan (30 and 35°C) than constant temperature counterparts. The observed response for *T. radiata* reflects that of other arthropods reared under variable temperature regimes (Williams et al. 2012; Colinet et al. 2015; Sánchez-Ramos et al. 2018). This pattern is likely a consequence of the asymmetrical structure of nonlinear functions as described by Jensen's inequality (Jensen 1906; Ruel and Ayres 1999). For thermal performance curves, the rate of physiological output accelerates with increasing temperature. As a result, insects are more sensitive to small temperature changes at the high end of the thermal gradient in comparison to the low end (Colinet et al. 2015). This effect is compounded by thermal oscillation in fluctuating regimes, where the metabolic "cost" of the heating period (i.e., time spent above the daily mean) exceeds the "savings" of the cooling period (i.e., time spent below the daily mean) (Martin and Huey 2008; Williams et al. 2012; Colinet et al. 2015). Consequently, fluctuating regimes are more energy-demanding environments than constant regimes held at equivalent mean temperatures and tend to produce divergent responses. The beneficial or deleterious impact of this imbalance is dependent on insect sensitivity, amplitude of incremental temperature ramping, and mean temperature (Colinet et al. 2015).

At high temperatures, insects have a narrow range of thermotolerance. Because fluctuating regimes expose insects to temperatures above the mean, there is greater risk of crossing critical thermal limits at which severe or catastrophic injury is sustained (e.g., systemic cell death induced by reduced respiration) (Colinet et al. 2015). Even if subsequently returned to permissible thermal conditions, insects may not survive damage sustained from previously experienced temperature shocks (Neven 2000). Recovery from acute thermal exposure (e.g., reestablishment of ion balance, upregulation of heat shock proteins, and DNA repair) is an energetically costly process that may further limit insect performance (Yocum 1992; Colinet et al. 2015). In the present study, this trade-off may have been expressed as increased development time and reduced longevity of *T. radiata* reared under fluctuating temperatures in comparison with those reared under equivalent constant regimes at high average temperatures (≥30°C) (**Table 2.2**).

In contrast to the adverse effects insects experience under high fluctuating temperatures, thermal variation at low average temperatures can produce favorable conditions for insect performance (Colinet et al. 2015). Warming intervals that interrupt periods of prolonged cold exposure activate biochemical pathways involved in damage repair and allow essential behaviors such as feeding to occur, thereby protecting against future injury (Denlinger et al. 1992; Yocum and Denlinger 1992; Neven 2000; Colinet et al. 2015). Consequently, fluctuating regimes may allow insect development to occur at temperatures outside the limits defined by constant regimes (Colinet et al. 2015). In this study, thermal variation reduced egg-to-adult development time for *T. radiata* reared under low temperatures and expanded its minimum developmental range (i.e., 12 – 35°C fluctuating vs. 15 – 35°C constant). This observation could be critical for accurately predicting the potential distribution range for *T. radiata*. Additionally, *T. radiata* reared under low fluctuating temperatures had greatly increased longevity in comparison with parasitoids reared under constant temperature counterparts (**Table 2.2**). Under the 15°C fluctuating profile, the maximum lifespan of *T. radiata* was 79 d, a value over four times greater than the 18 d recorded at 15°C constant and more than double the 32 d maximum recorded at 20°C constant. These findings have important implications for commercial production of *T. radiata*, as an extended shelf-life could facilitate releases of better-performing parasitoids or allow for greater flexibility in timing of deployments. Moreover, inclusion of a warming period during cold storage of mass-reared *T. radiata* would be an inexpensive, easily implementable management practice that would increase parasitoid longevity.

A significant number of mathematical functions have been developed to characterize insect development rates across thermal clines. While certain models consistently perform better within a specific context, there is no consensus on which are generally "best" across a wide range of applications. Model selection is generally left to author discretion and strongly subject to field-associated biases. Studies investigating arthropod temperature-dependent development typically examine the fit of a single or few models drawn from a limited pool standard for that particular taxonomic group, often without justification (Quinn 2017). Alternative models with potentially superior predictive power or other beneficial qualities (e.g., low complexity, inclusion of parameters with biological relevance, and consistency between observed and predicted values) may be overlooked. Consequently, the vast majority of insect developmental datasets are not fit with optimal functions, limiting the reliability of critical inferences and predictions drawn from these data (Quinn 2017). These criteria were considered in initial selection of nonlinear models fit to *T. radiata* developmental datasets and in the subsequent review of their performance. Functions evaluated included those with widespread usage in the entomological field (i.e., Lactin-2, Weibull, and Brière-2) and those not commonly used but with desirable properties (i.e., Beta, LRF, Ratkowsky, and Performance-2) (Shi et al. 2015).

All nonlinear models provided a relatively good fit to both constant and fluctuating datasets, a finding which could be partially attributed to the limited number of temperatures evaluated. However, differences in model performance were still apparent. The Ratkowsky, Beta, LRF, and Brière-2 consistently produced the lowest values of *RSS* while the Weibull, Lactin-2, and Performance-2 produced the highest (**Table 2.5**). While goodness-of-fit is an important measure of performance, it has been overly relied upon as an indicator of superior model function (Damos and Savopoulou-Soultani 2012; Régnière et al. 2012; Ratkowsky and Reddy 2017). For any given dataset, there are generally several models that will provide a good mathematical fit. High conformity between experimental and model-estimated values does not guarantee reliability of predicted thermal performance curves across the entire temperature cline range (Schwarz 1978; Damos and Savopoulou-Soultani 2012; Régnière et al. 2012; Mirhosseini et al. 2017). To ensure evaluation accurately represented overall model performance, other criteria such as inclusion of biologically interpretable model parameters and theoretical threshold estimates were examined in addition to goodness-of-fit.

In evaluating model predictions of T_{min} and T_{max}, it is important to emphasize that these measures are fixed, mathematical model extrapolations which represent the absolute minimum and maximum temperatures at which development is not theoretically observable. While empirical models cannot directly provide the actual developmental limits ($\text{i.e.}, MINt$ and $MAXt$), these "true" threshold values generally lie within the conceptual bounds delimited by T_{min} and T_{max} (McMeekin et al. 2013; Ratkowsky and Reddy 2017; Milosavljević et al. 2019). Although T_{min} and T_{max} are not equivalent to $MINt$ and $MAXt$, they may provide approximations of these points depending on the model structure, taxa examined, and sample size of the dataset (Shi et al. 2015). To minimize model extrapolation and improve accuracy in estimation of thermal bounds, experiments should be designed to examine a wide range of temperatures, especially those near T_{min} and T_{max} (Régnière et al. 2012; Quinn 2017; Rebaudo and Rabhi 2018). Regardless, theoretical thresholds should always be viewed as hypotheses which require experimental confirmation in the laboratory and field (Shi et al. 2015; Ratkowsky and Reddy 2017; Milosavljević et al. 2019). This step is particularly important, as pest management inferences made from erroneous estimates of development rates and thresholds can have economically significant consequences (Horn 1998; Roitberg et al. 2001; Hart et al. 2002; Régnière et al. 2012). Comparison of theoretical thermal limits across multiple models can facilitate delineation of the true tolerable thermal range for a species by producing a range of overlapping threshold values that may serve as a starting point for further experimental evaluation.

Estimates of the optimum development point for *T. radiata* were relatively consistent across models and regimes, ranging from 33.3 to 33.4°C for constant temperatures and 29.4 to 31.8°C for fluctuating temperatures (**Table 2.5**). Model-predicted values of T_{min} were generally higher for the constant regime (5.3 – 11.5°C) than the fluctuating regime (-6.2 to 7.3°C excluding the Beta model estimate). These model predictions are supported by experimental observations, as *T. radiata* was recorded to develop under the 12°C fluctuating profile but not under the 12°C constant counterpart. Regardless of regime, Lactin-2, Weibull, and Performance-2 produced higher estimates of T_{min} than Ratkowsky, Beta, LRF, and Brière-2. The sub-zero T_{min} values produced by the latter four models for the fluctuating dataset may not directly facilitate estimation of a range for $MINt$, as insect development is seldom reported to occur below 6°C (Damos and Savopoulou-Soultani 2012; Quinn 2017). This is particularly the case for the Beta model, which, despite providing the best mathematical fit to the fluctuating dataset, produced a T_{min} well outside the range of biological relevance (absolute zero; -273°C). As with T_{min} , predicted values of T_{max} were higher for the constant regime (42.1 – 52.5°C) than the fluctuating regime (35.2 – 40.5°C). Although the Weibull and Ratkowsky models consistently produced the highest estimates of T_{max} , for the constant regime dataset, all models predicted values of T_{max} that were well above the experimentally measured maximum developmental bound of 38°C. This example further illustrates the importance of treating theoretical thresholds as hypotheses which require empirical validation, particularly if estimations are based upon a limited number of temperature observations.

Percent deviation between measured and predicted development rates was relatively small for all models examined. The Ratkowsky and Performance-2 models produced values of zero for the constant dataset, and the Performance-2 and Weibull models produced values nearest zero for the fluctuating dataset. In addition to having biologically meaningful parameters, the Ratkowsky, Beta, and LRF models provided excellent fits to both the constant and fluctuating datasets and produced biologically realistic estimates for T_{opt}. The Beta model, however, was found to be unreliable with its T_{min} prediction of absolute zero and accompanying percent deviation of -0.11%, the largest recorded among models. The Brière-2 model was middling both in fit and estimation of theoretical developmental thresholds. The Performance-2 and Lactin-2 models provided poorer fits than the aforementioned models but gave higher estimates of T_{min} that could be potentially valuable in approximating $MINt$ (Shi et al. 2015). Additionally, the Performance-2 model includes parameters of direct biological relevance. The Weibull function was the worst performing of the group, consistently providing the poorest fit to datasets and lacking any parameters with biological meaning.

Of the seven functions evaluated, the Ratkowsky and LRF models were found to be consistently best for predictions associated with interpolation (i.e., estimation of T_{opt} or other development rates within the measured temperature range) as they produced small percent deviations and the best overall fit to the datasets. While the Performance-2 model (a simple modification of the Ratkowsky model [see **Table 2.3** for comparison]) provided a somewhat poorer fit to datasets, it produced the lowest percent deviation for both constant and fluctuating regimes and predicted theoretical thresholds within a potentially biologically realistic range. However, estimates derived from such extrapolative methods have limited reliability. Future studies which examine the temperature-dependent development of insects should consider inclusion of the Ratkowsky, LRF, and Performance-2 functions for modeling thermal performance curves.

The meta-analysis presented in **Table 2.4** represents a literature review pertaining to development of geographically disparate populations of *T. radiata* reared under constant temperatures. In general, estimates of development time were similar across different source populations of *T. radiata*. *Tamarixia radiata* was reported to always complete development between 15 and 35°C when these temperatures were evaluated. A notable exception is the Taiwan-sourced *T. radiata* which failed to complete development at 35°C (Chien et al. 1993). In comparison to other published studies, the experimental work reported here covered a wider range of temperatures that better-defined developmental bounds at thermal cline peripheries (12 and 38°C). Inclusion of additional lower and upper temperatures in future studies investigating *T. radiata* development could better facilitate comparison of thermal limits among populations from different geographic regions. Such studies would further our understanding of the importance of climate matching in the development of foreign exploration programs for biocontrol agents.

Although average development time varied among source populations of *T. radiata*, values reported for California and Réunion were nearly identical. Linear regression provided similar estimates of degree-days (137 – 179) and T_{min} (7.6 – 11.8°C) for *T. radiata* from all regions except China (333 degree-days and T_{min} of 0.8°C). Interestingly, *T. radiata* with similar parameter estimates (i.e., California, Réunion, and Taiwan) were originally introduced from Punjab, Pakistan. While the source of introduction for the Brazilian *T. radiata* is unknown (Torres et al. 2006), degree-day and theoretical minimum threshold estimates are strongly divergent from China-sourced parasitoids and similar to that of Pakistan-sourced parasitoids. Molecular studies could help resolve the initial area of origin for *T. radiata* found in Brazil by investigating its genetic relatedness to Pakistan-sourced populations.

A comparison of *T. radiata* populations sharing a Punjab, Pakistan origin could provide valuable insight into the impacts of genetic diversity on *T. radiata* performance. The population of *T. radiata* established in southern California is genetically diverse, as it was seeded by thousands of Pakistan-collected individuals accumulated over multiple collecting trips and maintained in 17 isocage lines to limit the effects of genetic drift in mass-production (Hopper et al. 1993; Roush and Hopper 1995; Hoddle and Hoddle 2013; Hoddle et al. 2014). By comparison, *T. radiata* populations currently present in Taiwan underwent two extreme genetic bottlenecks, first from the eight individuals released to establish the population on Réunion Island (Etienne and Aubert 1980) and again from the 62 individuals shipped from Réunion Island to propagate colonies released in Taiwan (Chiu et al. 1988; Chien and Chu 1996). Examination of developmental biology for all three *T. radiata* populations was conducted around the time of collection and introduction. These populations could be re-evaluated to compare performance among genetically poor (Réunion and Taiwan) and rich (California) populations and check for evidence of local and regional adaptation. An investigation of the effects of varying temperatures on the developmental biology of *T. radiata* established in major citrus producing regions within the continental United States (e.g., Texas and Florida) and Mexico could provide further insight into evolution of regional thermal tolerance variation among established parasitoid populations.

In addition to development rates and longevity, this study examined the biocontrol performance of *T. radiata* under fluctuating temperature regimes. Across all experimental temperatures, estimates of *D. citri* total mortality and mortality unrelated to visually identifiable parasitism in *T. radiata*-exposed *D. citri* cohorts (e.g., host feeding, failed oviposition attempts, etc.) were significantly higher than mortality rates for control *D. citri* cohorts (**Fig. 2.5**). In relation to temperature, parasitism of *D. citri* by *T. radiata* (**Fig. 2.3**) followed a dome-shaped pattern and non-parasitism related *D. citri* mortality followed a "U"-shaped pattern. Despite low parasitism rates at 12 and 35°C (<15%), total *D. citri* mortality was relatively high (57 and 68%, respectively) as a consequence of increased non-parasitism mortality (48 and 53%, respectively). Non-parasitism mortality was lower (21 – 34%) at temperatures optimal for parasitism (20, 25, and 30°C; 51 – 70%), but combined factor contributions increased total mortality to generally high levels (85 – 92%). Thus, the effects of non-parasitism mortality were important across all temperatures, contributing the highest proportion of total mortality near temperature cline ends (84 and 79% at 12 and 35°C, respectively) and the lowest at intermediate temperatures (23% at 25°C). These results suggest *T. radiata* inflicts substantial *D. citri* mortality through mechanisms other than successful parasitism (e.g., host feeding), the effects of which are pronounced near lower and upper temperature bounds.

In many regions where *T. radiata* has been introduced for *D. citri* biological control, considerable suppression of *D. citri* populations has been reported following parasitoid releases (Étienne and Aubert 1980; Chien and Chu 1996; Étienne et al. 2001; Pluke et al. 2008; Kistner et al. 2016; Flores and Ciomperlik 2017). However, the efficacy of this parasitoid has been questioned because *T. radiata* parasitism is spatiotemporally variable in the field (Hall et al. 2013; Grafton-Cardwell et al. 2013; Kistner et al. 2016; Milosavljević et al. 2018; Milosavljević and Hoddle 2019). This inconsistency, along with the results presented here, suggests that other sources of mortality inflicted by *T. radiata* (i.e., host feeding) may contribute significantly to total *D. citri* mortality. Thus, the parasitism estimates currently relied upon for verification of *T. radiata* effectiveness may underestimate its impact in the field, with prevailing temperature as an important but overlooked co-factor affecting parasitoid inflicted mortality on *D. citri* nymphs.

In summary, this study complements a growing body of literature demonstrating the important role of temperature variation in predicting insect life history responses that are transposable to field conditions (Colinet et al. 2015; Rebaudo and Rabhi 2018). The fluctuating regimes under which *T. radiata* performance was measured accurately represent the thermal conditions experienced by this parasitoid in inland southern California, improving our general understanding of this parasitoid's developmental biology. Incorporation of these results into biological control programs for *D. citri* may streamline mass-rearing, enhance release efforts, improve accuracy of predictions pertaining to parasitoid-host establishment and spread produced by climate forecasting models, and enable performance comparisons between Pakistan-sourced *T. radiata* and other geographically distinct populations.

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Figures and Tables

Hour	Mean Temperature (°C)							Photoperiod
	12	15	20	25	30	35	38	
0100	10	11	16	21	26	30	33	dark
0200	9	11	16	20	26	30	32	
0300	9	11	17	20	26	30	33	
0400	8	10	17	20	26	30	33	
0500	8	10	16	20	25	30	32	
0600	7	9	15	19	25	30	32	light
0700	8	9	15	20	26	31	34	
0800	9	10	17	23	28	33	36	
0900	12	14	20	25	29	35	38	
1000	15	18	22	27	32	36	40	
1100	17	20	24	29	33	38	42	
1200	18	22	25	30	34	40	43	
1300	19	23	26	31	35	40	44	
1400	19	23	27	32	35	40	45	
1500	19	23	27	32	36	41	45	
1600	18	23	27	32	36	41	45	
1700	16	20	25	31	35	41	45	
1800	13	16	22	30	34	40	44	
1900	11	14	20	27	32	37	41	
2000	10	13	18	24	30	36	38	dark
2100	9	13	18	23	30	35	37	
2200	9	13	17	22	28	33	35	
2300	7	11	16	21	26	31	32	
2400	7	11	16	21	26	31	32	
Total Steps	18	13	16	17	15	14	18	

Table 2.1 Fluctuating temperature profiles utilized for rearing *Tamarixia radiata*.

	Development time (mean days ± SE)					
	12^{o}C	15^{o}C	20^{o}C	25^{o}C	30^{o}C	35^{o}C
Constant	No emergence	33.87 ± 0.31^{a} (96)	16.64 ± 0.11^{a} (80)	10.45 ± 0.06^{a} (80)	8.49 ± 0.04^{a} (80)	8.14 ± 0.06^{a} (53)
Fluctuating	48.50 ± 0.81 (6)	23.57 ± 0.34^{b} (28)	17.02 ± 0.18^{a} (53)	10.98 ± 0.09^{a} (58)	9.28 ± 0.23^{a} (40)	12.14 ± 0.34^{b} (7)
P	N/A	<0.0001	1.0000	0.9999	0.9833	0.0431
	Adult longevity (mean days ± SE)					
Constant	No emergence	18.41 ± 1.57^{a} (95)	32.45 ± 1.74^{a} (80)	29.34 ± 1.07^{a} (80)	23.14 ± 1.02^{a} (80)	6.99 ± 0.46^{a} (53)
Fluctuating	70.50 ± 10.50 (6)	79.36 ± 3.35^{b} (25)	62.63 ± 2.06^{b} (51)	31.14 ± 1.32^{a} (50)	17.33 ± 1.25^{b} (40)	1.17 ± 0.17^{b} (6)
P	N/A	<0.0001	< 0.0001	0.9988	0.0378	0.0014

Table 2.2 Mean development times and adult longevity (days ± SE) of *Tamarixia radiata* under constant and fluctuating temperature regimes. For development time and longevity, means followed by the same letter within the same temperature are not significantly different α = 0.05 (estimated marginal means), (*n*) denotes number of individuals.

Model	Model Equation	Parameter	Parameter Estimate		Reference
			Constant	Fluctuating	
Ordinary Linear	$D_r = a + bT$	a b	-0.0592 0.0060	-0.0290 0.0047	Campbell et al. (1974)
Lactin-2 (Logan-Lactin)	$D_r = \lambda + e^{\rho T} - e^{(\rho T_u - (T_u - T)/\delta)}$	λ ρ T_u Δ	-1.0689 0.0063 51.1326 4.9804	-1.0286 0.0044 40.7663 1.8892	Logan et al. (1976) Lactin et al. (1995)
Weibull	$D_r = a\left(\frac{d-1}{d}\right)^{\frac{1-d}{d}}\left[\frac{T-b}{c}+\left(\frac{d-1}{d}\right)^{\frac{1}{d}}\right]^{d-1} e^{\left[-\left(\frac{T-b}{c}+\left(\frac{d-1}{d}\right)^{\frac{1}{d}}\right)^{d}+\frac{d-1}{d}\right]}$	a b c d	0.0763 40.2620 32.9344 7.8314	0.0544 36.1118 30.5081 17.7261	Angilletta Jr. (2006)
Brière-2	$D_r = aT(T - T_{min})(T_{max} - T)^{1/b}$	T_{min} T_{max} a b	0.0000 8.8934 43.3516 1.4193	0.0001 -6.1967 35.1672 6.0321	Brière et al. (1999)
Beta	$D_r = r_m\left(\frac{T_2 - T}{T_2 - T_m}\right)\left(\frac{T - T_1}{T_m T_1}\right)^{\frac{T_m - T_1}{T_2 - T_m}}$	T_1 T_2 T_m r_m	5.2869 46.1509 33.3844 0.1245	-273 38.2160 30.6802 0.1098	Yin et al. (2003) Auzanneau et al. 2011 Shi et al. (2015)
Lobry-Rosso-Flandrois	$D_r = \mu_{opt}\frac{(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right]}$	T_{min} T_{max} T_{opt} μ_{opt}	6.1237 46.0568 33.4138 0.1245	-3.8214 36.5141 31.5669 0.1123	Lobry et al. (1991) Rosso et al. (1993)

Ratkowsky	$D_r = \left(b(T - T_{min})(1 - e^{c(T-T_{max})})\right)^2$	T_{min}	5.8497	-5.0733	Ratkowsky (1983)
		T_{max}	51.6464	38.9538	
		b	0.0236	0.0100	
		c	0.0428	0.3175	
Performance-2	$D_r = b(T - T_{min})(1 - e^{c(T-T_{max})})$	T_{min}	10.7657	6.7013	Shi et al. (2011) Wang et al. (2013)
		T_{max}	42.128	36.731	
		b	0.00344	0.00484	
		c	0.1885	0.5356	

Table 2.3 Mathematical models and parameter estimates for eight performance functions describing the relationship between development rate (D_r) and temperature for *T. radiata* reared under constant and fluctuating temperature regimes. See corresponding references for full description of models and parameters.

Development time (mean days ± SE)														K (degree-days)	T_{min}	R^2	*T. radiata* studied country	Reference	*T. radiata* origin country	Reference
12°C	15°C	18°C	20°C	22°C	25°C	26°C	27°C	28°C	30°C	32°C	34°C	35°C	38°C							
-	-	-	-	-	12.6	-	-	-	-	-	-	-	-	-	-	-	Southeast China (Fujian)	Xu and Tang 1993	China	Tang 1989; 1990
-	-	19.6	-	14.5^{ad}	-	13.4	-	-	11.3^{d}	-	10.2^{d}	-	-	333.3	0.8	0.9980	South China (Guangdong)	Li et al. 2018	China	Li et al. 2018
-	20.3^{b}	-	18.8^{b}	-	15.5^{b}	-	-	-	11.8^{b}	-	-	10.4^{b}	-	-	-	-	Brazil	Gomez-Torres et al. 2012	Unknown	Torres et al. 2006
-	-	-	-	-	-	-	-	10.4	-	-	-	-	-	-	-	-	Brazil	Baños et al. 2013	Unknown	Torres et al. 2006
-	-	17.3	14.2	12.4	10.3	-	-	10.1^{a}	7.6	7.6	-	-	-	178.6	7.6	0.9951	Brazil	Gomez-Torres et al. 2014	Unknown	Torres et al. 2006
-	-	-	-	-	10.0	-	-	-	-	-	-	-	-	-	-	-	Brazil	Beloti et al. 2015	Unknown	Torres et al. 2006
-	-	-	-	-	11.6^{c}	-	-	-	-	-	-	-	-	-	-	-	Brazil	Alves et al. 2016^{c}	Unknown	Torres et al. 2006
-	-	-	16.8	-	10.1	-	9.1	-	8.5^{a}	-	-	-	-	137.0	11.8	0.9945	Réunion	Quilici and Fauvergue 1990	Pakistan	Etienne and Aubert 1980
-	-	-	-	-	11.4	-	-	-	-	-	-	-	-	-	-	-	Taiwan	Chien et al. 1991	Réunion (Pakistan)	Chiu et al. 1989
-	36.6	-	20.3^{a}	-	11.6	-	-	-	8.7	7.8	-	No emerg.	-	169.5	10.4	0.9998	Taiwan	Chien et al. 1993	Réunion (Pakistan)	Chiu et al. 1989
No emerg.	33.87	-	16.6	-	10.5	-	-	-	8.5	-	-	8.1^{a}	No emerg.	166.7	9.9	0.9923	California	This study (constant)	Pakistan	Hoddle et al. 2014
48.5^{a}	23.6	-	17.0	-	11.0	-	-	-	9.3	-	-	12.1^{a}	-	217.4	6.0	0.9813	California	This study (fluctuating)	Pakistan	Hoddle et al. 2014

Table 2.4 Comparison of development times, degree-days, theoretical minimum developmental thresholds, and R-squared values for *Tamarixia radiata* populations from China, Brazil, Réunion Island, Taiwan, and California. ^{a}Outliers excluded from *K*, *T*min and *R*2 calculations. ^{b}Development times provided for female *T. radiata* only. ^{c}Values averaged across all citrus varieties investigated in the study. ^{d}Values estimated from figures.

Model	Residual Sum of Squares (RSS)		Percent Deviation (%)		Theoretical Minimum Developmental Threshold ($T_{\min}$)		Optimum Developmental Temperature (T_{opt})		Theoretical Maximum Developmental Threshold ($T_{\max}$)	
	Constant	Fluctuating	Constant	Fluctuating	Constant	Fluctuating	Constant	Fluctuating	Constant	Fluctuating
Lactin-2	0.0000092	0.0000410	0.0023	-0.0182	10.66	6.35	33.32	31.65	42.35	36.83
Weibull	0.0000130	0.0000450	0.0117	0.0052	11.54	7.33	33.28	31.65	52.54	40.52
Brière-2	0.0000050	0.0000340	-0.0047	-0.0415	8.89	-6.20	33.39	29.43	43.35	35.17
Beta	0.0000038	0.0000220	-0.0047	-0.1064	5.29	-273^{a}	33.38	30.68	46.15	38.22
LRF	0.0000040	0.0000300	-0.0117	-0.0337	6.12	-3.82	33.41	31.57	46.06	36.51
Ratkowsky	0.0000036	0.0000260	0.0000	-0.0337	5.85	-5.07	33.44	31.01	51.64	38.95
Performance-2	0.0000098	0.0000430	0.0000	-0.0052	10.77	6.70	33.33	31.76	42.13	36.73

Table 2.5 Comparison of goodness-of-fit, percent deviation, and theoretical developmental thresholds among eight thermal performance functions fitted to the development rate of *Tamarixia radiata* under constant and fluctuating temperature regimes.

[a] Beta model achieved convergence at absolute zero

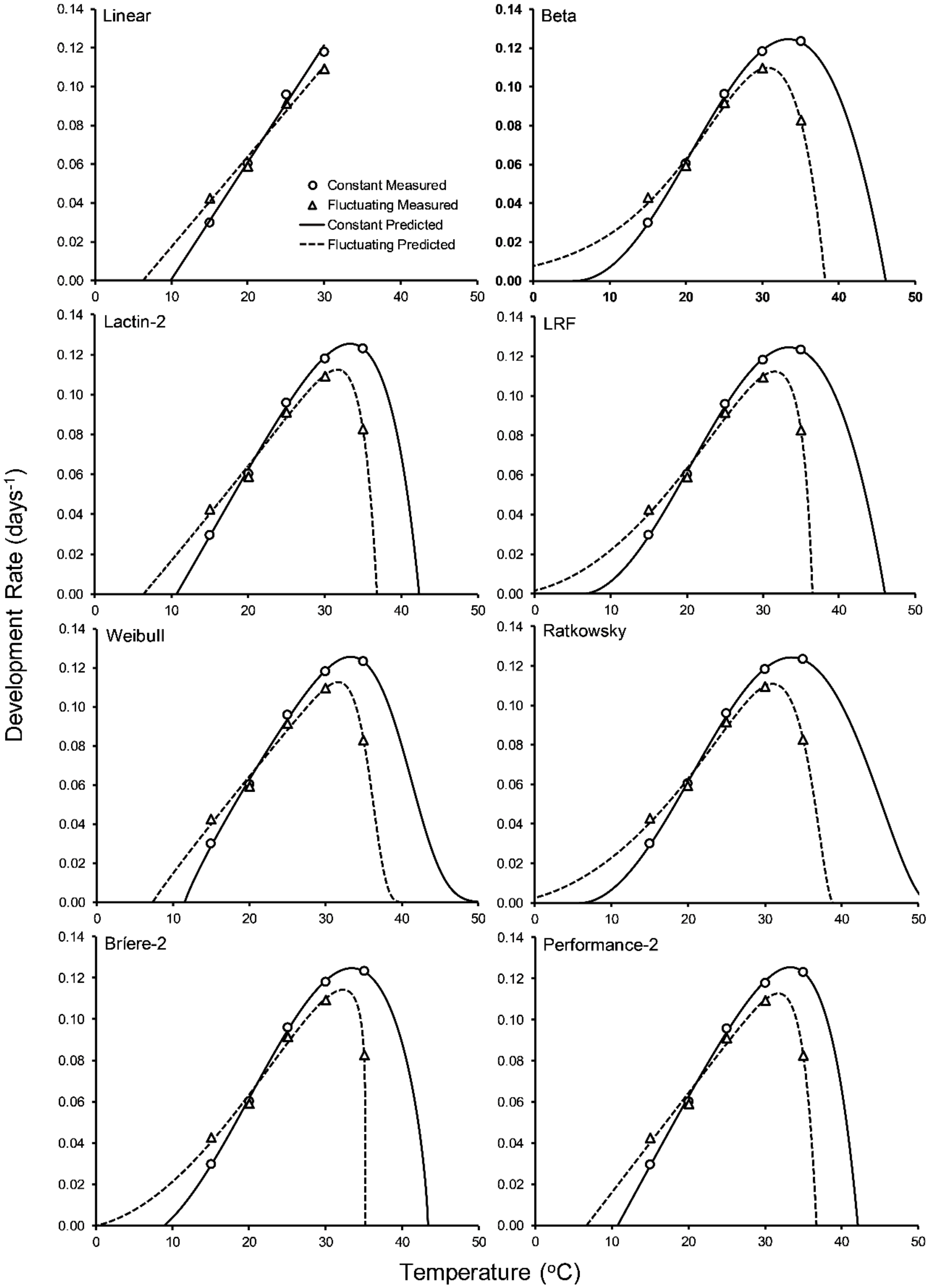
Linear
Beta
Lactin-2
LRF
Weibull
Ratkowsky
Bríere-2
Performance-2
Constant Measured
Fluctuating Measured
Constant Predicted
Fluctuating Predicted
Development Rate (days^{-1})
Temperature (°C)
0.00
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0
10
20
30
40
50

Figure 2.1: Relationship between temperature (^{o}C; x-axis) and development rate (days^{-1}; y-axis) of *Tamarixia radiata* as described by eight thermal performance functions. Solid lines represent model predictions for constant temperatures and dashed lines for fluctuating temperatures. Experimentally measured values are represented by circles (constant) and triangles (fluctuating). The 35^{o}C data point in both the constant and fluctuating dataset has been omitted from the linear model due to outlier status. The 12^{o}C data point (fluctuating) has been omitted from both the linear and nonlinear models to facilitate direct comparison between constant and fluctuating datasets.

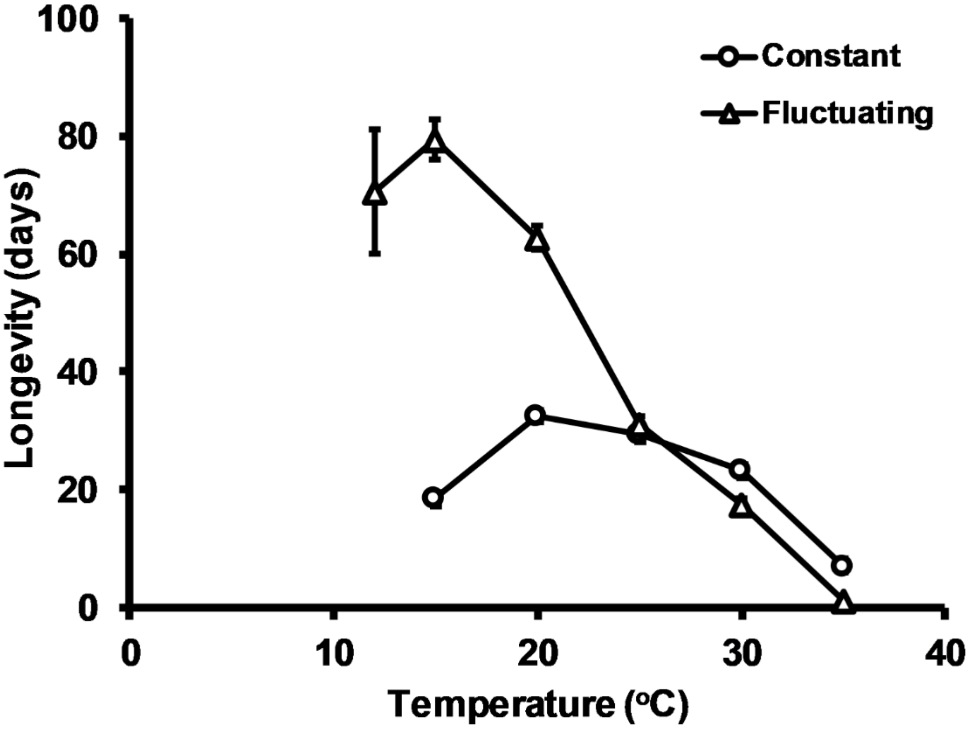

Figure 2.2: Average longevity (days) of adult *Tamarixia radiata* maintained under various constant (circle) and fluctuating (triangle) average daily temperatures (°C).

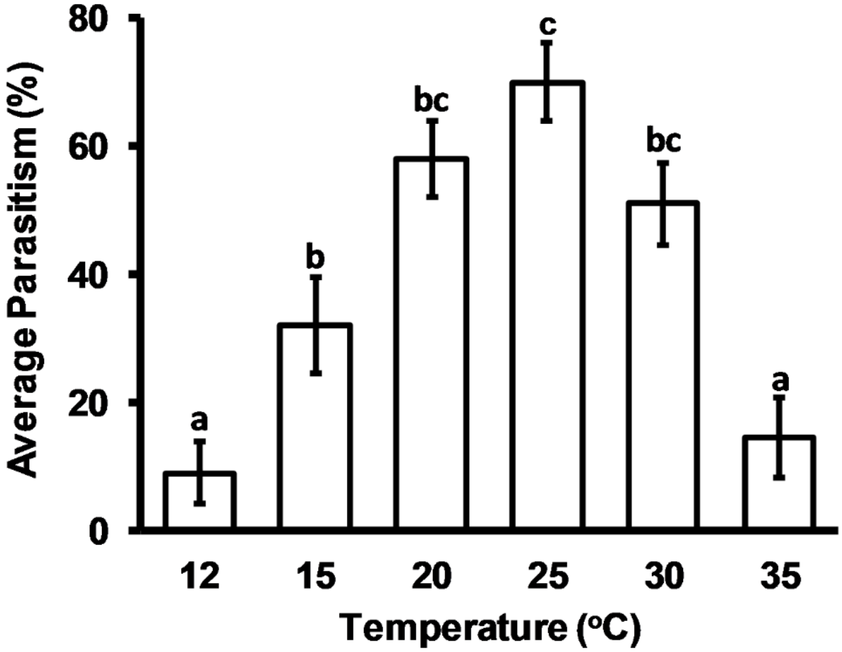

Figure 2.3: Comparison of parasitism rates of *Diaphorina citri* by *Tamarixia radiata* in fluctuating regime cohorts across average daily temperatures (^{o}C). Means followed by the same letter are not significantly different at $\alpha = 0.05$.

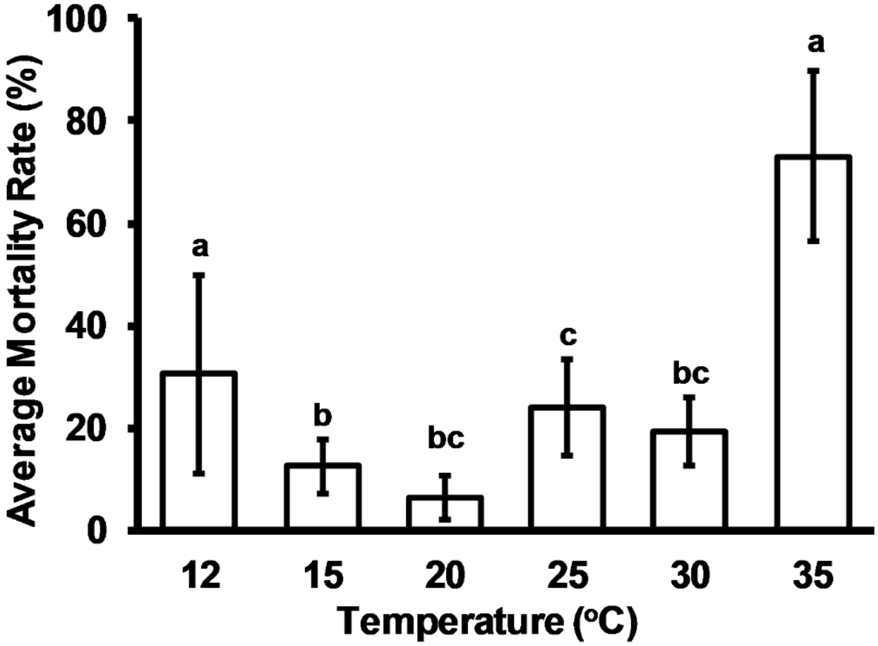

Figure 2.4: Developmental mortality of *Tamarixia radiata* in fluctuating regime cohorts compared across average daily temperatures (^{o}C). Means followed by the same letter are not significantly different at $\alpha = 0.05$.

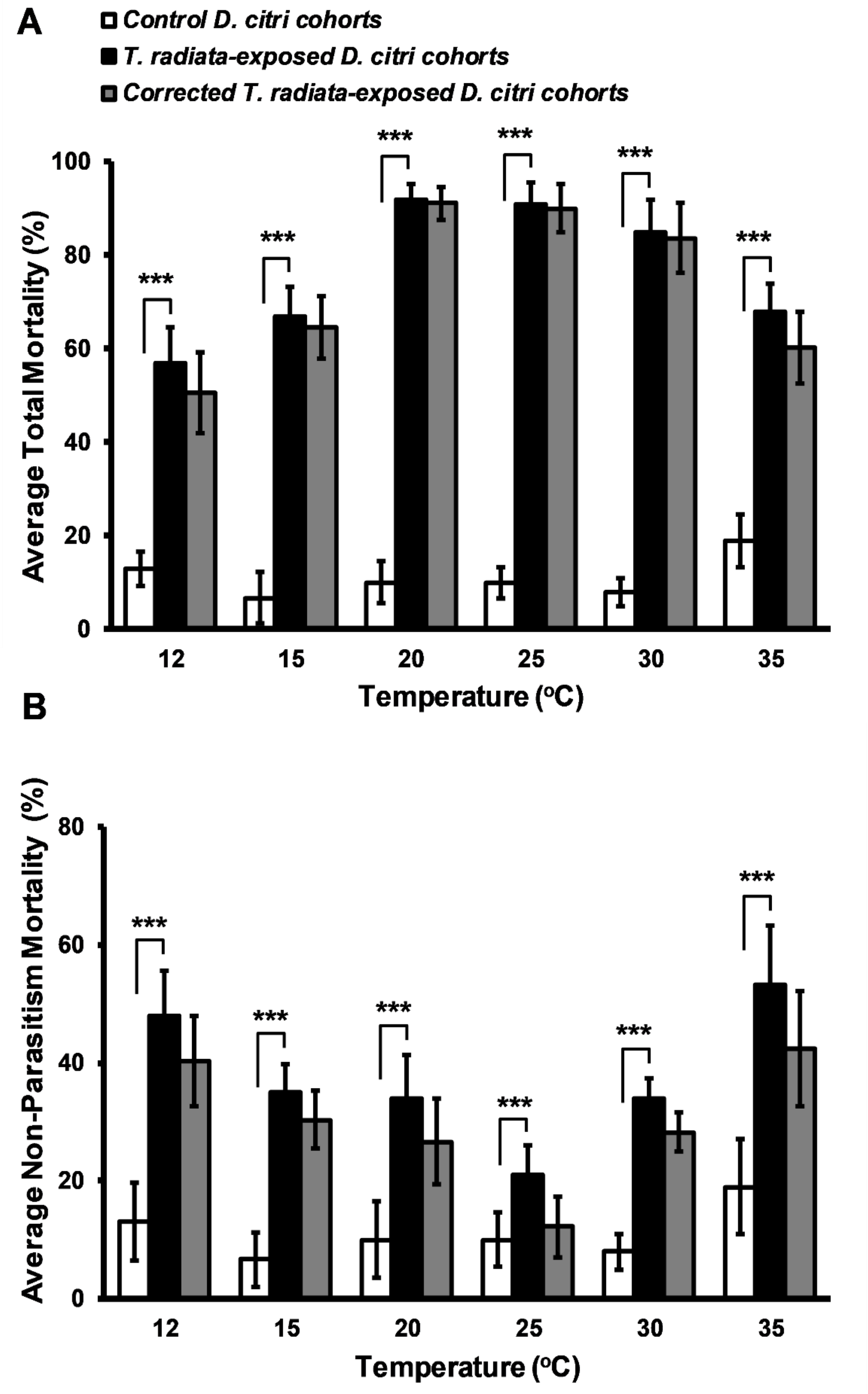
A
◻ Control D. citri cohorts
■ T. radiata-exposed D. citri cohorts
▨ Corrected T. radiata-exposed D. citri cohorts
Average Total Mortality (%)
0
20
40
60
80
100
***
***
***
***
***
***
12
15
20
25
30
35
Temperature (°C)
B
Average Non-Parasitism Mortality (%)
0
20
40
60
80
***
***
***
***
***
***
12
15
20
25
30
35
Temperature (°C)

Figure 2.5: Average total mortality rate (A) and average mortality rate excluding parasitism (B) of *Diaphorina citri* nymphs reared under fluctuating temperature regimes for control cohorts (white) and *Tamarixia radiata*-exposed cohorts (black). Grey bars represent total mortality in *T. radiata*-exposed cohorts corrected for baseline mortality with the Schneider-Orelli formula. Asterisks indicate a significant difference in *D. citri* mortality between control and parasitoid-exposed cohorts at $\alpha = 0.05$.

Chapter 3: A low-toxicity baiting program precipitates collapse of Argentine ant and ant-associated hemipteran pest populations in commercial citrus

Abstract

The Argentine ant, *Linepithema humile* Mayr (Hymenoptera: Formicidae), disrupts biological control agents which induces population explosions of the honeydew-producing hemipteran pests infesting agroecosystems. A multi-season liquid baiting program was evaluated for control of *L. humile* and ant-associated hemipteran pests in six commercial citrus orchards in southern California. A 92% reduction in *L. humile* activity was achieved within 2 months of treatment and activity was 95% lower when compared to trees in untreated control plots. Ant control resulted in the near elimination of hemipteran pest populations on trees in treated plots. Pre- and post-baseline estimates indicated that a 97, 84, and 99% decrease in twig, flush, and fruit infestation levels by pest hemipterans was achieved one year after treatment. Furthermore, cumulative hemipteran pest infestation frequency was 10 times lower and cumulative colony size was 5 times lower on treated trees than control trees during the final year of study. Examining individual hemipteran pest species, the frequency of infestation by *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) was 75% lower, *Coccus hesperidum* L. (Hemiptera: Coccidae) 95% lower, *Planococcus citri* Risso (Hemiptera: Pseudococcidae) 98% – 100% lower, and *Aonidiella aurantii* Maskell (Hemiptera: Diaspidae) 70 – 80% lower in treated trees relative to control trees. A temporary surge in predator activity shortly after treatment application may explain the rapid decrease in pest populations. These results suggest that *L. humile* management in commercial citrus can

drastically improve biological control of several economically important ant-associated hemipteran pests and, consequently, should be considered a critical constituent of integrated pest management programs targeting these pests.

Introduction

Argentine ant, *Linepithema humile* Mayr (Hymenoptera: Formicidae), pervades natural and anthropogenic environments globally, causing significant ecological and economic damage (Silverman and Brightwell 2008). Throughout its introduced range, *L. humile* forms sprawling, interconnected nest districts which exhibit tremendous growth capacity (Heller et al. 2008). A cooperative, high-density colony structure along with efficient foraging strategies and highly aggressive behavior facilitates rapid monopolization of resources (Holway 1999; Suarez et al. 2001; Silverman and Brightwell 2008; Rowles and Silverman 2009). Consequently, *L. humile* invasions are often characterized by the displacement of native ant and arthropod species (reviewed in Holway et al. 2002). These effects cascade through food webs, fundamentally altering community structure and function, and disrupting economically important ecosystem services such as pollination and biological control (Holway et al. 2002; LeVan and Holway 2015).

A mounting body of evidence suggests that access to plant- and insect-derived carbohydrate, especially monosaccharides (e.g., sucrose, glucose), are the primary macronutrients supporting *L. humile* populations in invaded environments (Grover et al. 2007; Helms and Vinson 2008; Kay et al. 2010; Wilder et al. 2011; Helms 2013; Shik and Silverman 2013; Shik et al. 2014; Wills et al. 2015; Wittman et al. 2018). In agroecosystems, sugar is widely available in the form of honeydew, an abundant and perpetually available exudate produced from sap-feeding hemipteran pests (Vega and Rust 2001). Consequently, aggregations of honeydew-producing hemipterans (HPHs) are commonly tended by ant workers (Way 1963; Ness and Bronstein 2004). To maintain access to a reliable supply of honeydew, ants, especially invasive ants, defend pest colonies from predators and parasitoids and may selectively cull parasitized individuals (DeBach et al. 1951; Frazer and van den Bosch 1973; Holway et al. 2002; Ness and Bronstein 2004). The effect of sugar feeding ants on natural enemies of HPHs includes mortality resulting from ant attacks, exclusion from hemipteran prey required for feeding and reproduction, and reduced access to hemipteran honeydew (the predominant sugar source available year-round in citrus) which supplies energy for host-searching and increased longevity (Tena et al. 2013, 2016, 2018; Calabuig et al. 2015). As sap-feeding hemipterans present in agricultural systems are generally considered pests, many of which are invasive, these negative interactions often impede the efficacy of biological control programs (Vega and Rust 2001; Ness and Bronstein 2004; Helms 2013). Independent of the effects of natural enemy protection, ant attendance may also improve mutualist development rate, body size, reproductive output, and survivorship (reviewed in Yoo et al. 2011 and Karami-Jamour et al. 2018). The mechanisms through which these individual-level benefits are gained include increased uptake and assimilation of phloem (stimulated by antennation by ants), dispersal of pests to new feeding sites, and removal of honeydew waste which reduces drowning risk and fungal contamination of nearby feeding and oviposition sites (Bartlett 1961; Way 1963; Buckley 1987; Mensah and

Madden 1992; Yoo et al. 2011). In combination with natural enemy suppression, these ant-mediated effects can facilitate population growth and spread of tended pests and increase associated plant damage (Holway et al. 2002; Ness and Bronstein 2004).

As a numerically and behaviorally dominant sugar-feeding ant species, *L. humile* readily forms food-for-protection mutualisms with HPHs in agroecosystems (Newell and Barber 1913; Vega and Rust 2001). *Linepithema humile* are physiologically and behaviorally adapted for rapid collection and transport of liquid sugar (Eisner 1957; Davidson 1998). Consequently, high-density populations process vast quantities of honeydew from hemipteran mutualists, which forms the majority of their diet (Markin 1970; Abril et al. 2014). This specialized honeydew foraging strategy in combination with a high patrolling tempo and level of aggression affords *L. humile*-tended hemipteran colonies greater protection from natural enemies than that provided by the native ants *L. humile* displaces (Holway 1999; Powell and Silverman 2010; Novgordova and Gavrilyuk 2012). Consequently, *L. humile* drives HPH pest outbreaks and concomitant economic injury (i.e., plant damage, pathogen transmission, and yield loss) in agricultural systems (Bartlett 1961; Way 1963; Moreno et al. 1987; James 1997; Ness and Bronstein 2004; Daane et al. 2007, 2008; Calabuig et al. 2014; Milosavljević et al. 2017; Schall and Hoddle 2017). Therefore, *L. humile* control is a critical constituent of integrated pest management programs targeting infestations of HPHs in commercial agriculture, particularly in grapes and citrus (Moreno et al. 1987; Cooper et al. 2008; Martinez-Ferrer et al. 2017; McCalla et al. in press).

The southern California citrus agroecosystem provides optimal proliferative conditions for *L. humile*: abundant honeydew producing mutualists (e.g., invasive mealybugs, scales, aphids, and psyllids), year-round moisture from irrigation, and a mild Mediterranean climate (Vega and Rust 2001). Consequently, infestations of *L. humile* are severe (Schall and Hoddle 2017; McCalla et al. in press). One *L. humile*-tended hemipteran pest, the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), threatens the long-term viability of California's $7.1 billion-per-year citrus industry. *Diaphorina citri* is the vector of the phloem-limited bacterial pathogen, *Candidatus* Liberibacter asiaticus (*C*Las), that causes huanglongbing, a lethal disease responsible for the decline of citrus industries globally (Batool et al. 2007; Grafton-Cardwell et al. 2013; Hall et al. 2013; Babcock 2018). The majority of *D. citri* colonies in southern California are tended by *L. humile*, and the presence of tending ants has been associated with reduced densities of natural enemies, fewer oviposition and predation attempts, and lower rates of parasitism (Tena et al. 2013, 2017; Kistner et al. 2017). For example, Schall and Hoddle (2017) reported that 95% of *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), a parasitoid of *D. citri* nymphs, were killed or harassed by workers when attempting to forage on *L. humile*-tended *D. citri* colonies. Tena et al. (2013) found that *D. citri* colonies actively tended by *L. humile* had significantly lower rates of parasitism and were more than twice the size of those with no ant attendance. In perturbation studies where *L. humile* was controlled with a liquid baiting treatment or sticky barrier in citrus trees, parasitism of *D. citri* was 70 to 800% higher and predator activity on colonies was 1 to 4 times greater when compared to trees lacking ant control

(Milosavljević et al. 2017; Schall and Hoddle 2017). These studies have investigated the relationship between biological control of *D. citri* and *L. humile* attendance of colonies primarily in urban citrus. However, large-scale manipulative field experiments in commercial citrus orchards are needed to quantify the effect of *L. humile* control on the abundance and density of *D. citri* and infestations of other species of HPH pests.

A well-accepted paradigm is that *L. humile* disrupts biocontrol and induces population increases of citrus-infesting, sap-feeding hemipteran pests. In a classic series of laboratory and field experiments published by Bartlett (1961), it was revealed that oviposition by parasitoids of brown soft scale (*Coccus hesperidum* L.; Hemiptera: Coccidae), a polyphagous pest infesting citrus, was reduced in the presence of *L. humile* by as much as 98.4%. When *L. humile* was chemically eliminated in the field, *C. hesperidum* colonies were extirpated by natural enemies in 2.5 months. Similarly, Moreno et al. (1987) reported that *L. humile* treatment with an insecticidal band or ground spray significantly reduced infestation of oranges in California by *L. humile*, and within 2 months natural enemies suppressed populations of citrus mealybug, *Planococcus citri* Risso (Hemiptera: Pseudococcidae), and wooly whitefly, *Aleurothrixus floccosus* Maskell (Hemiptera: Aleyrodidae). In eastern Spain, Calabuig et al. (2014) reported a significant reduction of 26 to 40% in infestation rates of *A. floccosus* on ant-excluded shoots in comparison with control shoots on navel orange and clementine mandarin. DeBach et al. (1951) reported that colonies of *P. citri* and *C. hesperidum* infesting lemons were virtually absent on ant-free trees in comparison to the high densities observed in *L. humile*-infested trees. This study also provided the first report that densities of the non-honeydew producing California red scale, *Aonidiella aurantii* Maskell (Hemiptera: Diaspidae), were lower (by up to 150 times) in ant-free trees. Several studies have since investigated the positive association between ant activity and densities of *A. aurantii* (DeBach et al. 1958; Moreno et al. 1987; Murdoch et al. 1995; James et al. 1997; Ferrer et al. 2003; Pekas et al. 2010; Yoo et al. 2013; Calabuig et al. 2014). The proposed mechanism is inadvertent disturbance of *A. aurantii* natural enemies by ant workers as they search for and collect honeydew from nearby co-occurring hemipteran pests (DeBach 1958; Reeve and Murdoch 1986; Heimpel et al. 1997; Ferrer et al. 2003).

Despite the rich historical record of research investigating the relationship between *L. humile* and hemipteran pests in citrus, new studies are needed to address the methodological limitations of previous work (e.g., localized ant exclusion with barriers vs. population-level suppression at orchard scale) and regional differences in pest composition due to the acquisition of new invasive insects (e.g., *D. citri*) within the modern citrus agroecosystem. First, the current understanding of the relationship between *L. humile* and *D. citri* is limited, and no studies have investigated the long-term impact of ant control on populations of *D. citri*. This information is highly relevant, as *D. citri* is currently the most economically significant insect pest in citrus worldwide. In addition, most of the aforementioned studies have been spatially or temporally limited and utilized treatments that are economically impractical (i.e., labor-intensive sticky barriers to exclude ants from pest colonies) or ineffective for biocontrol programs in commercial settings (i.e., broad-spectrum insecticides that extirpate natural enemy populations).

Much of this work has also focused on individual taxa rather than hemipteran pest assemblages (overlooking important competitive interactions) or emphasized the effect on biological control agents without subsequently examining pest infestation levels in orchards, a more direct measure of economic impact. To address the shortcomings of previous studies investigating the effects of *L. humile* on pest hemipteran populations and biocontrol services, this study: (1) was replicated across plots within commercial orchards over a multi-season period, (2), utilized a population-level ant control treatment that preserved biocontrol agents and could be reasonably applied in conventional settings, (3), evaluated treatment effects with multiple measures of biocontrol efficacy, and (4), accounted for complex, multi-species interactions by simultaneously monitoring the most prevalent hemipteran pest specie s in the southern California citrus agroecosystem.

Specifically, the efficacy of an orchard-wide, low-toxicity liquid baiting program for control of *L. humile* was evaluated in six commercial citrus groves across an 18-month treatment period. Following confirmation that the treatment adequately suppressed *L. humile* populations, the effect of this control on fruit, flush, and twig infestation by four ant-associated, economically significant pests (i.e., *D.* citri, *P. citri*, *C. hesperidum*, and *A. aurantii*) was examined. Multiple measures were collected for each pest species: frequency of niche infestation (i.e., twigs, flush, and fruit) by pests, pest colony size, parasitism rate, and presence and abundance of natural enemies categorized by morphotaxa (i.e., predators or parasitoids). In addition, the relationship between *L. humile* and hemipteran pest species was investigated by comparing absolute and relative ant attendance. Collectively, these measures allowed the evaluation of the net benefit of *L. humile* control for management of hemipteran citrus pests in commercial citrus orchards.

Materials and methods

Field site and plot selection. This study was conducted across an 18-month-period from August 2016 to January 2018 in six commercial navel orange groves located in southern California (i.e., Redlands, Mentone, Highland, and Pauma Valley, CA, USA). Selected groves were at minimum 1.5 Ha in size, spatially separated by at least 2 km, drip irrigated, and chemically untreated for pests the year prior to experiment start and for its duration. Trees were similar in height and possessed canopies that did not contact the ground or adjacent canopies.

A nested experimental design was utilized, with trees nested within four-by-four tree plots and plots within sites. In each experimental plot, three of the four innermost trees were randomly selected for monitoring of ants and ant-tended hemipteran pests. Initially, 12 plots per site were monitored for baseline *L. humile* activity with one-minute visual estimates of foragers ascending and descending tree trunks. The six plots with the most similar starting ant activities were selected for continued monitoring. To ensure treatment effects remained localized, plots were separated by 50 to 65 m, an interval exceeding the maximum recorded movement of liquid bait by *L. humile* foragers in distance marking studies (Ripa et al. 1999; Vega and Rust 2003, Greenberg et al. 2006; Cooper et al. 2008; Hogg et al. 2018; McCalla et al. in press). Across all sites, 108 trees in 36 plots (i.e., 54 trees in 18 plots for each treatment group) were surveyed monthly.

Treatment set-up and maintenance. At each site, plots were randomly assigned to receive an ant treatment program or no treatment for the experimental duration. Ant control in treatment plots was achieved with the deployment of six plastic dispensers (KM Ant Pro LLC., Nokomis, FL, USA) loaded with 0.5 L of low-toxicity liquid bait (i.e., 0.0001% thiamethoxam in 25% sucrose solution). To increase the rate of bait discovery by *L. humile* and potentially increase target specificity, 1 mL of the putative *L. humile* trail pheromone, (Z)-9-hexadecenal, was mixed into the bait in each reservoir (microencapsulated formulation, 5.6 mg/mL; Suterra, LLC., Bend, OR) (Welzel and Choe 2016). Previous studies have reported that this bait composition provides excellent suppression of *L. humile* populations in managed settings (Cooper et al. 2008; Milosavljević et al. 2017; Tay et al. 2017; Schall et al. 2018; McCalla et al. in press). Bait stations were placed centrally in each plot, spaced evenly around the four innermost trees. To minimize the loss of efficacy or palatability associated with extended bait deployment (e.g., evaporative loss, sugar fermentation, crystalline residue build-up) (McCalla et al. in press), bait was replenished, and reservoirs cleaned on a monthly basis.

Field sampling. All 108 experimental trees were monitored monthly (Aug 2016 – Jan 2018) for infestations of ants and ant-associated hemipteran pests. Ant activity was assessed with simultaneous one-minute visual counts of all foraging ants ascending and descending across a transverse cross-section of each tree trunk ~0.5 m from the ground. Visual counts were collected between 8.00 and 12.00 h to minimize estimation bias in ant activity related to daily thermal fluctuations. Tree canopies were surveyed for infestation of twigs, flush, and fruit by the four most predominant and economically important ant-associated hemipteran pests present in monitored groves: Asian citrus psyllid, *Diaphorina citri*, citrus mealybug, *Planococcus citri,* brown soft scale, *Coccus hesperidum,* and California red scale, *Aonidiella aurantii*. To determine the frequency of niche infestation by each hemipteran pest species, tree canopies were divided into quadrants based on cardinal direction, each of which was inspected visually for five minutes. Within this assessment period, five twigs, flush, and fruit per quadrant were randomly selected for pest screening at a canopy height of 1 – 2 m. In total, 20 twigs, 20 flush, and 20 fruit were examined per tree. Twig tips (30 cm) were inspected for *C. hesperidum* and *A. aurantii*, flush growth (i.e., immature leaves on green twigs) for *D. citri* and *P. citri,* and fruit for *P. citri* and *A. aurantii*. The percentage of samples infested by each pest species within each niche category (i.e., twigs, flush, and fruit) was recorded. In addition, a subset of four colonies of each pest species within niche was collected in quart-sized Ziploc plastic bags (S.C. Johnson & Son Inc., Racine, WI, USA). If fewer colonies were present, all were collected. To collect pest colonies on twigs, pruning shears were used to excise 5.5 cm samples from the tree, beginning at the edge of the colony nearest the twig tip. Whole fruit measuring at least 4 cm in diameter were collected with 0.5 cm of stem intact. Flush was removed at the node. Samples were stored in an ice-packed cooler in the field and transferred to a -5^{o}C freezer in the laboratory for up to two weeks before processing.

Laboratory processing of samples. Field collected samples were thawed in the refrigerator for ~2 h prior to assessing colony size, parasitism rate, and the presence of ants and natural enemies. The length of flush and twig sections and the diameter of halved fruit were recorded so that colony density could be standardized by niche surface area. Samples were examined under a dissecting microscope to estimate the number of individuals per colony as well as their life stage, size class (i.e., based on length in mm), and parasitism status. Pest species were further categorized into ‘parasitism-susceptible’ and ‘non-susceptible’ groups based on the host size or life stage preference of their dominant parasitoids (see **Table 3.3** for additional detail). Parasitism rate was calculated as the number of parasitized individuals per colony divided by the number of parasitized and non-parasitized susceptible individuals per colony. To ensure estimates of parasitism were accurate, all individuals in susceptible life stages not appearing visually parasitized (as indicated by mummification or the presence of parasitoid exit holes) were examined on both dorsal and ventral sides for the presence of parasitoid eggs, larvae, and pupae. For *Aonidiella aurantii* colonies, individuals in all life stages were additionally inspected for melanized mutilation marks on the cuticle, an indication of probing by parasitoids during host feeding and attempted oviposition. Because the resultant physical trauma and potential envenomation nearly always results in mortality, mutilized individuals were considered parasitized (Forster et al. 1995).

Finally, the presence and number of ants, predators, and adult parasitoids in pest colonies was recorded. Natural enemies were separated into morphotaxa (i.e., coccinellids, lacewings, parasitoids, predatory thrips, and syrphids). The number of ant workers collected in hemipteran colonies was used to determine the strength of their association with respective pest species. Three ratios were calculated: the proportion of pest colonies tended by ants and “absolute ant attendance” (number of ants per hemipteran colony), indicators of overall ant interest in hemipteran colonies, and "relative ant attendance" (number of ants divided by number of hemipteran individuals per colony), a measure of the level of protection ants afforded hemipteran individuals within colonies (Tena et al. 2013).

Statistical analyses. All analyses were conducted in R (version 3.6.1, R Core Team [2019]) at the $\alpha = 0.05$ level of significance. Model distributions (e.g., Poisson, zero-inflated Poisson, binomial, and negative binomial with a log link function) were selected by comparing AICc scores for the highest value with likelihood ratio tests ("*lmtest*" function in the lmtest package). To determine the optimal model for each dependent variable, all descriptor variables of interest were "dredged" to produce every possible model combination ("*dredge*" function in the MuMln package). Delta AICc was compared across all model candidates, and the model with the lowest value was selected for further analysis. Generalized linear mixed models (GLMMs) with the Laplace approximation ("*glmer*" and "*glmmTMB*" functions in the lme4 and glmmTMB packages, respectively) were used exclusively as they allowed definition of the nested error structure used in this study (i.e., trees within plots within sites). This was done to account for the pseudoreplication inherent in such hierarchical experimental designs (Spurgeon 2019). For all analyses, the random error term was defined as tree nested within plot nested within site. Although data were collected from August 2016 to January 2018, data collected before February 2017 were excluded from most analyses. The reasoning for this decision was twofold: (1), the liquid baiting program deployed in treated plots required a few months to exert its full effect on *L. humile* populations, and

(2), to ensure ant, hemipteran pest, and natural enemy phenological data were evenly weighted across a single 12-month sampling period. Following GLMM analyses, pairwise comparisons were made with Tukey-adjusted estimated marginal means ("*emmeans*" function in the emmeans package).

Ant activity. *Linepithema humile* was the dominant ant species observed at all sites across all monitoring dates, with 1,305 total sightings on individual trees in total. Although *Solenopsis invicta* Buren (Hymenoptera: Formicidae) and *Brachymyrmex patagonicus* Mayr (Hymenoptera: Formicidae) were observed infrequently (5 and 22 sightings, respectively), these species were excluded from analyses due to the rarity of sightings and low activity level when present. Differences in *L. humile* activity in baited and control trees were assessed with a GLMM (negative binomial error), where treatment, month, and the interaction between treatment and month were fixed factors. Contrasts were made between treated and untreated trees across corresponding months. GLMMs were also used to compare absolute (negative binomial error) and relative (zero-inflated Poisson error) ant activities on hemipteran pest colonies. Because ants were rarely found in pest colonies from baited trees, only colonies from control trees were included in related analyses. Hemipteran pest species within plant niche, month, and their interaction were the fixed factors for absolute ant activity and the proportion of ant-tended pest colonies. The same factors without the interaction were included in the model for relative ant activity. Follow-up tests (emmeans) compared absolute and relative ant activities among hemipteran pest species within niche.

Infestation rate. The infestation rate of twigs, flush, and fruit by all hemipteran pests and for each pest species individually (i.e., *A. aurantii* and *C. hesperidum* on twigs, *D. citri* and *P. citri* on flush, and *P. citri* and *A. aurantii* on fruit) were compared between ant suppression and control treatments with GLMMs (Poisson, zero-inflated Poisson, or negative binomial error). Final models included a combination of the following factors: treatment, month, and treatment: month as fixed factors and air and soil temperature as covariates (see **Table 3.1** for models and distributions). Infestation rate was further compared between treatments across months with estimated marginal means. Baseline (August 2016) and post-baseline (August 2017) infestation rates of twigs, flush, and fruit were compared between ant suppression and control treatments with GLMMs (negative binomial error). Treatment, pre- or post-baseline month, and their interaction were the fixed factors for all models, and the model for flush additionally included the covariate soil temperature. Infestation rates for combined pests within plant niche were compared between dates and treatments with estimated marginal mean contrasts.

Colony density and parasitism. The relative severity of pest infestations was evaluated by comparing hemipteran colony size (i.e., number of individuals per colony) across species and between treatments with a GLMM (Poisson error). The fixed factors were treatment, species within niche, and their interaction. The percent parasitism of pest colonies was also evaluated with a GLMM (binomial error weighted to the number of individuals per colony). Treatment, species within niche, and their interaction were fixed factors. Date was a covariate. Post-hoc comparisons of colony size and parasitism rate were made between ant control treatments across hemipteran pest species within niche using estimated marginal means.

Cumulative infestation frequency and severity. The overall burden of infestation by ant-associated hemipteran pests on experimental trees was examined by converting mean number of infestations and colony size to insect days (ID). These data were then summed across the February 2017 to January 2018 study period to calculate the cumulative infestation burden (cumulative insect days; CID) with the following formulas:

$$ID\ (colony\ density) = 0.5(D_a + D_b)(T_a - T_b)$$

$$ID\ (infestation\ frequency) = 0.5(I_a + I_b)(T_a - T_b)$$

$$CID = \sum ID$$

Here, D_a and D_b are hemipteran pest colony densities and I_a and I_b are hemipteran pest infestation frequencies which correspond with adjacent sampling dates T_a and T_b (i.e., a 30-day-period in the case of this study) (Ruppel 1983). Cumulative insect days, a measure of the total hemipteran pest infestation load per tree across the study duration, were calculated by summing insect days per tree across all sampling dates. Hemipteran pest infestation frequency (i.e., mean number of infestations per tree) and severity (i.e., mean colony size per tree) CIDs were compared between treatments with linear mixed models ("*lmer*" function in the lme4 package). Ant control treatment, month, and their interaction were fixed factors.

Natural enemies. GLMMs (Poisson and binomial error) were used to evaluate the proportion of hemipteran pest colony samples containing natural enemies and natural enemy abundance between ant control treatments. Treatment and hemipteran pest species within plant niche were fixed factors. Treatments were compared with hemipteran pest species within niche using estimated marginal means. The frequency of natural enemies in pest colonies was also compared across morphotaxa groups (i.e., coccinellids, lacewings, parasitoids, predatory thrips, and syrphids) between ant control treatments with a GLMM (Poisson error). Treatment and natural enemy species within niche were fixed factors. Natural enemy functional groups were further compared across treatment with estimated marginal means. Because syrphids were found to be highly prevalent in flush samples, GLMMs (Poisson error) were used to determine if colony densities of *D. citri* and *P. citri* were predicted by syrphid abundance on flush samples. Treatment designation (i.e., baited trees vs. untreated control trees) had a significant effect on hemipteran pest colony size. Consequently, analyses were conducted with untreated control data only. Syrphid presence, date, and their interaction were fixed factors.

Results

Ant activity. Ant control treatment ($\chi^2 = 17.18$, d.f. = 1, $P < 0.0001$), sampling month ($\chi^2 = 429.5$, d.f. = 11, $P < 0.0001$), and the interaction between treatment and month ($\chi^2 = 92.66$, d.f. = 11, $P < 0.0001$) significantly affected mean *L. humile* activity on trees (Feb 2017 – Jan 2018). Activity in untreated control trees was strongly seasonally variable, with peaks recorded mid-summer to mid-fall and minimums from winter to early spring (**Fig. 3.1**). In treated trees, ant activity declined rapidly following deployment of liquid bait-loaded dispensers with reduction from baseline estimates (Aug 2016) of 66%, 91%, and 96% in the first three months post-treatment (Sept, Oct, and Nov 2016, respectively). Ant activity remained 90 to 99.8% lower than pre-treatment levels for the experimental duration, indicating that excellent long-term suppression of *L. humile* populations was achieved with baiting. Estimates of *L. humile* activity were significantly lower in treated trees than control trees in across all concurrent months (Feb 2017 – Jan 2018; $P < 0.01$). Overall, *L. humile* was present in 93.83% of control trees with an average activity of 212.19 ± 8.81 (per min/tree). In treated plots, just 33.02% of trees had ants with an average activity of 11.12 ± 1.53 (per min/tree).

Ant attendance of hemipteran pest colonies. *Linepithema humile* was present on just 5 of the total 276 (1.81%) hemipteran pest colonies collected from treated trees. Consequently, estimates of absolute (total number of ants per hemipteran colony) and relative (number of ants per individual hemipteran within a colony) ant activity were based on samples from control trees, where *L. humile* was present on 720 of the total 2268 (31.75%) hemipteran pest colonies collected. The proportion of hemipteran pest colonies tended by *L. humile* varied by species nested within niche. The pest species with the greatest proportion of *L. humile* tended colonies was *P. citri* on fruit and flush, followed by *C. hesperidum* on twigs (**Fig. 3.2A**). Colonies of *A. aurantii* on fruit were tended more frequently than *D. citri* colonies on flush and *A. aurantii* on twigs. It is important to note that these values likely underestimate the true proportion of ant-tended pest colonies as they are based on a snapshot of ant presence at the time of colony collection in the field. However, the primary goal in recording *L. humile* attendance of hemipteran pest colonies in this study was to establish a hierarchy of *L. humile* preference for all monitored mutualist species, rather than accurately estimating the total proportion of ant-tended colonies.

Hemipteran species within plant niche ($\chi^2 = 203.15$, d.f. = 5, $P < 0.0001$; $\chi^2 = 53.04$, d.f. = 5, $P < 0.0001$) and month ($\chi^2 = 8.83$, d.f. = 1, $P = 0.003$; $\chi^2 = 174.44$, d.f. = 1, $P < 0.0001$) significantly affected the absolute and relative ant attendance of hemipteran pest colonies on control samples, respectively. For absolute *L. humile* activity, the interaction between hemipteran species and month was also a significant model factor ($\chi^2 = 33.21$, d.f. = 1, $P < 0.0001$). *Coccus hesperidum* colonies received significantly greater absolute but not relative ant attendance than *A. aurantii* on twigs ($P = 0.0031$; $P = 0.92$) and *P. citri* received significantly more absolute and relative attendance than *D. citri* on flush ($P < 0.01$) and *A. aurantii* on fruit ($P < 0.05$; **Fig. 3.2A** and **B**). Although there was significantly greater absolute ant attendance of *P. citri* on fruit than flush ($P = 0.0009$) and of *A. aurantii* on fruit than twigs ($P < 0.0001$), when standardizing by the number of individuals per colony (i.e., relative ant activity), these differences were not statistically significant ($P > 0.05$). *Diaphorina citri* were tended by *L. humile* significantly less intensively than all other HPH pest species on a per-individual basis ($P < 0.05$), a finding consistent with previous studies (Tena et al. 2013). Interestingly, individual *D. citri* were also tended significantly less intensively than the non-honeydew producer *A. aurantii* on fruit ($P < 0.0001$).

Pre- and post-baseline comparison. Prior to deployment of bait stations (Aug 2016), there were no significant differences in the mean infestation rate of fruit, flush, and twigs between control and treated trees ($P > 0.05$; **Fig 3.4**). One year later (Aug 2017), the infestation rate of all niches was significantly lower in treated trees in comparison to control trees ($P < 0.001$). Comparing pre- and post-treatment means (i.e., Aug 2016 vs. Aug 2017), a drastic reduction in infestation rate was observed in ant-treated trees ($P < 0.0001$) but not control trees ($P > 0.05$; **Fig. 3.4**). In this one-year-period, infestation rate decreased by 97.29% on twigs, 83.71% on flush, and 99.11% on fruit of treated trees.

Cumulative infestation burden. Pooling all hemipteran pest species within niche and examining across the final year of study (i.e., Feb 2017 – Jan 2018), the mean cumulative infestation frequency (i.e., number of infestations per tree) and mean cumulative infestation severity (i.e., number of individuals per hemipteran pest colony) based on cumulative insect days were significantly affected by treatment ($\chi^2 = 45.72$, d.f. = 1, $P < 0.0001$; $\chi^2 = 25.98$, d.f. = 1, $P < 0.0001$), date ($\chi^2 = 1684.63$, d.f. = 11, $P < 0.0001$; $\chi^2 = 1145.64$, d.f. = 11, $P < 0.0001$), and their interaction ($\chi^2 = 1173.25$, d.f. = 11, $P < 0.0001$; $\chi^2 = 447.56$, d.f. = 11, $P < 0.0001$). On the final sampling month, cumulative infestation frequency was 10 times lower in trees treated for ants (174.72 ± 21.5) than in control trees (1755.07 ± 157.61) ($P < 0.0001$; **Fig 3.3A**). Similarly, cumulative colony density was 4.5 times lower in ant-treated trees (2414.29 ± 402.49) than in control trees (11004.94 ± 1153.09) ($P < 0.0001$; **Fig 3.3B)**.

Overall infestation rate by niches. Across the February 2017 to January 2018 sampling period, a total of 25,856 twigs, 10,616 flush, and 22,116 fruit were examined in the field for the presence of hemipteran pest colonies. In sum, 10 times fewer pest infestations were found on treated trees (324) than control trees (3296). The mean infestation rate was significantly lower in treated trees than control trees for all niches ($\chi^2 = 154.39$, d.f. = 11,

$P < 0.0001$ for twigs; $\chi^2 = 253.50$, d.f. = 11, $P < 0.0001$ for flush; and $\chi^2 = 99.83$, d.f. = 11, $P < 0.0001$ for fruit) (**Table 3.1**). Mean twig infestation was 2.50 ± 0.30% and 0.55 ± 0.09%, mean flush infestation was 16.68 ± 1.19% and 3.33 ± 0.59%, and mean fruit infestation was 17.15 ± 0.95% and 0.86 ± 0.2% in control and treated trees, respectively (**Table 3.2**). In examining individual sampling months, significant differences between treatment groups were observed from May through September 2017 and January 2018 for twigs ($P < 0.01$; **Fig. 3.5A**), March through December 2017 for flush ($P < 0.05$; **Fig. 3.5B**), and May 2017 through January 2018 for fruit ($P < 0.0001$; **Fig. 3.5C).**

Niche infestation rate, colony density, and parasitism of hemipteran pests by species on treated and control trees.

Brown soft scale on twigs. The mean infestation rate of twigs by *C. hesperidum* was significantly lower in treated trees (0.04 ± 0.02%) than control trees (0.83 ± 0.19%) ($\chi^2 = 334.48$, d.f. = 22, $P < 0.0001$; **Table 3.1 and 3.2**). *C. hesperidum* was the least common pest monitored in the study, comprising only 2.28% of all infestations recorded in control trees and 0.84% in treated trees (**Table 3.2**). Infestation rates were highest May through August 2017 in control trees (1.57 – 2.57%) but nearly absent across concurrent months in treated trees (0.00 – 0.28%) (**Fig. 3.6A**). Despite this disparity in means, comparing treatment groups across individual months was not possible due to the lack of variance in treated trees (i.e., 0.00 ± 0.00% infestation in May, July – Aug; N = 54 trees/month).

Mean *C. hesperidum* colony size (i.e., number of individuals) was drastically lower in treated (1.36 ± 0.13) than untreated trees (57.48 ± 8.36; $P < 0.0001$; **Table 3.3)**. Parasitism of *C. hesperidum* in control trees averaged 55.33 ± 4.10% across the last year of study, with peak rates observed May through September 2017 (56.53 – 72.00%) (**Table 3.3)**. No parasitism was recorded in treated trees due to the absence of parasitoid-susceptible life stages in the few collected *C. hesperidum* colonies (N = 3).

California red scale on twigs. The average rate of twig colonization by *A. aurantii* was significantly lower in treated trees (0.51 ± 0.09%) than control trees (1.67 ± 0.24%) (χ^2 = 39.65, d.f. = 11, P < 0.0001; **Table 3.1 and 3.2**). In comparison with other hemipteran pests, *A. aurantii* represented just 4.60% and 10.74% of all infestations observed in control and treated trees, respectively (**Table 3.2**). While the infestation rate in both treatment groups followed a similar trend from February through August 2017, the peak observed from October 2017 through January 2018 in control trees (1.76 – 3.06%) was not present in treated trees (0.00%) (**Fig. 3.6B)**. As with *C. hesperidum*, treatment groups could not be compared across these months due to a lack of variance in treated trees.

On average, *A. aurantii* colonies on twigs from treated trees were less than a quarter of the size of colonies in control trees (3.91 ± 1.21 and 19.64 ± 3.08, respectively) (P < 0.0001; **Table 3.3**). Although mean percent parasitism was significantly higher in control trees (67.62 ± 4.11%) than treated trees (22.93 ± 7.53%; P = 0.011), nearly three-and-a-half times fewer *A. aurantii*-infested twigs were recovered from treated trees (**Table 3.3**). Percent parasitism in control trees was highest from September 2017 through January 2018 (67.73 – 100%).

California red scale on fruit. The average percent of fruit infested by *A. aurantii* was substantially lower in treated trees (0.7 ± 0.13%) than control trees (7.02 ± 0.65%) (χ^2 = 289.6, d.f. = 1, P < 0.0001; **Table 3.1 and 3.2**). *Aonidiella aurantii* fruit infestations comprised a similar proportion of all hemipteran pest infestations in treated (14.74%) and control (19.32%) trees (**Table 3.2)**. The highest rate of *A. aurantii* infestation on fruit was observed from September 2017 through January 2018 in control trees (8.89-15.23%). The rate of infestation present in treated trees across this period was close to zero (0.11-1.76%) ($P < 0.01$; **Fig. 3.6C)**.

The average number of *A. aurantii* individuals per colony was significantly higher on fruit from control trees (98.48 ± 5.84) than fruit from treated trees (36.81 ± 6.79) ($P < 0.0001$; **Table 3.3**). In addition, mean parasitism rate was the highest of all monitored pest species for both control (77.19 ± 1.78%) and treated trees (72.30 ± 5.78%), with no significant difference found between treatments ($P = 1.0$; **Table 3.3**). Parasitism peaked from September 2017 through January 2018 for *A. aurantii* in both treatment groups, with a combined mean rate of 94.21 ± 5.18%. For both treatment groups, colony size and parasitism were significantly higher in *A. aurantii* colonies present on fruit than on twigs ($P < 0.0001$; **Table 3.3**). Previous studies have also reported greater parasitism of *A. aurantii* colonies infesting fruit than twigs. This effect has been attributed to higher exposure of pests to parasitoids on fruit near the canopy perimeter (Moreno et al. 1987; Pekas et al. 2010; Yoo et al.2013; Calabuig et al. 2014).

Citrus mealybug on fruit. The mean percent of fruit colonized by *P. citri* was strongly affected by ant control treatment, with exceptionally low rates observed in treated trees (0.17 ± 0.10%) in comparison to control trees (10.13 ± 0.67%) ($\chi^2 = 1805.5$, d.f. = 22, $P < 0.0001$; **Table 3.1 and 3.2**). *Planococcus citri* on fruit was the second most common pest infestation observed for control trees (27.88% of all infestations), but the fourth for treated trees (3.58% of all infestations) (**Table 3.2**). The high rate of infestation present in control trees from June 2017 through January 2018 (6.88 – 22.69%) was not observed in treated trees (0 – 0.37%) (**Fig. 3.6D)**. The absence of *P. citri* fruit infestation in treated trees examined in Sept, Oct, and Dec prevented comparison of treatment means across these months. However, significant differences in infestation between control and treated trees were present across the rest of the sampling period ($P < 0.0001$ for Jun – Aug, Nov, and Jan).

On average, *P. citri* colonies on fruit in treated trees (17.90 ± 6.12 individuals) were approximately half the size of colonies in control trees (34.01 ± 2.62 individuals) ($P = 0.0002$; **Table 3.3)**. The mean parasitism rate was significantly lower in treated trees (30.74 ± 12.93%) than control trees (55.67 ± 1.44%; $P = 0.0164$), an effect which could possibly be attributed to the low number of colonies retrieved from treated trees (N = 10) relative to those collected from control trees (N = 743) (**Table 3.3**). The highest rates of parasitism were observed in control trees between September 2017 and January 2018 (58.36 – 96.40%).

Citrus mealybug on flush. Mean flush infestation by *P. citri* was higher in control trees (3.27 ± 0.52%) than treated trees, where no colonies were found across all sampling months (0.00 ± 0.00%) (**Table 3.1 and 3.2**). In control trees, *P. citri* infestations comprised 9.0% of all pest infestations and 19.6% of infestations in flush (**Table 3.2**). Infestation by *P. citri* was most prevalent between June and December 2017 where the infestation rate ranged from 3.63 to 6.96% (**Fig. 3.6E)**. Despite the observable difference between treatment groups, the absence of variance in treated trees across the entire sampling period produced challenges in model fitting. Although convergence was achieved with a reduced model fit to a weighted binomial distribution, treatment groups could not be compared reliably (**Table 3.1**).

For *P. citri* infesting flush in control trees, the average number of individuals per colony was 19.21 ± 2.48 and the average parasitism rate was 28.47 ± 2.49% **(Table 3.3)**. Peak parasitism rate was observed between July 2017 and January 2018 and ranged from 16.40 to 43.33%. Both colony size and percent parasitism were significantly lower for *P. citri* infestations in flush than fruit ($P < 0.0001$).

Asian citrus psyllid on flush. On average, colonization of flush by *D. citri* was four times lower in treated trees (3.33 ± 0.59%) than control trees (13.41 ± 1.09%) ($\chi^2 = 251.8$, d.f. = 11, $P < 0.0001$; **Table 3.1 and 3.2**). Despite considerably low levels of infestation in treated trees, *D. citri* was the most common pest observed in both treatment groups. It was responsible for 80.40% of flush infestations in control trees and 100% in treated trees (**Table 3.2**). In addition, *D. citri* represented 36.92% of all pest infestations in control trees and twice that in trees treated for ants (70.1%) (**Table 3.2**). The *D. citri* infestation rate was consistently high in flush from control trees between July and December 2017 (20.5 ± 1.75%), with peaks observed in July (35.15 ± 6.63%) and October (24.38 ± 4.01%). Infestation rate was not as high in treated trees, which averaged 4.49 ± 0.91% from July to December 2017 and peaked at 2.01 ± 1.61% and 10.33 ± 3.93% in July and October, respectively **(Fig 3.6F**). Significant treatment differences were present March through May 2017 and July through December 2017 ($P < 0.01$).

The average number of *D. citri* per colony was not significantly different between control (32.24 ± 1.79) and treated trees (37.45 ± 3.86) ($P = 0.562$; **Table 3.3**). *Diaphorina citri* densities peaked in October and November 2017, with a mean colony size of 52.12 ± 5.19 and 83.77 ± 11.08, respectively. Mean percent parasitism of *D. citri* colonies was also not significantly different between control (4.47 ± 1.13%) and treated trees (2.97 ± 1.9%) ($P = 1.0$), but it was the lowest overall rate recorded for any monitored pest (**Table 3.3**). Despite generally low levels of parasitism, a notable increase was observed in November 2017 for both treatment groups, with a combined mean rate of 20.09 ± 7.82%.

Natural enemy presence and density in pest colonies. Beneficial insects were present in 3.3 ± 0.37% of hemipteran pest colonies collected from control trees and 3.23 ± 1.06% of colonies from treated trees. Natural enemy prevalence (i.e., percent of pest colonies containing natural enemies) and density (i.e., number of natural enemies recovered per pest colony) were not significantly different between treatment groups ($\chi^2 = 2.1$, d.f. = 1, $P = 0.148$; $\chi^2 = 1.68$, d.f. = 1, $P = 0.196$). However, prevalence and density did vary by pest species within niche ($\chi^2 = 88.89$, d.f. = 5, $P < 0.0001$; $\chi^2 = 57.212$, d.f. = 5, $P < 0.0001$). Natural enemies were present in a greater proportion of *D. citri* (8.36 ± 1.08%) and *P. citri* (7.18 ± 1.85%) colonies infesting flush in comparison to all other pest species ($P < 0.0001$), although these differences were not significantly different for *C. hesperidum* or *A. aurantii* on twigs ($P > 0.05$; **Fig 3.7A**). In addition, the density of natural enemies was significantly higher on *D. citri* (0.14 ± 0.02) and *P. citri* (0.13 ± 0.04) colonies infesting flush than all other pests ($P < 0.05$) except *C. hesperidum* on twigs ($P > 0.05$). There was no significant difference in the prevalence or density of natural enemies between *D. citri* and *P. citri* colonies on flush ($P > 0.05$).

Five distinct morphotaxa groups were observed in hemipteran pest colonies: coccinellids, lacewings, parasitoids, predatory thrips, and syrphids. Frequency of recovery significantly differed among these morphotaxa ($\chi^2 = 77.69$, d.f. = 4, $P < 0.0001$). Syrphids were the most common group, accounting for 70.71% of all collected specimens ($P < 0.0001$). Predatory thrips accounted for 12.14% of specimens, lacewing larvae 8.57%, parasitoids 5.0%, and coccinellids 2.86%. Syrphid larvae were collected entirely from *D. citri* (6.69 ± 0.97%) and *P. citri* (6.67 ± 1.79%) colonies infesting flush (**Fig 3.7B**). In control trees across the full study duration (Aug 2016 – Jan 2018), syrphid prevalence was associated with colony size of *D. citri* (**Fig 3.8A**) and *P. citri* (**Fig 3.8B**), although synchronization appeared stronger with the former. Syrphid presence ($\chi^2 = 1276.04$, d.f. = 1, $P < 0.0001$; $\chi^2 = 46.99$, d.f. = 1, $P < 0.0001$), month ($\chi^2 = 4844.54$, d.f. = 15, $P < 0.0001$; $\chi^2 = 5243.47$, d.f. = 16, $P < 0.0001$), and their interaction ($\chi^2 = 427.01$, d.f. = 10, $P < 0.0001$; $\chi^2 = 204.0$, d.f. = 4, $P < 0.0001$) were all significant descriptive variables for colony size of both *D. citri* and *P. citri*, respectively. However, the directionality of this relationship is unknown.

Discussion

This study is the first to evaluate the effectiveness of a liquid baiting program for multi-season control of *L. humile* and ant-associated hemipteran pests in commercial citrus.

Densities of *L. humile* and all monitored hemipteran species (i.e., *D.* citri, *P. citri*, *C. hesperidum*, and *A. aurantii*) declined markedly during the first few months of treatment deployment and remained near zero for the study duration. By comparison, control trees continued to sustain heavy pest infestations, and a distinct hierarchy of *L. humile* preference for hemipteran pest colonies emerged. In addition to a greatly reduced infestation rate, the size of established colonies of most hemipteran pest species was considerably lower in trees treated for *L. humile* than control trees. However, the scarcity of pest colonies in treated trees made it difficult to determine explicitly the role of biological control in the level of pest suppression achieved.

Few treatments options are available for management of the exceptionally high population densities of *L. humile* present in the California citrus agroecosystem (McCalla et al. in press). Although a common experimental method to preclude *L. humile* from tree canopies is application of a sticky barrier around tree trunks, this process is highly labor intensive and impractical at the scale of commercial production. Furthermore, sticky barriers possess many of the same disadvantages as conventional barrier spray treatments (i.e., application of the broad-spectrum insecticide at the trunk base). Both sticky and chemical residue bands provide little to no control of subterranean ant colonies and may reroute workers to nearby unprotected areas and structures (McCalla et al. in press). Barriers also require continuous grove management (i.e., hedging, skirting, weed control, regular debris removal) to prevent ants from accessing alternate points of entry which circumvent protective barriers (McCalla et al. in press). Ground applications of chlorpyrifos, the only broad-spectrum insecticide registered for *L. humile* control in commercial citrus in California, have poor residual longevity (< 30 days) (Knight and Rust 1990 a,b; Rust et al. 1996). Consequently, frequent reapplications of chlorpyrifos are required which can cause high mortality of the natural enemies required for management of citrus pests (Bellows and Morse 1988; Thomson and Hoffmann 2006). Because of environmental and human health hazards, chlorpyrifos use was banned in California in 2019 (California Department of Pesticide Regulation 2019 a,b). Replacement ant control treatments for use in citrus are urgently needed.

Unlike ground sprays of insecticide, liquid baiting programs are relatively target-specific and exploit the social behavior of ants to provide extended, colony-wide control (Rust et al. 2004; McCalla et al. in press). An aqueous sucrose bait laced with a small amount of toxicant is ideal for targeting *L. humile*, as this formulation mimics the preferred honeydew food source and promotes rapid bait intake and relocation to subterranean colonies where it is redistributed to nestmates (Silverman and Roulston 2001; Silverman and Brightwell 2008; Sola and Josens 2016). Bait is deployed in a plastic reservoir with a narrow opening, which physically occludes potential non-target species. Although a number of studies have investigated the use of liquid bait-and-dispenser-based programs for control of *L. humile* in agricultural settings (Klotz et al. 2003; Tollerup et al. 2004; Daane et al. 2006, 2008; Greenberg et al. 2006), this study is the first to evaluate the long-term effectiveness of a liquid baiting program for control of *L. humile* in a commercial citrus setting.

Liquid bait was deployed at a rate of ~3 L (divided across 6 reservoirs) per four-by-four tree plot. Although the full suppressive effect of baiting was not apparent immediately, within two months of treatment, *L. humile* activity in treated trees dropped to less than one tenth of baseline estimates. This level of control (i.e., 90 - 99% reduction from baseline estimates) was maintained in treated trees for the study duration. In addition, mean *L. humile* activity (i.e., ants traversing trunk/min) was 20 times lower in treated trees (11 ants/min) than control trees (212 ants/min) across the final year of study. The suppression of *L. humile* in treated trees translated to strongly reduced worker presence in hemipteran pest colonies. On average, *L. humile* was recovered in 32% of pest colonies collected from control trees but just 2% of those collected from treated trees. Furthermore, when ants were present, the mean number of *L. humile* per pest colony (i.e., absolute ant activity) was 2.5 times higher in control trees than treated trees.

The considerable level of *L. humile* suppression achieved with liquid baiting is likely the result of multi-season treatment duration, area-wide application, and ant monitoring approach (see McCalla et al. in press for a detailed discussion of why visual monitoring may be a better indicator of ant foraging in tree canopies than baited vial monitoring). A high bait station density and rate of bait replenishment were necessary to effectively control populations of *L. humile* in small plot replicates surrounded by large areas of untreated orchard that were heavily ant infested. The high level of artificial *L. humile* reinvasion pressure produced by this experimental design would be absent in a realistic commercial scenario where the entire orchard would be treated. Consequently, the ‘true’ treatment application rate required to control *L. humile* is likely lower than what is reported here.

Because of the potentially high labor requirements for maintaining liquid bait-and-dispenser programs (i.e., retrieving, cleaning, and refilling of reservoirs in addition to initial deployment), the long-term viability of this treatment strategy in commercial agricultural settings has been questioned (Nelson and Daane 2014). However, novel bait disbursement technologies under development (i.e., hydrogels) may greatly improve the feasibility of baiting in commercial systems. Large-scale field trials conducted in commercial citrus groves indicated that biodegradable hydrogel beads loaded with liquid bait can produce a level of control similar to dispenser-based programs (Tay et al. 2017; McCalla et al. in press). Furthermore, hydrogels can be distributed by hand, mechanical spreaders, or aircraft to rapidly treat large areas, eliminating the labor requirements associated with plastic reservoirs (Rust et al. 2015; Merrill et al. 2018; McCalla et al. in press).

Suppression of *L. humile* populations had a profound effect on infestation of citrus orchards by ant-associated hemipteran pests. One year after treatment deployment (Aug 2017), the mean infestation rate of twigs, flush, and fruit from treated trees was 97%, 84%, and 99% lower (respectively) than baseline estimates collected in August 2016. Across this same period, no significant difference in infestation of niches from control trees was registered. At the final sampling point in January 2018 (18 months after treatment application), mean cumulative infestation frequency was 10 times lower and mean cumulative infestation severity (i.e., individuals per pest colony) was 4.5 times lower in treated trees relative to control trees. In addition, mean infestation rate was 4.5

times lower on twigs, 5 times lower on flush, and 20 times lower on fruit recovered from treated trees when compared to control trees.

The strength of association between *L. humile* and sap-feeding hemipteran pest species predicted the impact of ant control on pest prevalence. In control trees, *P. citri* commonly infested fruit and flush and was tended by *L. humile* more intensively than any other hemipteran pest monitored. Several studies have confirmed the high prevalence of this relationship in California citrus, and *P. citri* is thought to be the primary supplier of honeydew for *L. humile* (Markin 1970; Moreno et al. 1987; Tena et al. 2013; Yoo et al. 2013). *Planococcus citri* colonies secrete vast quantities of high-quality honeydew in comparison with other sap-feeding insects, a key determinant in ant mutualist preference (Moreno et al. 1987; Völkl et al. 1999; Mailleaux et al. 2000). In addition, *P. citri* are thigmotactic and form compact, high density aggregations along fruit junctions and within crevices. Because of this colony structure, tending ants can service and defend *P. citri* with high efficiency (Moreno 1987). The results presented here suggest that *P. citri* strongly relies upon *L. humile* protection from natural enemies, as colonies were completely absent in flush and very seldom observed on fruit collected from treated trees. In fact, the mean infestation frequency of *P. citri* on fruit was 98% lower and mean colony size was 47% lower on treated trees in comparison with control trees. A similar trend was observed for *C. hesperidum*, another prolific honeydew producer that was observed to be heavily tended by *L. humile*. Colonies of *C. hesperidum* were nearly absent in treated trees, with a 95% lower infestation rate than in control trees. Moreno et al. 1987 reported similar population reductions of *P. citri* and *C. hesperidum* following *L. humile* exclusion in citrus orchards.

Ant control treatment significantly impacted *D. citri* infestation, which was, on average, 75% lower in treated trees than control trees. However, no difference in mean *D. citri* colony size was observed between treatment groups. The weak association between *D. citri* and *L. humile* may explain why ant control had a smaller effect on populations of *D. citri* in comparison with *P. citri*, *C. hesperidum*, and even the non-honeydew producing *A. aurantii*. In control trees, the proportion of *D. citri* colonies tended by *L. humile* was the lowest of all HPH species, as was the average absolute and relative ant activity per colony. This observation corroborates findings from Tena et al. 2013, which found that *L. humile* workers responded significantly less intensively to colonies of *D. citri* than *P. citri* and *C. hesperidum*. Both *P. citri* and *C. hesperidum* produce copious amounts of liquid honeydew that can be rapidly ingested by *L. humile*. By contrast, *D. citri* produces wax-coated honeydew ‘ribbons’ that *L. humile* workers must harvest whole with their mandibles and transport to the nest for processing (Ammar et al. 2013; Tena et al. 2013). In addition, the rapid generation time of *D. citri* (often less than a month) means that colonies are a relatively unreliable source of honeydew, and ants generally respond more strongly to spatiotemporally stable food sources (Noë and Hammerstein 1994; Bonabeau et al. 1997; Tena et al. 2013). By comparison, *P. citri* and *C. hesperidum* colonies can persist for months to years, facilitating greater worker fidelity (Tena et al. 2013). The reduced foraging efficiency of *L. humile* for solid honeydew and ephemerality of colonies may explain why *D. citri* is tended less intensively than other HPH species.

In control trees, twig-infesting colonies of *A. aurantii* were tended by *L. humile* less frequently than any other hemipteran pest species in the study. This result is unsurprising, given that (1), *A. aurantii* is a diaspid scale that does not produce honeydew and receives protection inadvertently from ant surveillance of neighboring hemipteran colonies (DeBach 1958; Reeve and Murdoch 1986; Ferrer et al. 2003; Yoo et al. 2013), and (2), infestations of the honeydew-producing *C. hesperidum* and attending ants on twigs was rare (< 1% of twigs colonized). By comparison, fruit was heavily infested by the strongly ant-associated *P. citri*. Field observations indicated that *L. humile* workers commonly patrolled clusters of fruit co-infested by *P. citri* and *A. aurantii*. The proximity of high densities of *P. citri*-tending *L. humile* likely provided *A. aurantii* colonies on fruit greater protection from natural enemies than *A. aurantii* on twigs, where patrolling ants were less common. In support of this hypothesis, the effect of *L. humile* suppression on *A. aurantii* infestation was found to be more pronounced for colonies on fruit than on twigs. The mean infestation frequency was 90% lower in fruit from treated trees than control trees and 70% lower on twigs. Ant control also impacted *A. aurantii* colony size, which was 63% lower on treated fruit and 80% lower on treated twigs in comparison with samples from control trees.

Other studies have found the effect of *L. humile* exclusion on *A. aurantii* infestation to be niche-dependent. For example, Moreno et al. (1987) and Calabuig et al. (2014) reported lower densities of *A. aurantii* on fruit from *L. humile*-excluded trees than control trees, but neither study registered a significant effect of ant control on *A. aurantii* colonies on twigs. The authors attributed this difference to a higher foraging intensity of the *A. aurantii* parasitoid *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae) in the canopy periphery than in the interior of trees. However, several other studies have reported that ant exclusion can considerably reduce densities of *A. aurantii* colonizing twigs (DeBach et al. 1958; Murdoch et al. 1995; James et al. 1997; Pekas et al. 2010). Regional or temporal disparity in the composition of parasitoid communities could explain the discrepancy in results among studies. For example, *Encarsia perniciosi* Tower prefers to oviposit on twig-infesting *A. aurantii* but poorly tolerates extreme temperatures. Consequently, it is more prevalent in the mild California coast than in hotter inland areas. In contrast, *Aphytis melinus* and *Comperiella bifasciata* Howard (Hymenoptera: Encyrtidae) tolerate a wide temperature range and primarily target *A. aurantii* on fruit (Carol and Luck 1984; Luck et al. 1995). In areas where these parasitoids are highly abundant (i.e., the southern California interior), this preference could translate to increased control of *A. aurantii* colonizing fruit relative to twigs.

In control trees across the final year of study, the rate of parasitism was relatively high for hemipteran pests colonizing twigs and fruit (55 – 77%) in comparison with pests in flush (i.e., 28 and 4.5% for *P. citri* and *D. citri*, respectively). However, 82% of all natural enemies recovered in the study were found in flush-infesting *P. citri* and *D. citri* colonies. Syrphids, found exclusively in flush, were the most common predator recovered in flush (88%) and overall (71%), outnumbering the second most common predator morphotaxon (predatory thrips) by a ratio of 6:1 and the third most common (lacewings) by 9:1. Syrphid prevalence was associated with *D. citri* colony density, with both peaking in November and December 2017. Although coccinellids have been reported to be the most important predator guild member for *D. citri* in Florida (Kistner et al. 2016), only a single incidence of coccinellid presence in *D. citri* colonies (*Diomus pumilio* Weise [Coleoptera: Coccinellidae]) was observed in this study. Other predator surveys conducted in southern California residential and research citrus groves corroborate the observation in this study that syrphids are the most abundant and potentially important *D. citri* predator taxon in southern California (Kistner et al. 2016, 2017; Schall and Hoddle 2017). The relative importance of syrphids as *D. citri* predators may have been underestimated by other studies due, in part, to their nocturnal foraging behavior (Kistner et al. 2017). Kistner et al. 2016 reported that syrphid and lacewing larvae accounted for ~86% of predation events and could cause up to 93% mortality of field-deployed *D. citri* cohorts. Video surveillance revealed that *L. humile* frequently removed syrphid larvae from tended *D. citri* colonies and that *L. humile* exclusion could increase the number of *D. citri* predation events by syrphid larvae by 312% (Kistner et al. 2016, 2017). In addition to ant control, adult syrphids benefit from floral resources which provide shelter and energy required for biocontrol critical behaviors (e.g., prey-searching, egg-laying, etc.) (Kistner et al. 2016). Preliminary field studies conducted in southern California have shown that flowering potted alyssum, *Lobularia maritima* (L.) Desv., in organic citrus orchards increased syrphid oviposition by up to 245% and enhanced predator abundance by 155% compared with bare ground controls. This translated into significantly lower (11% lower) survival of *D. citri* nymphs in alyssum plots compared with controls (Irvin, Hoddle, and Pierce, unpublished data). The implementation of conservation biocontrol strategies in combination with ant control may increase generalist predator recruitment, resulting in greater control of *D. citri* and other hemipteran pests in citrus.

Although a strong suppressive effect of ant control on hemipteran pest populations was observed in this study, the extent of natural enemy contributions in achieving this result was unclear. Parasitism of *D. citri* and *A. aurantii* colonies in treated trees was either lower than or not significantly different from control colonies. Ant control treatment reduced infestations of *C. hesperidum* and *P. citri* to such a significant degree that percentage parasitism could not be estimated or was calculated from relatively few samples making estimates unreliable. Similarly, it is difficult to draw definitive conclusions about seasonal or between-treatment trends in predator activity. It is interesting to highlight, however, that the total predator density briefly surged in treated but not control trees one month after liquid bait deployment. In comparison with baseline estimates collected in August 2016, 9 times more predators were recovered from hemipteran pest colonies in treated trees in September 2016. Predator densities in treated trees returned to pre-treated levels the following month (Oct 2016) and no resurgence in natural enemy or pest activities in treated trees was observed for the study duration. This temporary increase was primarily the result of mealybug destroyer larvae *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae) in *P. citri* and *A. aurantii* colonies in fruit and is consistent with the rapid decline in fruit infestation observed shortly after treatment application. No *C. montrouzieri* were observed in *D. citri* colonies. High densities of unprotected hemipteran prey would have attracted *C. montrouzieri*, as adults and larvae display strong aggregative functional responses to *P. citri*. Additionally, predation efficacy likely would have been improved, as larvae are harassed by tending *L. humile* (Bartlett 1961; Way 1963; Daane et al. 2007; Abdollahi et al. 2012; Mansour et al. 2012). This observation suggests that ant suppression may create a spatial refuge containing large populations of vulnerable prey that are rapidly extirpated by natural enemies. Frequent monitoring intervals shortly after treatment application and supplemental sampling methods (e.g., pitfall traps, canopy aspiration, etc.) may provide better insight into the natural enemy population trends which accompany infestation declines of individual hemipteran pest species.

The negative impact of ant-hemipteran mutualisms on generalist predator populations and efficacy is supported by a large body of literature (reviewed in Karami-Jamour et al. 2018). In ant-excluded citrus orchards, Calabuig et al. (2014) reported that generalist predators were more abundant and parasitoids were less abundant, and Dao et al. (2014) reported a proportionately greater increase in the rate of hemipteran pest predation in comparison with parasitism. Consequently, the decline in hemipteran pest infestations observed following ant elimination may be more a function of increased predator activity than increased parasitoid activity (Dao et al. 2014; Calabuig et al. 2015). When examined individually under laboratory conditions, disruption of ant-hemipteran mutualisms generally increases the biocontrol efficiency of predators and parasitoids (i.e., predation, parasitism, and host-feeding). However, these studies do not account for the antagonistic interactions that result from predator and parasitoid niche co-occupation in the field (Karami-Jamour et al. 2018). Under field conditions, predator-induced host population declines and intraguild predation (i.e., consumption of parasitized hemipterans) may reduce the net benefit of ant control for parasitoids (Barzman and Daane 2001; Kaneko 2006; Calabuig et al. 2015). Many parasitoids have adaptations (i.e., short oviposition time, avoidance behavior patterns, or visual or chemical camouflage) which allow them to mitigate ant aggression and utilize ant-tended hosts with varying degrees of proficiency (Völkl 1997). Because ants facilitate and support larger host densities and reduce the intensity of intraguild predation, ant-resistant and even ant-sensitive parasitoids may be equally or more successful in ant-tended than ant-excluded trees (Calabuig et al. 2015; Karami-Jamour et al. 2018).

Antagonistic intraguild interactions may explain why ant control often produces little to no increase in parasitism in the field (Murdoch et al. 1995; Urbaneja et al. 2004; Pekas et al. 2010; Yoo et al. 2013; Calabuig et al. 2014, 2015) but does consistently in controlled laboratory studies (Bartlett 1961; Barzman and Daane 2001; Ferrer-Martinez et al. 2003; Mgocheki and Addison 2009; Dao et al. 2014; Schall and Hoddle 2017), even when examining the same species combinations. Understanding the impact of facultative mutualisms between *L. humile* and HPH pests on intraguild predation within the natural enemy community in citrus may provide insight as to how to improve field performance of parasitoids employed in biocontrol programs.

In conclusion, multi-season management of *L. humile* with a liquid baiting program produced significant decreases in populations of *D. citri*, *P. citri*, *C. hesperidum*, and *A. aurantii* in commercial citrus groves in southern California. Over the course of the 18-month treatment period, *L. humile* activity was reduced by 90 – 99.8% and infestation rates of hemipteran pests were 70-100% lower in treated trees in comparison with controls, including a notable 75% reduction for the major citrus pest *D. citri*. Although numerous species of natural enemies likely contributed to these observed declines, increased predator activity may have been the dominant cause driving observed declines. Additional studies should be conducted to determine the relative contributions of predators and parasitoids in population suppression of hemipteran pests under varying levels of ant suppression. The results presented here suggest that standard measures of natural enemy efficacy (i.e., parasitism) my considerably underestimate the potential value of natural enemies for suppressing *D. citri* and other major citrus pests. Citing low rates of *D. citri* parasitism, Florida *D. citri* management programs have largely abandoned biological control in favor of management regimes that are pesticide intensive. This has resulted in widespread insecticide resistance in populations of *D. citri* (Boina and Bloomquist 2015). The economic burden of continually increasing spray requirements (currently 8 – 12 applications/year) and concomitant secondary pest outbreaks is unsustainable for citrus growers in Florida (Boina and Bloomquist 2015). In California where the *D. citri*-*C*Las complex has yet to establish in major citrus producing regions (i.e., the San Joaquin Valley), ant management could be an invaluable tool for suppressing populations of *D. citri* and slowing the spread of *C*Las-induced huanglongbing (Milosavljević et al. 2017). Consequently, ant management should be considered a foundational support for integrated pest management programs of economically important hemipteran pests in both urban and agricultural systems. Further research is needed to develop a cost- and labor-efficient delivery system for liquid bait in commercial orchards (i.e., hydrogels) for controlling invasive sugar feeding ants such as *L. humile* (McCalla et al. in press).

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Figures and Tables

Plant niche	Hemipteran species	Factor	Distribution	df	χ^2	***P***
Twigs	All pests	Treatment	Poisson	1	0.17	0.684
		Month		11	191.49	< 0.0001
		Air temperature		1	5.11	0.0238
		Soil temperature		1	16.98	< 0.0001
		Treatment:month		11	154.39	< 0.0001
	Coccus hesperidum	Treatment	Poisson	1	0.0000	1.000
		Treatment:month		22	334.48	< 0.0001
	Aonidiella aurantii	Treatment	Zero-inflated Poisson	1	0.77	0.379
		Month		11	284.53	< 0.0001
		Air temperature		1	75.02	< 0.0001
		Treatment:month		11	39.65	< 0.0001
Flush	All pests	Treatment	Zero-inflated Poisson	1	0.0003	0.987
		Month		11	2257.51	< 0.0001
		Soil temperature		1	159.85	< 0.0001
		Treatment:month		11	253.5	< 0.0001
	Diaphorina citri	Treatment	Zero-inflated Poisson	1	0.0000	0.999
		Month		11	1605.29	< 0.0001
		Soil temperature		1	209.24	< 0.0001
		Treatment:month		11	251.8	< 0.0001
	Planococcus citri	Treatment	Binomial	1	0.0000	0.998
		Treatment:month		22	53.58	0.0002
Fruit	All pests	Treatment	Negative binomial	1	1.37	0.242
		Month		11	195.69	< 0.0001

	Soil temperature		1	2.72	0.099
	Treatment:month		11	99.83	< 0.0001
Planococcus citri	Treatment	Zero-inflated Poisson	1	0.0002	1.000
	Treatment:month		22	1805.5	< 0.0001
Aonidiella aurantii	Treatment	Poisson	1	2.57	0.109
	Month		11	1138.52	< 0.0001
	Air temperature		1	52.27	< 0.0001
	Soil temperature		1	42.84	< 0.0001
	Treatment:month		11	289.6	< 0.0001

Table 3.1 Results of generalized linear mixed models evaluating the effects of treatment and environmental factors on the infestation rate (%) of twigs, flush, and fruit by various hemipteran pest species. Model selection was based on comparison of AICc scores through likelihood ratio tests and the function "*dredge*" in the R package MuMln. Only variables retained in the final model are shown below.

Mean Infestation Rate and (Proportion of All Pest Infestations)

Niche	Twigs		Flush		Fruit	
Pest	*Coccus hesperidum*	*Aonidiella aurantii*	*Diaphorina citri*	*Planococcus citri*	*Planococcus citri*	*Aonidiella aurantii*
Control Trees	0.83 ± 0.19%^{a} (2.28%)	1.67 ± 0.24%^{a} (4.60%)	13.41 ± 1.09^{a} (36.92%)	3.27 ± 0.52* (9.00%)	10.13 ± 0.67^{a} (27.88%)	7.02 ± 0.65^{a} (19.32%)
Ant Treated Trees	0.04 ± 0.02%^{b} (0.84%)	0.51 ± 0.09%^{b} (10.74%)	3.33 ± 0.59^{b} (70.10%)	0.00 ± 0.00* (0.00%)	0.17 ± 0.10^{b} (3.58%)	0.70 ± 0.13^{b} (14.74%)

Table 3.2 Rate of infestation of niches by various hemipteran pest species in treated and control trees. Parentheses delineate the proportion of total infestation for each pest species within treatment categories. Within each column (control vs. treatment), means (± SEM) followed by the same lowercase letter are not significantly different at $\alpha = 0.05$ (estimated marginal means). On a monthly basis, N = 54 control trees and N = 54 treated trees (N = 648 per treatment group overall). Mean values calculated from the February 2017 through January 2018 are displayed. Models and p-values are provided in **Table 3.1**.
*Comparison cannot be made due to the absence of variance in the treated group.

Mean Colony Size (± SEM)

Niche	Twigs		Flush		Fruit	
Pest	*Coccus hesperidum*	*Aonidiella aurantii*	*Diaphorina citri*	*Planococcus citri*	*Planococcus citri*	*Aonidiella aurantii*
Control Trees	57.48 ± 8.36^a N = 89	19.64 ± 3.08^a N = 168	32.24 ± 1.79^a N = 502	19.21 ± 2.48 N = 195	34.01 ± 2.62^a N = 743	98.48 ± 5.84^a N = 571
Ant Treated Trees	1.36 ± 0.13^b N = 3	3.91 ± 1.21^b N = 49	37.45 ± 3.86^a N = 151	N/A N = 0	17.90 ± 6.12^b N = 10	36.81 ± 6.79^b N = 63
Mean Parasitism Rate (± SEM)						
Untreated Trees	55.33 ± 4.10% N = 76	67.62 ± 4.11%^{a} N = 109	4.47 ± 1.13%^{a} N = 290	28.47 ± 2.49 N = 169	55.67 ± 1.44%^{a} N = 690	77.19 ± 1.78%^{a} N = 458
Ant Treated Trees	N/A	22.93 ± 7.53%^{b} N = 27	2.97 ± 1.90%^{a} N = 74	N/A	30.74 ± 12.93%^{b} N = 9	72.30 ± 5.78%^{a} N = 46

Table 3.3 Comparison of mean colony size (number of individuals/colony) and percent parasitism (number of parasitized individuals/[parasitized + susceptible individuals]) of hemipteran pest colonies collected from treated and control trees (Feb 2017 – Jan 2018). Within each column (control vs. treatment), means (± SEM) followed by the same lowercase letter are not significantly different at $\alpha = 0.05$ (estimated marginal means). "N" represents sample size and "N/A" denotes that no comparison could be made due to the collection of no colonies or only colonies with life stages not susceptible to parasitism. In general, the sample size for calculating parasitism rate is lower than that of colony size because some colonies did not contain life stages susceptible to parasitism. The final model for colony size (Poisson error) was ant control treatment ($\chi^2 = 21.75$, d.f. = 1, $P < 0.0001$), pest species within niche ($\chi^2 = 5350.24$, d.f. = 5, $P < 0.0001$), and their interaction ($\chi^2 = 662.67$, d.f. = 4, $P < 0.0001$). The final model for parasitism rate (Poisson error) was ant control treatment ($\chi^2 = 4.78$, d.f. = 1, $P = 0.0289$), pest species within niche ($\chi^2 = 3165.25$, d.f. = 5, $P < 0.0001$), their interaction ($\chi^2 = 34.03$, d.f. = 3, $P < 0.0001$), and sampling month ($\chi^2 = 4929.34$, d.f. = 1, $P < 0.0001$).

Susceptible Host Range for Parasitoids of Hemipteran Pests

Pest Species	Susceptible Life Stages	Length or Other Distinguishing Criteria	Dominant Parasitoid(s) in CA	Reference(s)
Coccus hesperidum	3rd instar	≥ 1.8 mm	*Metaphycus* spp.	Gray 1954
	adult	3 - 4 mm		Kapranas et al. 2007, 2011
Aonidiella aurantii	2nd instar male/female and 3rd instar females	Determined visually by the presence of 1 - 2 molt rings	*Aphytis melinus*	Forster et al. 1995
	Preovipositioning adult females	Retracted pygidium, crawlers absent	*Comperiella bifasciata*	
Planococcus citri	2nd instar	only included if ≥ 0.8 mm	*Leptomastix dactylopii*	de Jong and Alphen 1989
	2nd, 3rd, and 4th instar	0.8 - 1.5 mm		Cadée and Alphen 1997
	Preovipositioning adult females (no egg sac)	> 1.5 mm, ovisac absent	*Leptomastix abnormis*	
Diaphorina citri	3rd, 4th, and 5th instar	0.7 – 1.6 mm, wing pads well-developed, and presence of 1 - 3 setae per antennae	*Tamarixia radiata*	Chien et al. 1991 Tsai and Liu 2000

Table 3.4 A compendium providing life stages of monitored hemipteran pests susceptible to parasitism (and used to calculate percent parasitism). Additionally provided are the criteria used to determine pest life stage and the parasitoids reported to be dominant in southern California.

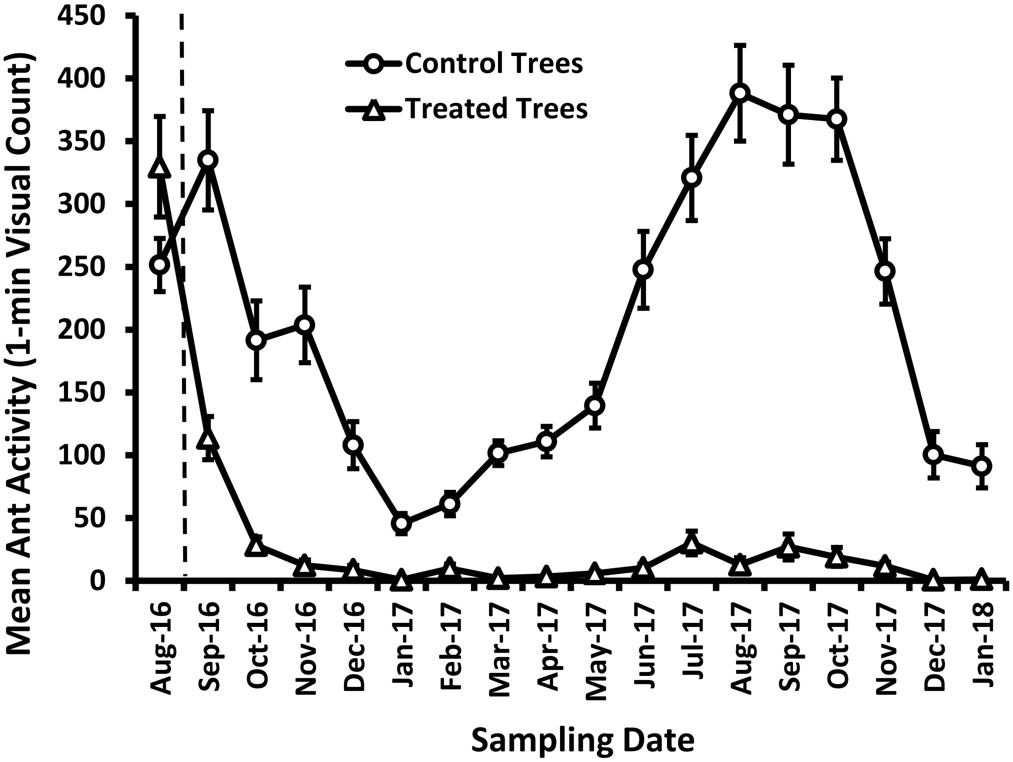
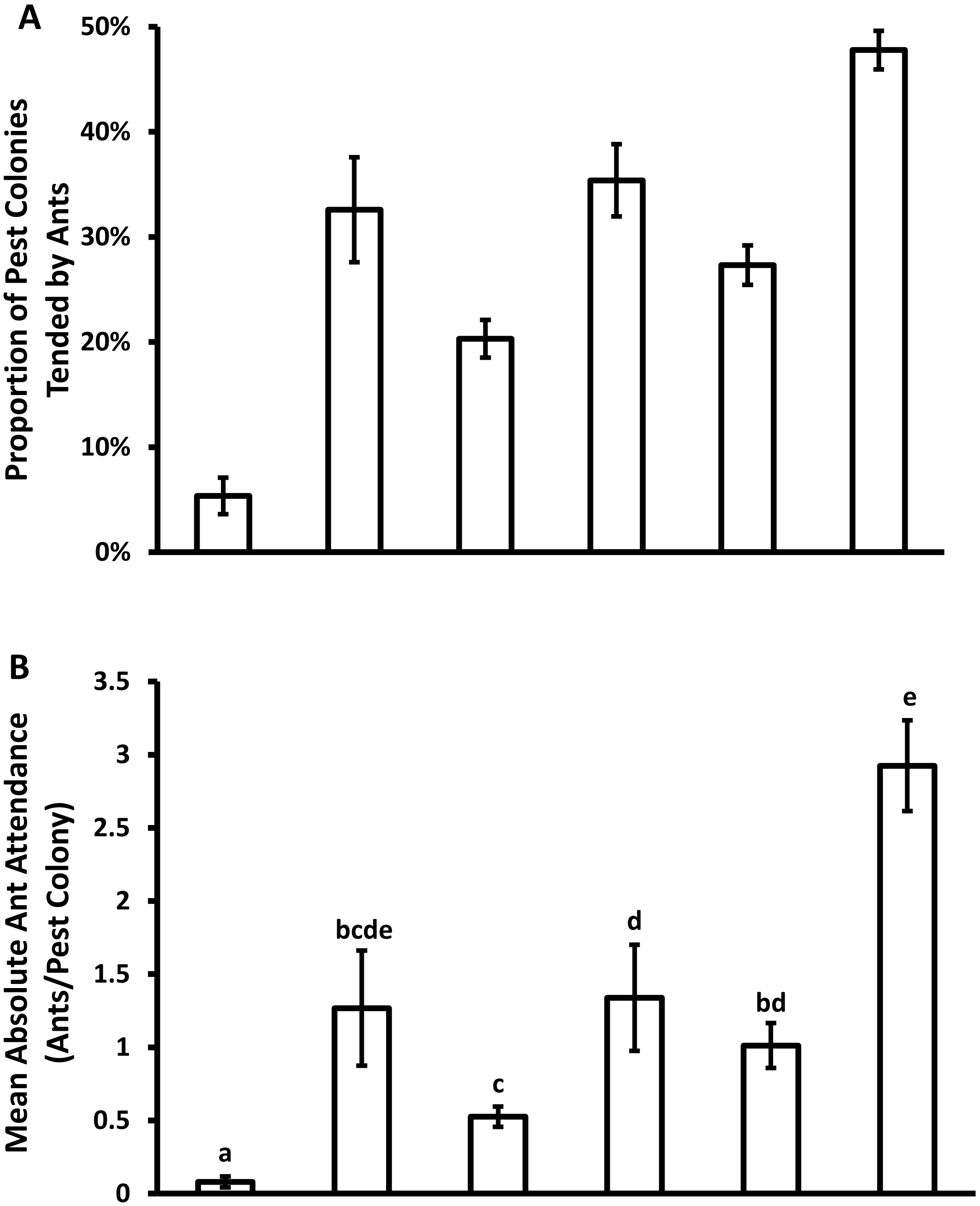


Figure 3.1 Mean visual estimates (± SEM) of *Linepithema humile* activity in treated and control trees across the 18-month experimental duration. The dashed line separates pre- and post- treatment sampling dates. Mean differences between treated and untreated trees across all analyzed months (Feb 2017 – Jan 2018) were statistically significant at $\alpha = 0.05$ (estimated marginal means).


A
Proportion of Pest Colonies Tended by Ants
50%
40%
30%
20%
10%
0%
B
Mean Absolute Ant Attendance (Ants/Pest Colony)
3.5
3
2.5
2
1.5
1
0.5
0
a
bcde
c
d
bd
e

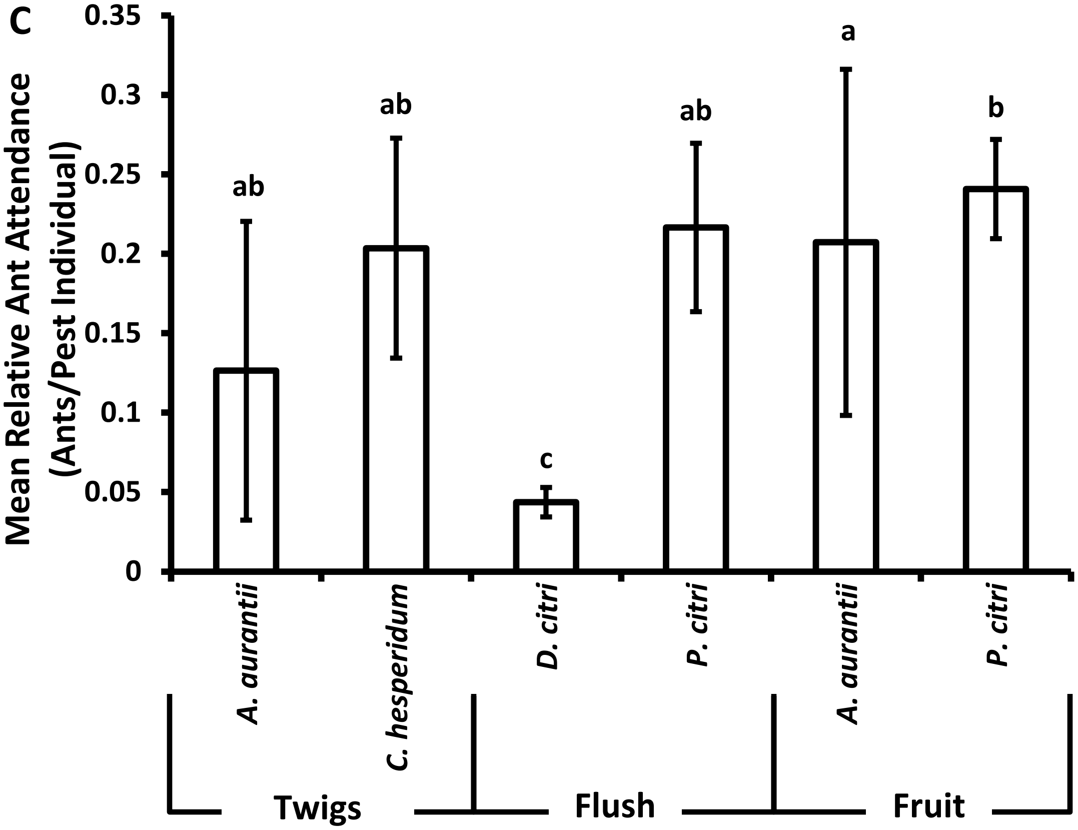


Figure 3.2 Relationship between *Linepithema humile* and four hemipteran pest species (i.e., *Aonidiella aurantii*, *Coccus hesperidum*, *Diaphorina citri*, and *Planococcus citri*) infesting twigs, flush, and fruit in control trees. The proportion of pest colony species tended by *L. humile* (A), absolute attendance (ants per colony; B) and relative attendance (ants per individual per colony; C) are shown. Means (± SEM) labeled with the same lowercase letter are not significantly different at α = 0.05 (estimated marginal means).

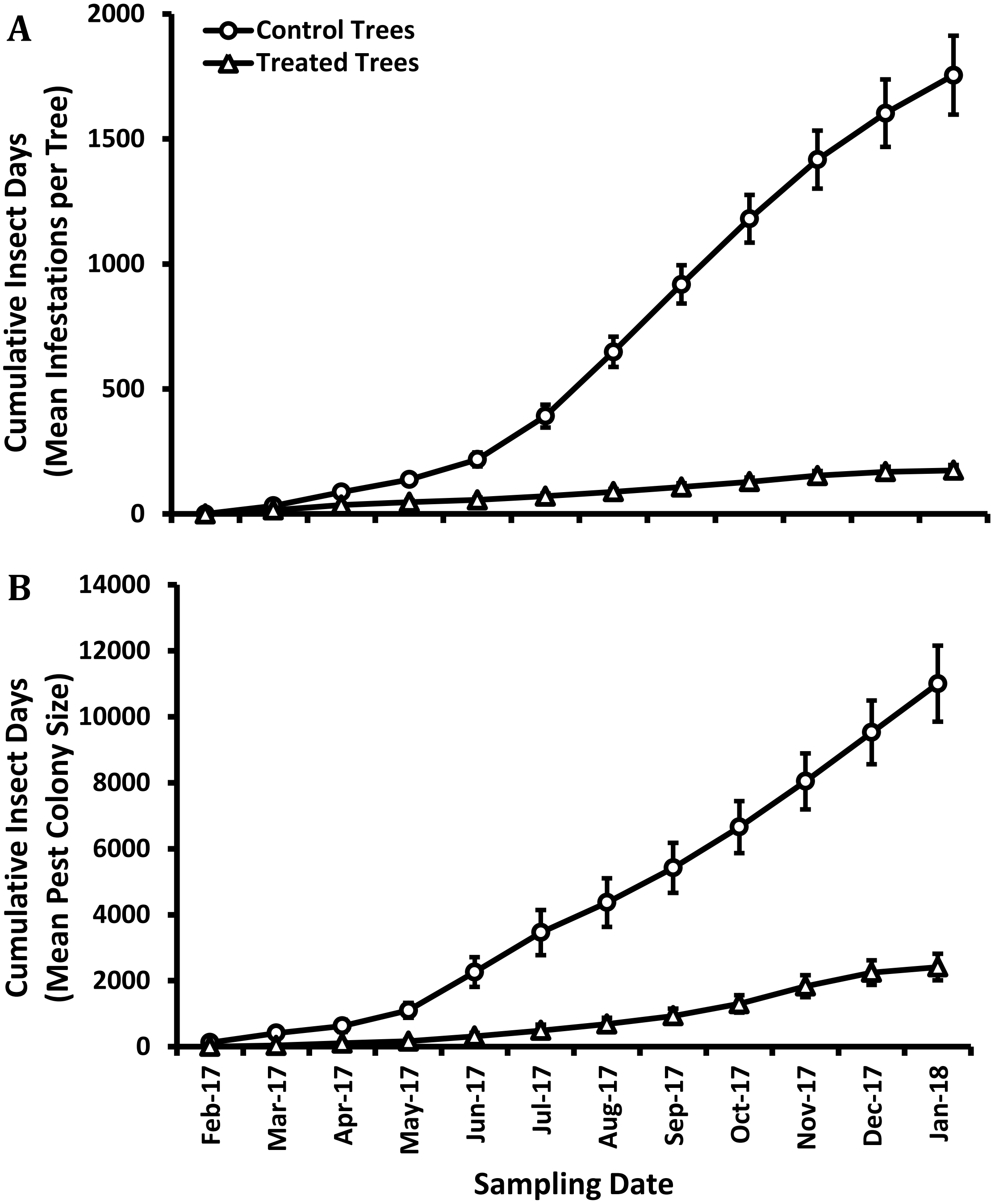


Figure 3.3 Mean (± SEM) cumulative infestation of combined niches in insect days (A) and colony size of hemipteran pests in insect days (B) in ant-treated and control trees across the final year of study (Feb 2017 – Jan 2018). Mean differences between treated and untreated trees across all analyzed months were statistically significant at $\alpha = 0.05$ (estimated marginal means).

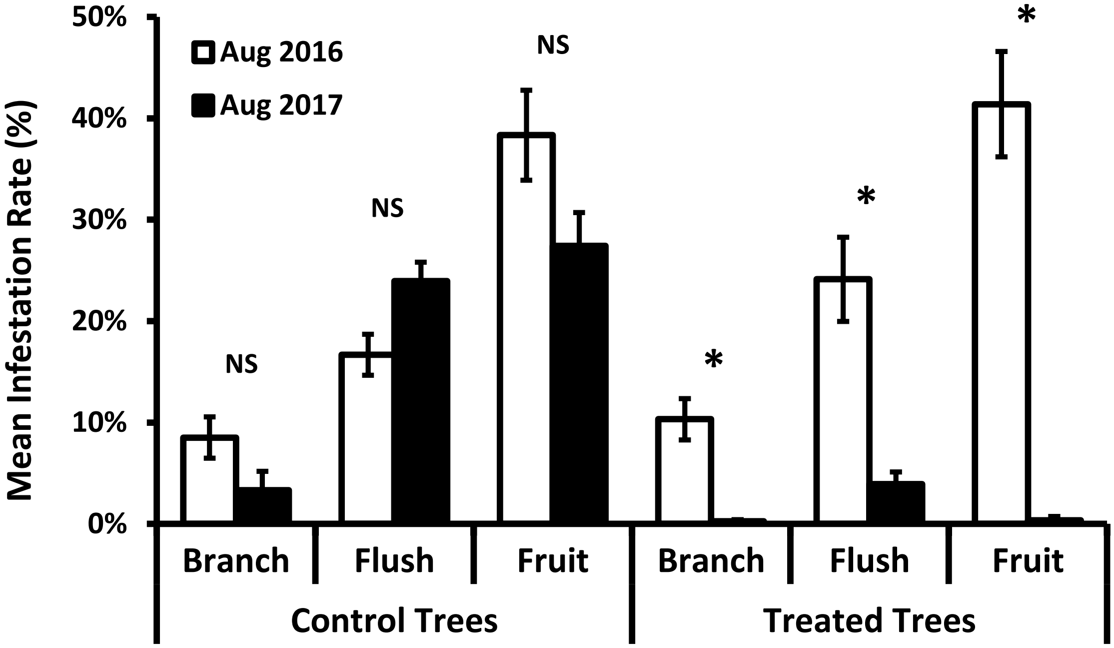
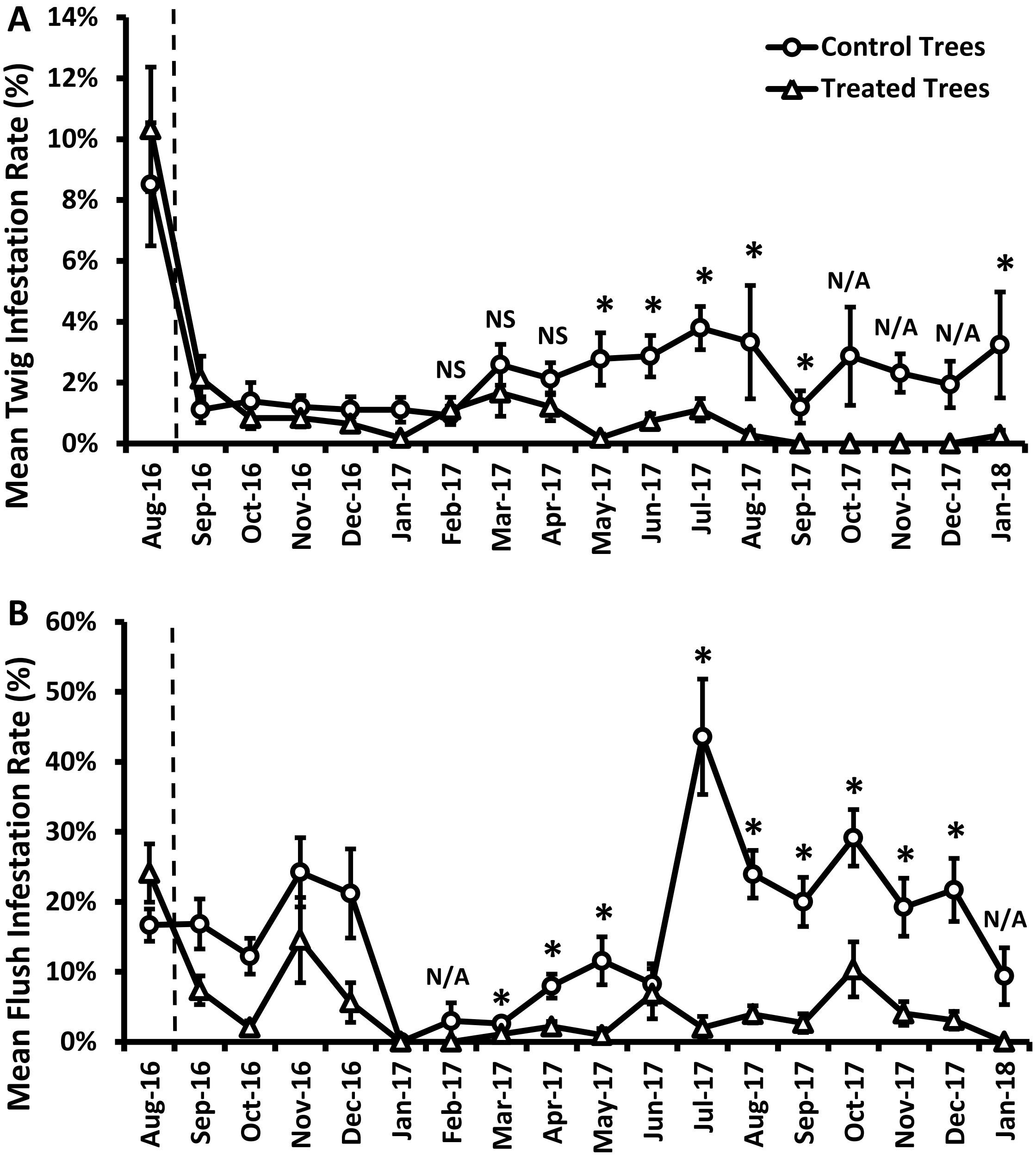


Figure 3.4 Mean (± SEM) pooled hemipteran pest infestation rate of twigs, flush, and fruit prior to (Aug 2016) and following (Aug 2017) liquid bait deployment in treated and control trees. Asterisks represent statistically significant differences between pre- and post-baseline estimates between treated and control trees and "NS" represents non-significant differences at $\alpha = 0.05$ (estimated marginal means).


A
B
Control Trees
Treated Trees
Mean Twig Infestation Rate (%)
Mean Flush Infestation Rate (%)
0%
2%
4%
6%
8%
10%
12%
14%
0%
10%
20%
30%
40%
50%
60%
Aug-16
Sep-16
Oct-16
Nov-16
Dec-16
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17
Jan-18
NS
NS
NS
*
*
*
*
*
N/A
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*
*
*
*
N/A

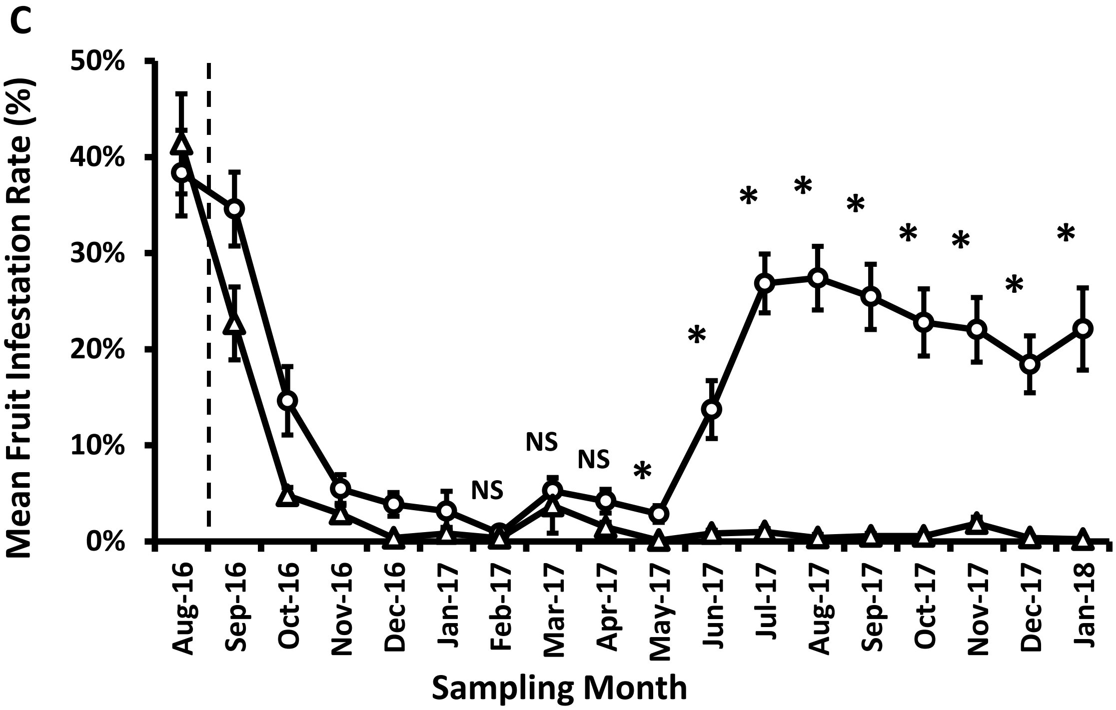
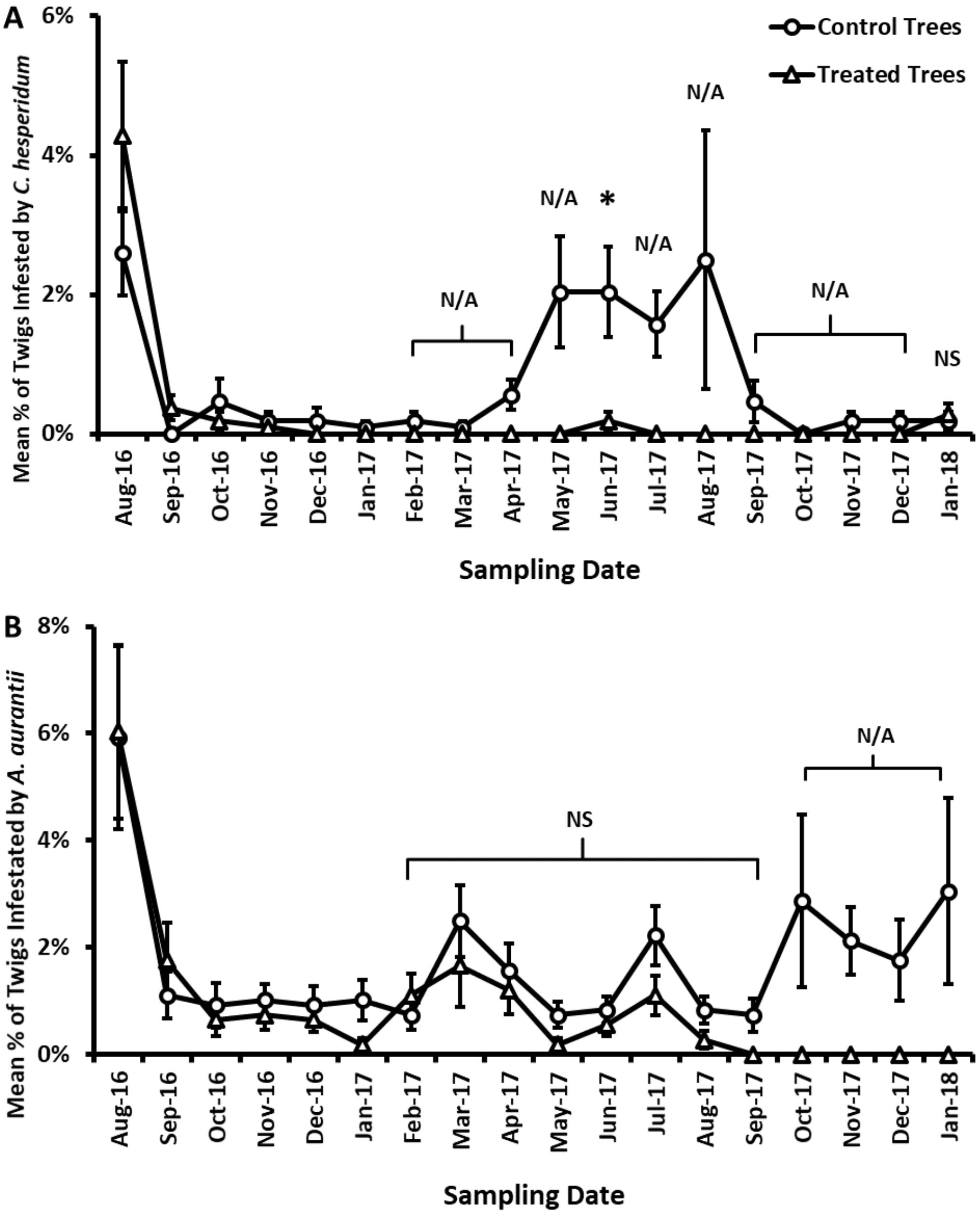
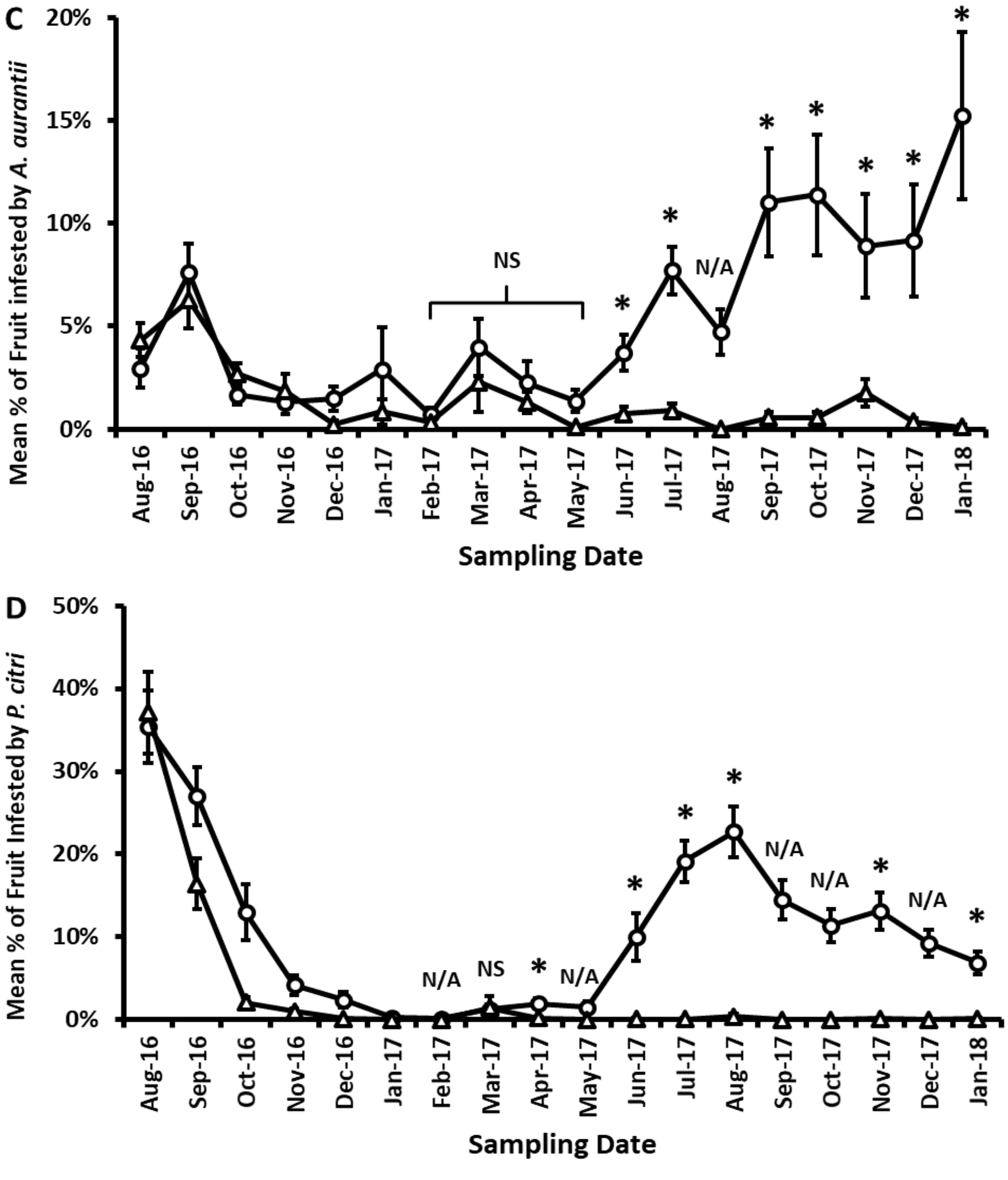


Figure 3.5 Mean (± SEM) infestation of twigs (A), flush (B), and fruit (C) by hemipteran pest species pooled (i.e., *Aonidiella aurantii*, *Coccus hesperidum*, *Diaphorina citri*, and *Planococcus citri*) in ant-treated and control trees across sampling date. The dashed line separates pre- and post- treatment sampling dates. Asterisks represent statistically significant differences between treated and control trees in concurrent sampling months and "NS" represents non-significant differences at $\alpha = 0.05$ (estimated marginal means). "N/A" denotes that infestation rates could not be compared across treatment due to the absence of variance in one or both groups.


A
6%
4%
2%
0%
Mean % of Twigs Infested by C. hesperidum
Control Trees
Treated Trees
N/A
N/A
*
N/A
N/A
N/A
NS
Aug-16
Sep-16
Oct-16
Nov-16
Dec-16
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17
Jan-18
Sampling Date
B
8%
6%
4%
2%
0%
Mean % of Twigs Infestated by A. aurantii
NS
N/A
Aug-16
Sep-16
Oct-16
Nov-16
Dec-16
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17
Jan-18
Sampling Date


C
Mean % of Fruit infested by A. aurantii
20%
15%
10%
5%
0%
NS
N/A
*
Aug-16
Sep-16
Oct-16
Nov-16
Dec-16
Jan-17
Feb-17
Mar-17
Apr-17
May-17
Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17
Jan-18
Sampling Date
D
Mean % of Fruit Infested by P. citri
50%
40%
30%
20%
10%
0%
N/A
NS
*
N/A
*
*
N/A
N/A
*
N/A
*
Aug-16
Sep-16
Oct-16
Nov-16
Dec-16
Jan-17
Feb-17
Mar-17
Apr-17
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Jun-17
Jul-17
Aug-17
Sep-17
Oct-17
Nov-17
Dec-17
Jan-18
Sampling Date

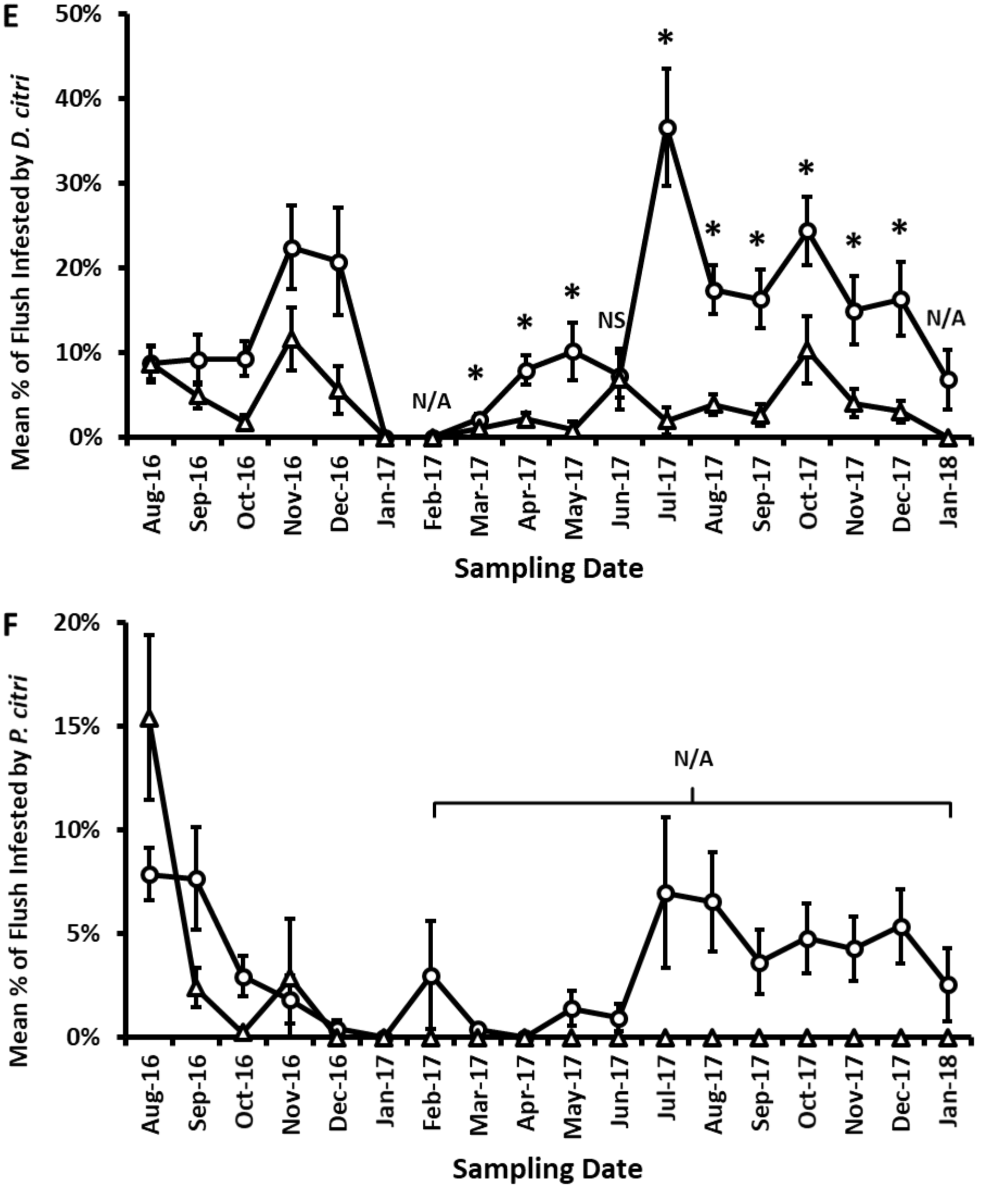

Figure 3.6 Mean (± SEM) infestation of niches by individual hemipteran pest species (i.e., *Aonidiella aurantii*, *Coccus hesperidum, Diaphorina citri*, and *Planococcus citri*) in ant treated and control trees across sampling date. The dashed line separates pre- and post- treatment sampling dates. Asterisks represent statistically significant differences between treated and control trees in concurrent sampling months and "NS" represents non-significant differences at $\alpha = 0.05$ (estimated marginal means). "N/A" denotes that infestation rates could not be compared across treatment due to the absence of variance in one or both groups.

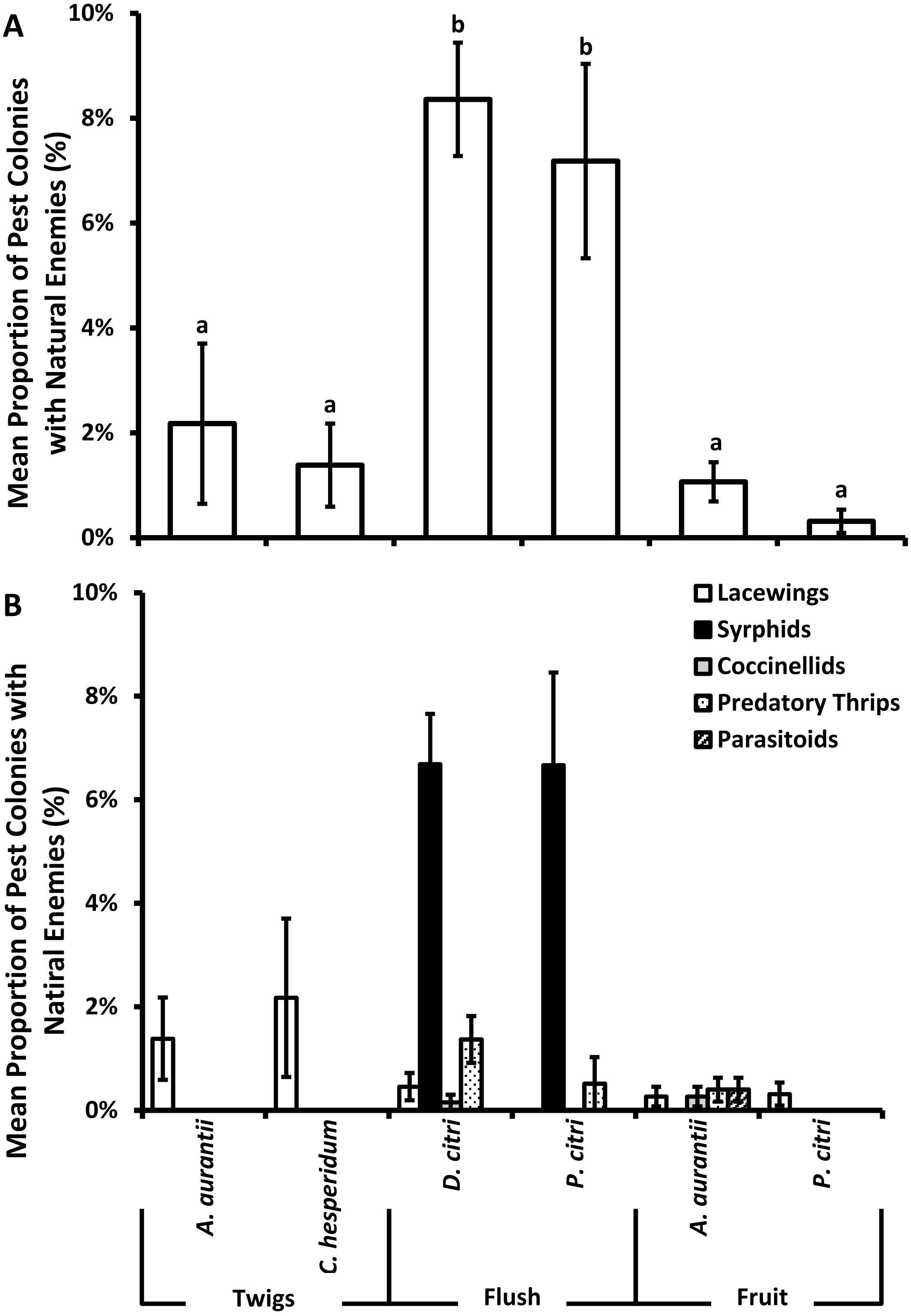
A
Mean Proportion of Pest Colonies with Natural Enemies (%)
10%
8%
6%
4%
2%
0%
a
a
b
b
a
a
B
Mean Proportion of Pest Colonies with Natiral Enemies (%)
Lacewings
Syrphids
Coccinellids
Predatory Thrips
Parasitoids
A. aurantii
C. hesperidum
D. citri
P. citri
A. aurantii
P. citri
Twigs
Flush
Fruit

Figure 3.7 Mean proportion (± SEM) of natural enemies (NEs) present in hemipteran pest species within twigs, fruit, and flush. Natural enemies are pooled (A) or separated by morphotaxa group (B). Means labeled with the same lowercase letter are not significantly different at $\alpha = 0.05$ (estimated marginal means).

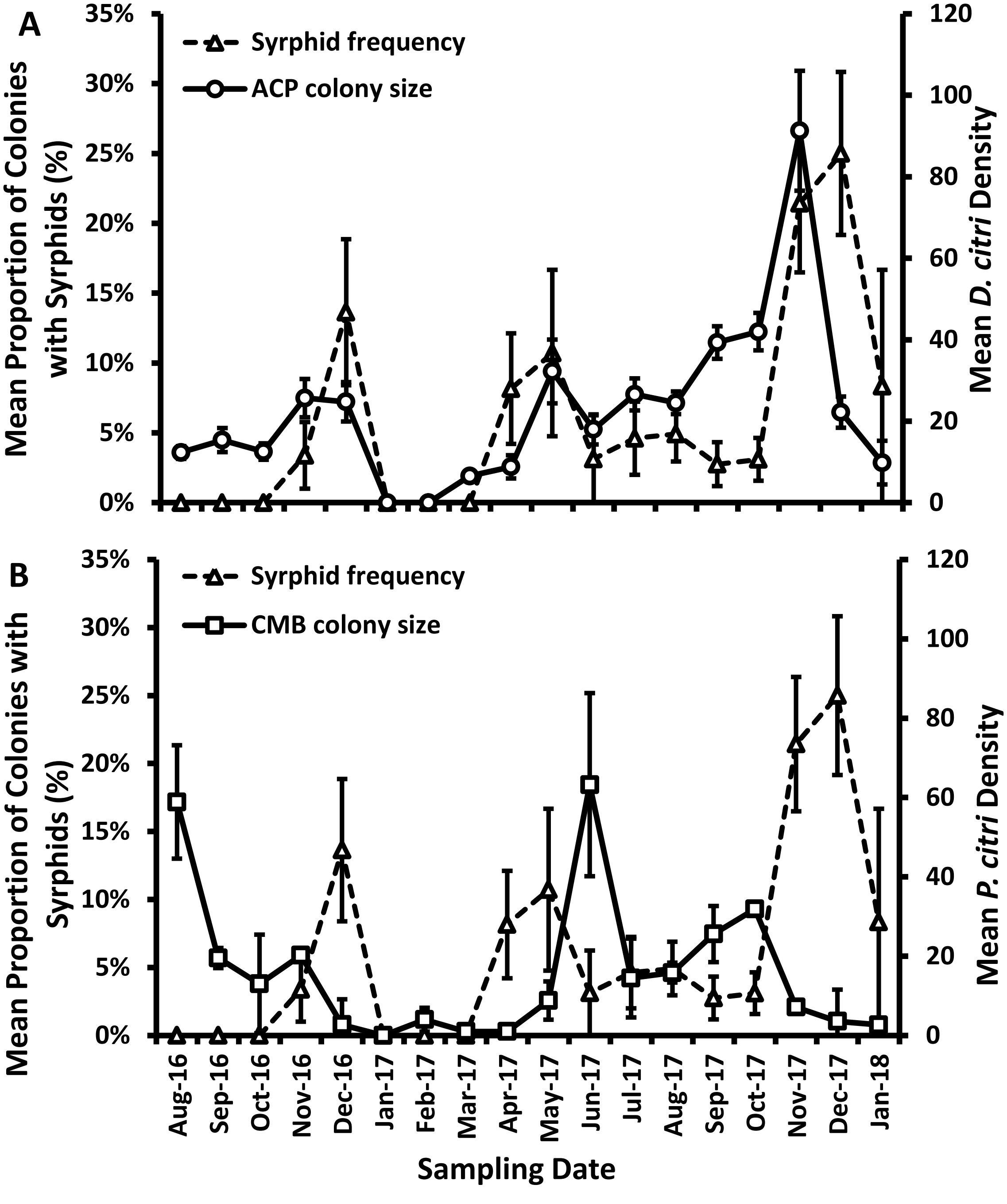

Figure 3.8 Association between syrphid prevalence in pest colonies (i.e., proportion of pest colonies with syrphids present) and densities (i.e., colony size) of *Diaphorina citri* (A) and *Planococcus citri* (B) over the full 18-month sampling period.

Chapter 4: A biodegradable alginate hydrogel bait delivery system effectively controls high-density populations of Argentine ant in commercial citrus

Abstract

The Argentine ant, *Linepithema humile* (Mayr), poses a significant economic threat to citrus production in southern California. Broad-spectrum insecticide sprays provide poor ant control and present a hazard to human and environmental health. Liquid sucrose bait infused with a low concentration of insecticide is an effective alternative treatment option, but current approaches require substantial economic investment in plastic dispensers and continual maintenance. To produce a baiting product for *L. humile* suitable for large-scale application, a biodegradable, broadcastable alginate hydrogel for delivery of aqueous low-dose thiamethoxam sucrose bait was developed. The efficacy of this baiting system for control of *L. humile* was evaluated in replicated field trials conducted in commercial citrus groves over a three-month-period. *Linepithema humile* activity was significantly reduced in hydrogel treated trees. Peak *L. humile* control was achieved 48 hours following the final hydrogel disbursement, with an estimated 91% reduction in activity from baseline estimates and a 17-fold lower activity in treated trees in comparison to untreated trees. Significant residual activity of the hydrogel treatments was recorded, with a nearly 70% reduction from pre-treatment levels persisting at least three weeks after the last application. We conclude that alginate hydrogels can provide a level of *L. humile* control equivalent to or exceeding that of commercially available barrier spray treatments while deploying 99.99% less insecticide into orchards. Alginate hydrogel treatments could be a highly effective component of integrated pest

management programs for sugar-feeding ants and ant-tended hemipteran pests in large-scale commercial agriculture.

Introduction

The invasive Argentine ant, *Linepithema humile* (Mayr) (Hymenoptera: Formicidae), is a highly destructive pest of natural and managed systems worldwide (Vega and Rust 2001; Silverman and Brightwell 2008). A collective colonial structure, numerical dominance, efficient resource collection and utilization, and a propensity to associate with humans in disturbed environments results in sprawling infestations of *L. humile* with devastating economic and ecological impacts (Holway 1999; Suarez et al. 2001; Silverman and Brightwell 2008). Although primarily recognized as an urban structural pest, persistent infestations of *L. humile* are also sustained in agroecosystems. In southern California citrus groves, an optimal Mediterranean climate in combination with access to year-round moisture from irrigation and honeydew from sap-feeding hemipteran pests supports large ant populations (Vega and Rust 2001). Individual citrus trees have been recorded to receive hundreds of thousands of visits from *L. humile* foragers in a single 24-hr period (Markin 1970; Milosavljević et al. 2017; Schall and Hoddle 2017; Schall et al. 2018). *Linepithema humile* obstruct irrigation piping, invade commercial beehives, disrupt native pollinators, and impede biological control of the sap-feeding pests they tend, resulting in pest outbreaks and concomitant plant damages (Buckley 1987; Vega and Rust 2001; Cooper et al. 2008; Silverman and Brightwell 2008; Hanna et al. 2015; LeVan and Holway 2015). Previous studies have demonstrated the importance of *L. humile* control for integrated pest management of economically important ant-tended pests in citrus (i.e., Asian citrus psyllid, *Diaphorina citri* Kuwayama) (Tena et al. 2013; Milosavljević et al. 2017; Schall and Hoddle 2017; Babcock 2018). Consequently, *L. humile* control is critical for preservation of California’s $7.1 billion-per-year citrus industry. Tools with which to implement an effective and economically feasible population-level suppression program for *L. humile* in commercial citrus, however, are limited.

Conventional treatment for *L. humile* typically consists of a barrier spray application of insecticide around the base of crop plants to limit access and kill workers upon contact with residue (Rust et al. 2004; Tollerup et al. 2004; Silverman and Brightwell 2008). In California commercial citrus production, formulations approved for *L. humile* control take the form of an emulsifiable concentrate (i.e., Lorsban-4E and Lorsban Advanced), wet powder (i.e., Lorsban-75WE), or granular application (i.e., Lorsban-15G), all of which contain the active ingredient chlorpyrifos. This broad-spectrum toxicant has been banned in California due to its potential to cause developmental neurotoxicological effects in children at low dose levels (Environmental Protection Agency 2016; California Department of Pesticide Regulation 2019a,b). All usage is mandated to cease by 2021 and replacement ant control products will be needed.

Chlorpyrifos sprays kill ants foraging on the soil surface but have little impact on the subterranean colony where the queens, brood, and majority of workers reside (Vega and Rust 2001). In addition, the moderate repellency of chlorpyrifos residues can result in forager avoidance of treated areas and redirection to nearby uncontaminated food or shelter resources (Knight and Rust 1990b; Silverman and Brightwell 2008). The poor performance of chlorpyrifos is further compounded by its susceptibility to environmental conditions. Residues degrade in high heat, are diluted and dispersed with irrigation water or rain, and may be circumvented via movement over untreated ground cover or use of alternate routes to access canopies (i.e., ground-contacting branches on unskirted trees) (Rust et al. 1996). The potentially low residual longevity (i.e., < 30 days) and efficacy of chlorpyrifos in combination with high availability of replacement nestmates and immigration of foragers from untreated areas often results in rapid resurgence shortly after treatment (Knight and Rust 1990a; Rust et al. 1996; Daane et al. 2008).

In addition to poor long-term performance and failure to provide colony-level ant control, as a potent broad-spectrum insecticide, chlorpyrifos applications can cause considerable mortality of natural enemies needed to manage infestations of HPHs and other pests (Bellows and Morse 1988; Thomson and Hoffmann 2006). Because *L. humile*-associated damage is primarily incurred through the disruption of biocontrol agents and subsequent outbreaks of HPH mutualists, ant control using contact broad-spectrum insecticides confers little net benefit and may even result in secondary pest outbreaks if populations of natural enemies are extirpated in the process (Schall and Hoddle 2017; Schall et al. 2018). Despite significant drawbacks, chlorpyrifos sprays are the prevailing method of ant control employed in citrus as they are easily applied, provide immediate albeit temporary suppression of high-density populations of worker ants, and few other cost-effective treatment options are available (Buczkowski et al. 2014b; Welzel and Choe 2016).

Baiting programs are excellent alternatives to conventional barrier sprays. They exploit the social behavior of ants to provide long-term, colony-wide suppression while preserving natural enemies and minimizing environmental contamination (Cooper et al. 2008; Milosavljević et al. 2017; Schall and Hoddle 2017). Ant baits are typically comprised of a low-concentration toxicant and a phagostimulant (e.g., sugar, oil, or protein) and can be delivered in a granular, gel, or liquid formulation. Unlike broad-spectrum insecticide sprays which kill upon contact, the delayed toxicity of baiting treatments provides sufficient time for mass-recruitment and fidelity to bait sources and trophallactic dissemination of the toxicant among colony members, resulting in population collapse (Rust et al. 2004; Silverman and Brightwell 2008). In addition to having a delayed-action effect, baits must be formulated to be more attractive than nearby food resources, non-repellent, easily transferrable to nestmates, and efficacious under field conditions (i.e., does not photodegrade in sunlight or lose palatability following evaporative loss) (Silverman and Brightwell 2008). For aqueous formulations, the incorporated toxicant must also be water-soluble (Rust et al. 2015).

Liquid sucrose bait compositions are ideal for control of sugar-feeding ants such as *L. humile* as they are inexpensive to produce and resemble honeydew, a preferred natural food source collected year-round (Markin 1970; Silverman and Brightwell 2008; Abril et al. 2014). In comparison with solid and gel formulations, liquid baits maximize *L. humile* forager feeding rate, toxicant intake, and colony mortality (Silverman and Roulston 2001). A sucrose concentration of 20-25% is optimal as this minimizes sugar crystallization in the field and promotes strong recruitment of *L. humile* to baits, high bait intake and crop load per individual, and rapid foraging cycles, which collectively maximize toxicant transfer and colony mortality (Sola and Josens 2016). The most commonly evaluated toxicants used in liquid bait formulations for control of *L. humile* are hydramethylnon, boric acid, fipronil, thiamethoxam, and imidacloprid (Klotz et al. 2003; Tollerup et al. 2004; Greenberg et al. 2006; Cooper et al. 2008; Silverman and Brightwell 2008). While these active ingredients vary in consistency of efficacy, thiamethoxam-based baits have been reported to reliably control *L. humile* populations (Cooper et al. 2008; Buczkowski et al. 2014a,b; Rust et al. 2015; Tay et al. 2017).

In addition to providing excellent area-wide control of *L. humile*, liquid baiting strategies minimize off-target effects. Narrow bait dispenser openings physically exclude larger arthropods and containment of bait in a reservoir minimizes runoff (Cooper et al. 2008). Furthermore, experimental baiting treatments have a thiamethoxam concentration that is 15,000 to 42,000-fold lower relative to the chlorpyrifos concentration used in conventional spray applications (**Table 4.1**). Consequently, off-target effects and potential for environmental contamination are likely to be substantially reduced. However, the standard liquid bait-and-plastic dispenser design has limited feasibility for large-scale agricultural production. Loaded dispensers must be deployed in high densities and continually cleaned and refilled to provide acceptable control of *L. humile* infestations (Nelson and Daane 2014). This expensive and labor-intensive process may be economically unfeasible for large-scale commercial operations (Daane et al. 2008; Buczkowski et al. 2014a,b). In addition, liquid bait deployed for extended periods of time is susceptible to fermentation and evaporative loss, both of which may render the bait unpalatable or ineffective (Silverman and Brightwell 2008; Buczkowski et al. 2014a,b). Despite the consensus that currently available products for control of *L. humile* in agricultural settings are inadequate, little progress in commercial development of liquid bait delivery systems has been made (Silverman and Brightwell 2008). Significant factors impeding development of additional ant control technologies may include time to develop and test products, cost and registration constraints, and uncertainties over market size and adoption rates (Buczkowski et al. 2014b; Rust et al. 2015).

Currently, no commercially available *L. humile* management treatments have proven to be both consistently effective and economically viable for commercial agricultural operations. Previous research has focused largely on the evaluation of liquid bait compositions and application rates rather than the development of new modes of delivery suitable for large-scale, area-wide control programs. A handful of recent studies have evaluated synthetic polyacrylamide hydrogels (i.e., water storing crystals) as an alternative liquid bait delivery option for *L. humile* control (Buczkowski et al. 2014a,b; Rust et al. 2015; Boser et al. 2017; Merrill et al. 2018). Hydrogels are conditioned in an insecticide-laced sucrose solution, increasing in size by up to 300 times as liquid is absorbed into a porous matrix (Rust et al. 2015). Saturated hydrogel beads individually act as micro-sized controlled-release bait dispensers (Tay et al. 2017). As with conventional sprays, hydrogels are mass deployed as a broadcast or aerial application, forgoing the high labor output and expense associated with plastic bait stations (Rust et al. 2015; Boser et al. 2017; Tay et al. 2017; Merrill et al. 2018; Schall et al. 2018). Although polyacrylamide hydrogel applications have been reported to be highly effective in controlling *L. humile* in natural, urban, and agricultural settings, they are not readily biodegradable and persist in the environment for 2-5 years (Buczkowski et al. 2014a,b;

Rust et al. 2015; Boser et al. 2017; Merrill et al. 2018). Through a variety of processes (e.g., mechanical or chemical stresses, biodegradation, exposure to sunlight or high heat > 35^{o}C), nontoxic polyacrylamide slowly degrades into its residual monomer acrylamide, a California state and World Health Organization registered neurotoxin and probable carcinogen (Xiong et al. 2018). Because of its high water solubility, acrylamide is highly mobile in the environment and is ingested by humans in drinking water (Environmental Protection Agency 2010; Xiong et al. 2018). Acrylamide can accumulate in the body by binding with proteins and, consequently, chronic daily oral intake of even low doses (i.e., 0.5 mg/L or above) is hazardous in mammals (Erkekoglu and Baydar 2014). Thus, while previous studies have demonstrated the excellent utility and potential of hydrogels for bait delivery, the polyacrylamide formulation may be unsuitable for commercial use due to environmental and human health risks.

To address the shortcomings of currently available treatment options for *L. humile* control, a non-toxic, biodegradable hydrogel was engineered from alginate, a naturally occurring polysaccharide found in brown seaweeds (Tay et al. 2017). In laboratory assays, the crosslinked calcium-alginate gel matrix absorbed and delivered the targeted concentration of thiamethoxam within a liquid sucrose solution to *L. humile* (Tay et al. 2017). Alginate hydrogels were evaluated for control of *L. humile* in laboratory and urban field settings and demonstrated to be highly effective (Tay et al. 2017). To determine if this alginate hydrogel bait delivery system could provide a comparable level of *L. humile* control in heavily infested citrus groves, a series of preliminary studies evaluating hydrogel application rates, application frequency, and dispersal methodology were conducted (Schall et al. 2018). These preliminary studies provided the basis for a three-month field study which evaluated alginate hydrogel efficacy for *L. humile* control in three commercial citrus operations in southern California. Results of this large-scale field trial are presented here.

Materials and Methods

Alginate hydrogel preparation. To produce calcium alginate hydrogels, 10 g L^{-1} sodium alginate (Na-Alg) solution was slowly poured into a 150 mm diameter funnel attached to a 100-nozzle shower head (AKDY AZ-6021 8-inch bathroom chrome shower head, CA, USA) clamped to a retort stand. As the solution passed through the showerhead nozzles, droplets of 10 g L^{-1} Na-Alg solution formed. Droplets were collected in a 17 L plastic container filled with 5 g L^{-1} calcium chloride ($CaCl_2$) crosslinking solution that was continuously stirred to prevent the aggregation of beads. The resultant 60 kg of calcium alginate hydrogels were separated from the crosslinking solution, divided equally between three 68 L plastic storage bins each filled with 20 L of bait solution (50% sucrose solution with 0.0002% thiamethoxam [2 mg L^{-1} thiamethoxam]; 1:1 ratio of alginate hydrogel to liquid bait), and conditioned for a 24-hour period to produce hydrated hydrogels containing a 25% sucrose solution with 0.0001% thiamethoxam (1 mg L^{-1}) (see Tay et al. 2017 for further details). Thiamethoxam (Thiamethoxam PESTANAL®, Sigma Aldrich) was the selected toxicant as it is dissolvable in sucrose solutions, non-repellant, consistently effective across a wide range of concentrations, and previously reported to be highly efficacious for ant control in a hydrogel-delivered liquid sucrose bait (Buczkowski et al. 2014a,b; Rust et al. 2015; Tay et al. 2017; Boser et al. 2017; Merrill et al. 2018; Schall et al. 2018).

Bait-saturated hydrogels were sieved from the remaining liquid and condensed into one sealed storage bin to minimize air exposure and desiccation. For each batch of 60 kg of hydrogel, 360 mL of putative *L. humile* trail pheromone, (Z)-9-hexadecenal (microencapsulated formulation, 5.6 mg/mL; Suterra, LLC., Bend, OR) was added. The mixture was vigorously stirred for several minutes to ensure even distribution across hydrogels. The addition of (Z)-9-hexadecenal to toxicant-laced liquid bait has been shown to increase the rate of bait discovery by *L. humile* workers and control achieved (Welzel and Choe 2016). Hydrogels were immediately weighed out into 62.5 g aliquots and stored in 286 mL disposable plastic containers in a cold storage room (12 ± 2^{o}C) for up to 48 hours before field deployment.

Field site and plot selection. Three commercial navel orange groves located in southern California (Redlands and Mentone, CA, USA) were selected for evaluation of the efficacy of alginate hydrogel applications for *L. humile* control. Twelve four-by-four (i.e., 16) tree plots were selected per site, for a total of 36 plots. Plots were spaced at least 45 m apart to minimize movement of *L. humile* between hydrogel treated and untreated plots. Distance marking studies conducted in natural settings, vineyards, and citrus groves have reported that the majority of liquid bait movement by *L. humile* foragers occurs within 25-35 m of bait stations and seldom exceeds 45-50 m (Ripa et al. 1999; Vega and Rust 2003, Greenberg et al. 2006; Cooper et al. 2008; Hogg et al. 2018).

Following baseline assessments (see below section “*monitoring*”), six of the twelve plots per site determined to have similar average *L. humile* activities were selected for experimental monitoring. The four central trees of each plot were monitored for *L. humile* activity, for a total of 18 plots (i.e., 72 trees) monitored in the study. Plots were randomly assigned to one of two treatments: hydrogel (i.e., 9 total plots; 36 total trees) or untreated control (i.e., 9 total plots; 36 total trees). Due to close proximity and potential contamination from an insecticide application at a nearby residence, ant monitoring in two plots (one treated, one control) at one site was discontinued. Monitoring in a third plot at a different site (treated) was discontinued due to an adjustment in irrigation schedule which offset watering and sampling of the plot from the other five. Maintenance of a consistent irrigation schedule for all plots within each site was critical to maintain consistency in hydrogel hydration following applications to soil (Schall et al. 2018). After these post-trial initiation adjustments, fifteen four-by-four tree plots and sixty trees (i.e., 32 trees and 8 plots in the control group and 28 trees and 7 plots in the treated group) were monitored for the full three-month study duration (August – October 2017).

Hydrogel applications. All sixteen trees in each plot randomly assigned to the treatment group received a series of three hydrogel applications each spaced three weeks apart at a rate of 250 g of hydrogel per tree, a rate determined optimal in preliminary experiments (Schall et al. 2018). Hydrogel baits were hand-distributed on recently irrigated soil (< 48 h) surrounding experimental trees. To ensure even application, a 1.5 m diameter circular hoop transect constructed from 3/4 in opaque vinyl tubing (Eastman Chemical Co., Kingsport, TN, USA) was divided into quarters with four pieces of flagging tape

(Grainger Inc., Lake Forest, IL, USA). The hoop was placed around the base of each tree trunk and flagging tape was extended from the margin of the hoop to the trunk to ensure consistency in treated area. Each flagging tape-delineated quadrant received one pre-weighed 62.5 g hydrogel aliquot, totaling 250 g of hydrogel across all four quadrants. After placement on soil, the hydrogel bead piles were hand spread to maximize even dispersal over soil surface. Summing all sites, 60 kg of hydrogel bait was applied per each of three treatment applications, for a total of 180 kg of hydrogel produced and applied throughout the experiment.

Monitoring. Monitoring was conducted over a three-month-period from August to October 2017. *Linepithema humile* activity was assessed in a subset of the four innermost trees of each plot across three baseline samplings (i.e., one week, two days, and one day prior to the first hydrogel application) and fifteen post-baseline samplings (i.e., 24 h, 48 h, one week, two weeks, and three weeks following each of the three hydrogel applications). Two methods were utilized to monitor *L. humile* activity: (1) one-minute visual estimations of the number of workers ascending and descending each tree trunk, and (2) 24-hour deployments of two monitoring vials each filled with 40 mL of 25% sucrose solution at the base of monitored trees. Monitoring vials were constructed from Falcon 50 mL conical centrifuge tubes (Corning Inc., Corning, NY, USA) fitted with a 40 x 40 mm square of weed block fabric (Easy Gardener Products Inc., Waco, TX, USA) secured between the vial opening and lid. A 2.5 mm diameter hole was made in each lid to allow ant access to bait which was imbibed through the weed block fabric. To estimate *L. humile* activity with baited monitors, the pre- and post-deployment weights of each vial were compared. After accounting for evaporative loss (i.e., liquid loss in deployed control vials with no ant access), the amount of liquid consumed was divided by 0.003 g (i.e., the average amount of liquid removed by each *L. humile* forager per visit) to determine the total number of ant visits (detailed methods in Reierson et al. 1998).

In addition to monitoring *L. humile* activity, soil temperature and moisture data were collected with a digital soil thermometer (Model S40P-V; Dr. Meter Co. Ltd., Kaohsiung City, Taiwan) and moisture meter (Model HSM50; Omega Engineering Inc., Norwalk, CT, USA). For each monitored tree on all sampling dates, meters were deployed in the soil near the trunk for 2 min at a depth of 2.5 cm.

Statistical methods. A linear mixed model ("*lme*" function in the lme4 package, R) was used to examine the effect of hydrogel treatment (fixed, categorical), monitoring date (fixed, continuous), their interaction, and the covariates soil temperature (fixed, continuous) and soil moisture (fixed, continuous) on *L. humile* activity (i.e., visual and baited vial estimations). A nested error structure (i.e., trees within plots within sites) was used to account for the pseudoreplication inherent in a hierarchically organized experimental design (Spurgeon 2019). Model selection was conducted through backwards elimination and comparison of AIC values, with lower values indicating superior model fit. Visual and baited vial estimations of *L. humile* activity data were power transformed to satisfy normality and homoscedascity assumptions prior to analysis. The overall difference in ant activity between hydrogel treated and control trees across combined sampling dates was examined using the Tukey-adjusted estimated marginal means function ("*emmeans*" in the emmeans package, R). Comparisons were considered significant if $P < 0.05$. All analyses were conducted in the statistical software R (version 3.5.2, R Core Team [2018]).

Mean baited vial and visual estimates of *L. humile* activity collected 48 hours after each hydrogel treatment and on the final sampling date were compared across treatment type (i.e., control or hydrogel treated) and to the baseline estimate (averaged across all baseline sampling days) using a linear mixed model. Ant activity data were power transformed to meet normality and homoscedascity assumptions prior to analysis. Hydrogel treatment, treatment period (i.e., baseline, 48 hours after the first, second, and third treatments, and the final sampling point), and their interaction were included as fixed categorical factors, soil temperature was included as a fixed continuous covariate, and trees within plots within sites was defined as the nested error structure. Tukey-adjusted estimated marginal means was used to compare ant activities between treatment groups and pre- and post-treatments. Tests were conducted in R at the 0.05 significance level.

To evaluate the burden of cumulative *L. humile* infestation, power transformed *L. humile* activity data (visual) were converted to insect days (ID) then summed across the entire sampling period (cumulative insect days; CID) with the following formulas:

$$ID = 0.5(D_a + D_b)(T_a - T_b)$$

$$CID = \sum ID$$

where D_a and D_b are corresponding *L. humile* activities at adjacent time points T_a and T_b (which ranged from 1-7 d in this study) (Ruppel 1983). Insect days were summed across all sampling dates to calculate cumulative insect days, a measure of the intensity of cumulative *L. humile* infestation on each tree over the study duration. Mean cumulative *L. humile* activities for hydrogel treated and untreated trees were compared with an unpaired t-test in R at the 0.05 significance level.

Results

For both visual and baited vial estimates of *L. humile* activity, soil moisture was not found to be a significant model covariate (T = 0.051, d.f. = 1, 816, $P = 0.61$ and T = 1.5, d.f. = 1, 767, $P = 0.13$ respectively) and was dropped from the final model. Hydrogel treatment (T = -3.73, d.f. = 1, 817, $P < 0.01$), the interaction between treatment and monitoring date (T = -7.70, d.f. = 1, 817, $P < 0.0001$), and the covariate soil temperature (T = -2.98, d.f. = 1, 817, $P < 0.05$) significantly affected visual estimates of mean *L. humile* activity. Monitoring date alone (T = -1.0, d.f. = 1, 817, $P = 0.28$) was not a significant model factor. Baited vial estimates of mean *L. humile* activity followed a similar pattern, with a significant effect of treatment (T = -2.98, d.f. = 1, 768, $P < 0.05$), monitoring date (T = 2.93, d.f. = 1, 768, $P < 0.01$), their interaction (T = -2.07, d.f. = 1, 768, $P < 0.05$), and soil temperature (T = 6.98, d.f. = 1, 768, $P < 0.001$). Mean *L. humile* activity was significantly lower in treated trees than untreated trees for both visual (T = 7.57, d.f. = 1, 817, $P < 0.001$) and baited vial (T = 4.72, d.f. = 1, 768, $P < 0.001$) estimates (**Fig. 4.1**).

A comparison of visual and vial estimations of *L. humile* activity prior to and following successive hydrogel applications is provided in **Fig. 4.1** and **Table 4.2**. Visual counts indicated a significant 40, 79, and 91% reduction from baseline activity 48 hours after the first (T = -4.66, d.f. = 4, 351, $P<0.001$), second (T = -7.75, d.f. = 4, 351, $P < 0.001$), and third hydrogel applications (T = -16.53, d.f. = 4, 351, $P < 0.001$), respectively (**Fig. 4.1A**). Concurrently, *L. humile* activity increased in untreated trees with a 34 (T = 2.85, d.f. = 4, 351, $P = 0.12$), 34 (T = -1.84, d.f. = 4, 351, $P = 0.71$), and 51% increase (T = 2.40, d.f. = 4, 351, $P = 0.33$) from baseline estimates following each application. These increases, however, were not significant. Three weeks after the final application, *L. humile* activity in treated trees was 67% lower than baseline estimates (T = -9.96, d.f. = 4, 351, $P < 0.001$) and activity in control trees was not significantly different from baseline estimates (T = -2.9, d.f. = 4, 351, $P = 0.11$), indicating considerable residual activity of hydrogel treatments.

Although baited vial estimates of *L. humile* activity in treated trees followed a trend similar to visual estimates, the greatest percent reduction from baseline values was seen at 24 hours (i.e., a 78, 77, and 81% reduction following the first, second, and third treatments, respectively) rather than 48 hours after hydrogel application (**Fig 4.1B)**. An insignificant 48% increase (T = -0.65, d.f. = 269, $P = 1.0$), significant 41% decrease (T = -4.18, d.f. = 269, $P < 0.01$), and insignificant 30% decrease (T = -0.59, d.f. = 269, $P = 1.0$) in ant activity were observed in treated trees 48 hours after the first, second, and third hydrogel applications, respectively. Concurrently, ant activity in control trees rose, with an observed 206 (T = 7.33, d.f. = 269, $P < 0.001$), 227 (T = 9.52, d.f. = 269, $P < 0.001$), and 120% increase (T = 6.00, d.f. = 269, $P < 0.001$) from baseline estimates (**Table 4.2**). Vial estimates of *L. humile* activity in treated trees on the final sampling date did not corroborate the residual bait efficacy indicated by visual estimates. Three weeks after the final treatment, vial estimates were 53 and 235% higher than baseline values in treated (T = 5.22, d.f. = 269, $P < 0.001$) and control trees (T = 9.30, d.f. = 269, $P < 0.001$), respectively.

Baseline *L. humile* activities were not significantly different between treated and control trees for visual ($T_{4,\ 351} = 0.60$, $P = 1.0$) or vial estimates (T = -0.78, d.f. = 269, $P = 1.0$) (**Fig. 4.1, Table 4.2**). Visual observations of ant activities were significantly lower in treated trees than control trees 48 hours following all hydrogel applications (T = 5.54, d.f. = 4, 351, $P < 0.01$; T = 4.94, d.f. = 4, 351, $P < 0.05$; T = 13.79, d.f. = 4, 351, $P < 0.001$) and on the final sampling date (T = 5.67, d.f. = 4, 351, $P < 0.01$). Estimated ant activities from monitoring vial data for treated trees were significantly lower than control trees following the first two hydrogel treatments (T = 4.53, d.f. = 269, $P < 0.05$; T = 8.92, d.f. = 269, $P < 0.001$) but not significantly different after the final treatment (T = 3.77, d.f. = 269, $P = 0.06$) or on the last sampling date (T = 2.32, d.f. = 269, $P = 0.45$).

The average number of cumulative *L. humile* days (i.e., cumulative infestation burden as determined by visual estimates) in hydrogel treated trees (9020.64 ± 650.45 CID) was less than half that of control trees (22714.83 ± 1967.40 CID) (T = 6.26, d.f. = 1, 58, $P < 0.001$) (**Fig. 4.2**).

Discussion

Current treatment options for management of Argentine ant in commercial citrus are inadequate. Chlorpyrifos barrier sprays repel or provide temporary knockdown of *L.*

humile foragers but are ineffective in controlling subterranean ant colonies, require frequent reapplication, and are hazardous to human, natural enemy, and environmental health (Knight and Rust 1990a; Rust et al. 1996; Thomson and Hoffmann 2006; Daane et al. 2008; California Department of Pesticide Regulation 2019 a,b). Highly targeted toxic liquid baiting programs preserve biological control services and integrated pest management practices and produce long-term, area-wide control of *L. humile* (Cooper et al. 2008; Schall and Hoddle 2017). However, plastic bait stations must be placed in high densities to be effective, and the cost to purchase and time to deploy and service dispensers makes baiting a potentially undesirable management option for growers maintaining large operations (Nelson and Daane 2014; Schall et al. 2018). To generate industry interest and end-user adoption of research-developed bait and bait delivery products, treatments must be targeted, highly effective, economically viable, and easy to deploy for large-scale commercial citrus production.

Hydrogels are a recently explored format for mass-delivery of toxicant-laced aqueous bait to pestiferous sugar-feeding ants such as *L. humile*. However, previously studied matrix compositions (i.e., polyacrylamide) degrade into toxic components (Xiong et al. 2018), and consequently, are unlikely to be registered for commercial use (Tay et al. 2017). To produce a product suitable for *L. humile* control in commercial agriculture, a biodegradable alginate hydrogel was engineered and shown to be highly effective in laboratory assays, field trials in urban areas (Tay et al. 2017), and preliminary field trials in citrus orchards (Schall et al. 2018).

In the present study conducted in commercial citrus groves, both visual and baited vial estimates indicated applications of alginate hydrogels provided excellent control of *L. humile* despite the presence of heavy infestations in neighboring untreated plots. Excluding the baseline and averaging across all sites and sampling dates, mean *L. humile* activities (visual and vial) and cumulative insect days in hydrogel treated trees were significantly lower than that of untreated control trees (**Figs. 4.1 and 4.2**). Forty-eight hours after each hydrogel application, mean *L. humile* activities (visual and vial) were lower in hydrogel treated trees than in control trees (**Table 4.2**). All of these trends were significant with the exception of baited vial estimates following the final hydrogel application. However, the overall trend for pre- and post-treatment ant activities diverged for visual and vial estimates.

According to visual estimates, successive hydrogel treatments increased overall *L. humile* control achieved (**Table 4.2**) and reduced the rate of rebound in treated trees (**Fig. 4.1**). A significant 40% reduction in ant activity from baseline estimates was observed following the initial hydrogel application. However, ant activity rapidly increased after the 48-hour point, returning to pre-treatment levels within a week. The second hydrogel application further reduced ant activity (a significant 79% lower than baseline estimates) and resulted in a slower activity resurgence. Peak control was achieved 48 hours after the third and final hydrogel application, with a recorded 17-fold difference in *L. humile* activity between treated and control trees and a 91% reduction in activity from baseline estimates. In treated trees, ant activity was significantly lower (nearly 70%) than baseline levels three weeks after the final treatment, a residual lifespan similar to that of chlorpyrifos barrier sprays (Knight and Rust 1990a; Rust et al. 1996). In comparison to treated trees, visual ant activity in control trees increased from pre-treated levels across this sampling period (i.e., 34, 34, and 51% following successive hydrogel applications) (**Table 4.2**). These trends, however, were not statistically significant.

The improved residual efficacy of the second and third hydrogel applications relative to the first is unsurprising, given the extraordinarily high densities of *L. humile* reported to infest southern California citrus groves (Markin 1970; Schall and Hoddle 2017). As local nests are eradicated in bait treatment areas, *L. humile* from neighboring colony districts reinvade unoccupied zones for resources, resulting in rapid population rebound (Vega and Klotz 2003; Nelson and Daane 2014; Schall et al. 2018). Waves of reinforcement foragers and their respective colonies are eliminated with subsequent treatments, producing long-lasting area-wide control. Thus, initial hydrogel applications may act as a "sink," the extent of which is dependent on the severity and extent of *L. humile* infestation and spatial resource saturation. Resurgence in ant activity is likely to be more rapid in heavily infested areas (e.g., large, homogenous swathes of untreated citrus production area). This may warrant a shorter reapplication interval between the first and second hydrogel applications to eliminate colonies in reinvaded treatment areas. In the current study, the treatment of small plots surrounded by heavily infested, untreated areas artificially increased reinvasion pressure from neighboring colonies. Replication and the inability to mass-produce hydrogels in quantities sufficient for area-wide application necessitated this experimental design. However, in a real-world scenario, hydrogels would be dispersed across entire groves, eliminating the majority of

L. humile reservoir populations. Consequently, area-wide applications may reduce rebound amplitudes, provide superior control for extended periods of time, and require fewer reapplications (Schall et al. 2018).

In comparison to visual counts, vial sampling methods recorded smaller improvements in *L. humile* control with successive hydrogel treatments and a more rapid resurgence in activity following all applications. Vial estimates of activity between treated and control trees were significantly different 48 hours after the first two hydrogel applications (**Table 4.2**). Ant activity was recorded to be the lowest 24 hours after each application (i.e., 78, 77, and 81%, respectively) and began to rebound 48 hours after each application (**Fig. 4.1**). The consistent level of reduction observed at the 24-hour point is likely the result of forager disruption by hydrogel applications, as the bait evaluated has a delayed toxic effect requiring two to three days to produce high worker mortality (Tay et al. 2017). The divergence in activity trend estimated by baited vial and visual estimates 24 and 48 hours after treatment applications may be related to fundamental differences between these monitoring techniques.

Although baited vial estimates are commonly used to corroborate visual estimates in studies where ant activity is monitored, these sampling measures provide estimates of different niches (i.e., visual estimates measure canopy activity and vial estimates measure ground activity) and are subject to method-inherent biases. Manually counting workers traversing irrigation pipes or tree trunks is subject to human error and provides only a "snapshot" of activity based on the duration of sampling time and the time of day at which observations are made. Consequently, variation in activity may be missed (i.e., at night when more than half of *L. humile* activity occurs) (Agosti et al. 2000; Kistner et al. 2017). While sampling with baited vial monitors can account for changes in ant activity over time (Reierson et al. 1998), provision of an additional food source artificially inflates estimates as *L. humile* foragers proportionally recruit to resources (Agosti et al. 2000; Silverman and Brightwell 2008). This is particularly problematic when sampling alongside other competing attractants such as the hydrogels used in the present study. The addition of a large, highly attractive food source (i.e., hydrogels laced with putative trail pheromone) may have both attracted new workers and redirected nearby workers collecting sugar water away from baited vials. However, following hydrogel depletion (i.e., ~86% of the weight of field-deployed hydrogels is lost within a 24-hour period [Schall et al. 2018]), all residual foragers may have aggregated to baited vials, inflating estimates of activity at the 48-hour mark. Consequently, ground-deployed baited vials may be an unreliable sampling method for monitoring *L. humile* activity in tree canopies and for corroborating visual estimates. The development of ant monitoring technologies (e.g., infrared sensors) capable of capturing hourly fluctuations in *L. humile* activities over a 24-hour period without artificially inflating counts could replace baited vial monitoring.

The results presented here suggest alginate hydrogels loaded with thiamethoxam-laced aqueous bait are a highly effective and sustainable alternative treatment option to chlorpyrifos spray programs in California citrus for *L. humile* control. California has recently banned chlorpyrifos due its designation as a toxic air contaminant and complete cessation of use will occur within 2 yr (i.e., 2021) (Environmental Protection Agency

2016; California Department of Pesticide Regulation 2019 a,b). The mammalian toxicity of thiamethoxam is 4.5 - 19 (oral) and > 6.7 (inhalation) times lower than chlorpyrifos (National Center for Biotechnology Information PubChem Database). Furthermore, the concentration of thiamethoxam in an application of bait-loaded hydrogels is over 15,000 times lower than that of chlorpyrifos in a Lorsban-4E application and in these amounts, presents a negligible human health risk (**Table 4.1**). Even if the thiamethoxam in a single hydrogel application at the study rate (i.e., 70 kg/ha of hydrogel and 0.07 g/ha of thiamethoxam) leeched into the soil, was absorbed by tree roots, and was expressed in the fruit without any loss, the total 0.00084 ppm of thiamethoxam is just 0.21% of the Environmental Protection Agency's (EPA) 0.4 ppm fruit tolerance level in conventional citrus production. At such trace quantities, this treatment would even meet organic citrus production regulations (i.e., one application registers at just 4.2% of the EPA's 0.02 ppm fruit tolerance level) where a small quantity of pesticide is expected from contaminated water or soil sources and pesticide drift.

Although neonicotinoids including thiamethoxam have been implicated in pollinator and natural enemy declines in agroecosystems (Prabhaker et al. 2011; Blacquiere et al. 2012; Stanley et al. 2015), the small quantity of thiamethoxam used in hydrogel applications is unlikely to affect beneficial arthropods present in citrus. Furthermore, the inclusion of putative *L. humile* trail pheromone (Z-9-hexadecenal) in the bait formulation, the short one-to-three-day period hydrogels remain hydrated and attractive, the guarding of hydrogels by *L. humile* workers, and the transport of bait to inaccessible subterranean ant colonies all minimize potential off-target effects (Schall et al. 2018). Consequently, hydrogel treatments likely have a negligible impact on biological control and could be a highly effective component of integrated pest management programs for sugar feeding ants and the HPH pests they tend.

The economic feasibility of hydrogel transport of aqueous bait is an improvement over traditional liquid baiting programs for *L. humile*, which necessitate initial investment in bait dispensers and continuous labor costs for servicing. The material costs for a year of hydrogel applications is less than half that of commercial liquid baiting programs utilizing dispensers (**Table 4.1**). Alginate hydrogels are produced from natural, inexpensive, commercially available materials and require minimal labor input and maintenance, as beads can be rapidly hand-distributed or potentially broadcasted through a mechanical spreader or aerial drops (Tay et al. 2017; Merrill et al. 2018; Schall et al. 2018). Alginate hydrogels have ease-of-use comparable to chlorpyrifos barrier insecticide sprays but vastly outperform them in efficacy using toxicant concentrations several orders of magnitude lower. These features make the alginate hydrogel an excellent candidate for commercial mass-production and agricultural application where control of sugar-feeding pest ants is needed.

The alginate hydrogel baiting system joins the area-wide, colony-level ant control and integrated pest management synergy of standard bait-and-dispenser strategies with the low-labor and cost of broad-spectrum insecticide sprays to provide a comprehensive, sustainable baiting product suitable for control of *L. humile* in commercial citrus production. Future studies could evaluate the efficacy of alginate hydrogels for use against other pestiferous sugar-feeding ant species or in other crops such as grapes which suffer economically significant damage from ants and ant-tended HPH pests.

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Figures and Tables

Application Type	Trade name and company	Availability	Active ingredient and concentration	Dilution and concentration	Application rate; total product applied per ha	Amount of active ingredient per application per ha	Fold change in active ingredient vs. chlorpyrifos in Lorsban-4E	Approximate cost of treatment[a]		
								Per kg or unit	Per month (one application)/ha	Per year/ha
Barrier spray	Lorsban-4E (Dow)	Commercial	44.9% chlorpyrifos	-	2.34 L	1050.66 ml	-	$14.53/L	$34.00	$408.00
Liquid bait	Gourmet Liquid Ant Bait (Innovative Pest Control Products)	Commercial (organic)	1.0% disodium octaborate tetrahydrate	0.5% disodium octaborate tetrahydrate in solution	50 dispensers/ha[b] 0.5 L bait/dispenser 25 L bait/ha	0.125 ml	- 8.41	Bait: $5.47/L Dispensers: $17.50/unit	Bait: $136.75 Dispensers: $875.00	Bait: $1641.00 Dispensers: $875.00 Total: $2516.00
Liquid bait	Optigard Flex[c] (Syngenta)	Experimental	21.6% thiamethoxam	0.0001% thiamethoxam in 25% sucrose solution	50 dispensers/ha[b] 0.5 L bait/dispenser 25 L bait/ha	0.025 ml	- 42026	Bait: $0.19/L Dispensers: $17.50/unit	Bait: $4.75 Dispensers: $875.00	Bait: $57.00 Dispensers: $875.00 Total: $932.00
Bait-loaded alginate hydrogels	Optigard Flex[c] (Syngenta)	Experimental	21.6% thiamethoxam	Hydrogels conditioned in a 0.0002% thiamethoxam, 50% sucrose solution to produce hydrogels containing 0.0001% thiamethoxam, 25% sucrose solution	0.25 kg hydrogel/tree[d] 280 trees/ha 70 kg hydrogel/ha	0.07 g	- 15,009.43	Bait: $0.38 Hydrogel: $0.71/kg	Bait: $26.60 Hydrogel: $49.70	Bait: $319.20 Hydrogel: 596.40 Total: $915.60

Table 4.1 Comparison of active ingredient concentrations, product application rates, and treatment costs for various Argentine ant control strategies used in California commercial citrus production. Calculations are based on lowest-cost material sources available as of 20 May 2019. [a] Cost of treatment only; does not include labor expenses. One application per month is assumed. [b] Application rate based on successful control of heavily Argentine ant infested commercial citrus groves in southern California (94-99% reduction from baseline estimates for 18 months; McCalla unpublished data). [c] Optigard Flex was substituted for analytical standard thiamethoxam in calculations for more cost-effective production of liquid bait. [d] Based on study rate.

Mean Argentine Ant Activity (Visual Estimates)					
	Average of all baseline sampling dates	48 hours after first application	48 hours after second application	48 hours after third application	Final sampling date; three weeks after third application
Control – Visual ant activity	295.33 ± 14.28^{aA}	395.69 ± 29.20^{aA}	395.06 ± 44.48^{aA}	445.97 ± 60.79^{aA}	277.63 ± 33.91^{aA}
(Percent change from baseline)		(+33.98%)	(+33.77%)	(+51.01%)	(-6.00%)
Treatment – Visual ant activity	281.61 ± 18.66^{aA}	169.39 ± 17.12^{bB}	59.14 ± 7.88^{bB}	25.89 ± 3.59^{bB}	91.57 ± 12.22^{bB}
(Percent change from baseline)		(-39.85%)	(-79.00%)	(-90.81%)	(-67.48)
Mean Argentine Ant Activity (Baited Vial Estimates)					
Control – Vial ant activity	88376.36 ± 9593.92^{aA}	270311.51 ± 39450.78^{aA}	289210.86 ± 34805.22^{aA}	194178.75 ± 20759.58^{aA}	295814.47 ± 37784.38^{aA}
(Percent change from baseline)		(+205.86%)	(+227.25%)	(+119.72%)	(+234.72%)
Treatment – Vial ant activity	113429.92 ± 13593.44^{aA}	167726.32 ± 44585.26^{bA}	66488.29 ± 23072.65^{bB}	79362.91 ± 15449.85^{aA}	173108.49 ± 12556.10^{aA}
(Percent change from baseline)		(+47.87%)	(-41.38%)	(-30.03%)	(+52.61%)

Table 4.2 Comparison of mean Argentine ant activities (± SEM) as estimated visually and with baited vials on hydrogel treated and control citrus trees across all sites following applications. Within each column (control vs. treatment), means (± standard error) followed by the same lowercase letter are not significantly different at $\alpha = 0.05$ (estimated marginal means). Within each row (pre- vs. post-treatment), means followed by the same uppercase letter are not significantly different at $\alpha = 0.05$ (estimated marginal means). Values in parentheses represent percent change from baseline estimates. Visual estimates of ant activity indicate the number of workers observed to traverse each tree trunk across a 1-min-period. Baited vial estimates of ant activity indicate the number of worker visits to monitors in a 48-hour-deployment-period and is based on the total amount of liquid removed (each worker consumes 0.003g of bait/visit).

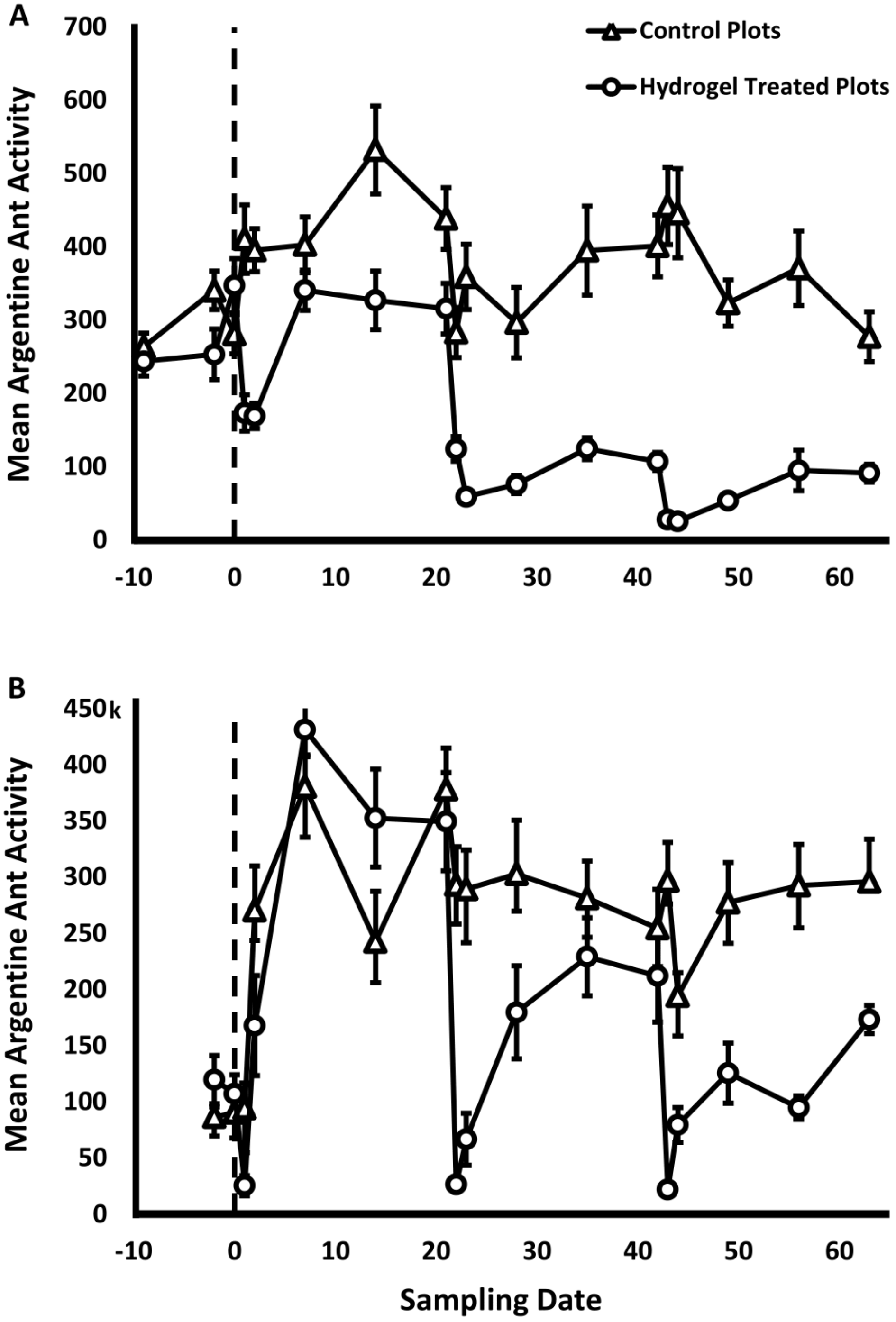

Figure 4.1 Mean visual (A) and 24-hour baited vial (B) estimates (± SEM) of foraging Argentine ants in hydrogel treated and control trees averaged across all sites. The dashed line separates pre- and post-baseline samplings.

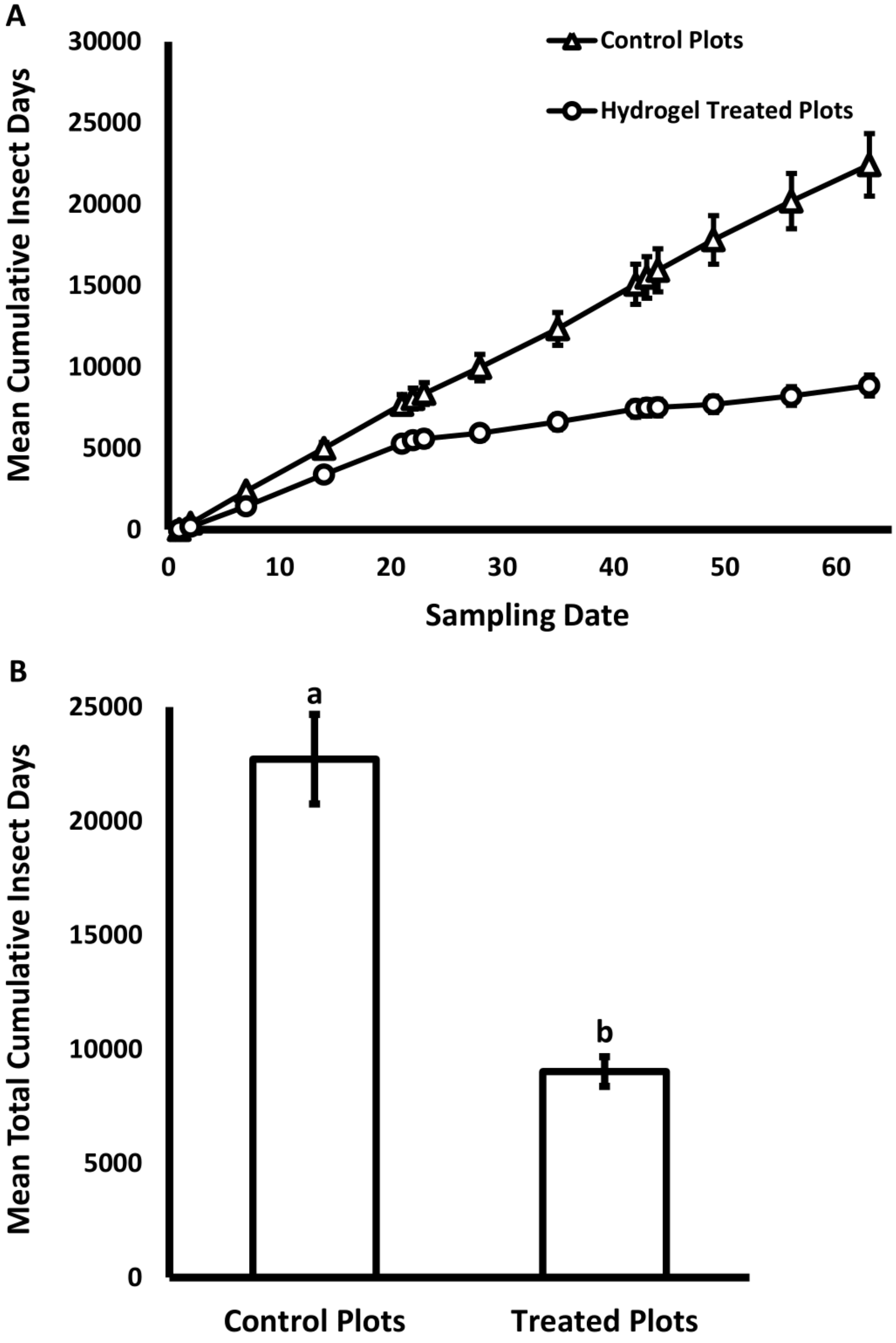

Figure 4.2 Mean cumulative insect days (± SEM) across all sampling dates (A) and mean total cumulative insect days averaged across all sampling dates (B).

Chapter 5: Conclusion

Chapter 2 examined the impact of constant and variable temperature profiles (12 – 38°C) on the life history (i.e., development, longevity, and host-killing capacity) of the Punjab, Pakistan-sourced *Tamarixia radiata* released in southern California for biological control of *Diaphorina citri* (McCalla et al. 2019). To produce thermal performance curves, seven nonlinear models (i.e., Lactin-2, Weibull, Brière-2, Beta, Lobry-Rosso-Flandrois, Ratkowsky, and Performance-2) were fit to egg-to-adult *T. radiata* development rate data. All models predicted lower theoretical minimum and maximum developmental thresholds for parasitoids reared under fluctuating regimes than under constant regimes. Comparative analysis of nonlinear functions indicated high model goodness-of-fit and low deviation between model-predicted and empirically estimated development rates. However, considerable discrepancy in predictions of theoretical minimum and maximum developmental bounds was observed among models and between constant and fluctuating temperature regimes. Overall, the Ratkowsky and Lobry-Rosso-Flandrois models provided the best fit to the datasets while the Performance-2 model produced the most realistic theoretical threshold estimates. Both mean temperature and regime type significantly affected the development and longevity of adult *T. radiata*. Parasitoids reared under variable temperature regimes developed at lower temperatures overall (12°C) and at a more rapid rate (15°C) and survived longer (15 – 20°C) in comparison with conspecifics reared under constant temperature profiles with corresponding means. However, *T. radiata* reared under high average temperatures had a longer development period (35°C) and shorter lifespan (30 – 35°C) in fluctuating regimes. Additionally, degree-day requirements for completion of development were significantly higher (30%) in the fluctuating regime than the constant regime. Finally, *T. radiata* was observed to inflict substantial total host mortality under fluctuating temperature profiles (i.e., 85 – 92% at 20, 25, and 30^{o}C) through a combination of parasitism and non-parasitism (i.e., host feeding) damages. Consequently, field estimates of parasitism may undervalue the contributions of *T. radiata* to total *D. citri* mortality. A comparison of the results of this study with other published *T. radiata* development datasets indicated convergence in estimates of degree-days and theoretical minimum development thresholds among geographically isolated parasitoid populations (i.e., California, Brazil, Réunion Island, and Vietnam). These results emphasize the importance of examining parasitoid life history under field-realistic temperature conditions (i.e., across a range of average temperatures with daily thermal cycles) and are of significance in improving *T. radiata* mass-rearing and release programs, *T. radiata-D. citri* synchrony predictions produced by climate modeling software, and comparison of spatiotemporally disparate *T. radiata* populations.

Antagonistic interactions between the Argentine ant, *Linepithema humile*, and natural enemies of the honeydew-producing hemipteran insects it tends are well-documented in agroecosystems. Preliminary studies conducted in urban citrus gardens indicated that *L. humile* control increased generalist predator activity in *D. citri* colonies and parasitism by *T. radiata* (Tena et al. 2013; Schall and Hoddle 2017). In chapter 3, this work was expanded upon. The efficacy of an 18-month liquid bait-and-dispenser program for control of *L. humile*, *D. citri*, and other ant-associated hemipteran pests in southern California commercial citrus orchards was assessed. Within 2 months of treatment, *L. humile* activity was reduced by 92% and it remained 90 – 99% lower than baseline estimates for the study duration. In addition, average ant activity was approximately 20 times lower in treated than control trees. Supresssion of *L. humile* nearly eliminated hemipteran pest infestations in treated trees. A 97, 84, and 99% decrease in twig, flush, and fruit infestation was achieved after one year of treatment. Across the final year of study, the cumulative frequency and severity of pest infestation was 10 and 5 times lower (respectively) in treated trees than control trees. Relative to control trees, the mean infestation rate of *D. citri* was 75% lower, *C. hesperidum* 95% lower, *P. citri* 98% – 100% lower, and *A. aurantii* 70 – 80% lower in treated trees. The mean number of individuals per colony was also significantly lower in treated trees for most hemipteran pest species. Interestingly, predation may have played a larger role in control of pest populations than parasitism, which was significantly lower than or not significantly different between treatments across all pest species. Syrphid flies were the most common predator morphotaxa observed in the study (i.e., comprising >70% of all natural enemies collected) and were recovered exclusively from *D. citri* and *P. citri* colonies in flush. Furthermore, syrphid prevalence was strongly associated with peaks in *D. citri* colony size, although the directionality of this relationship cannot be determined. The results of this study and others indicate syrphids may be a dominant predator guild for biological control of *D. citri* in southern California. Regardless of the importance of individual natural enemy groups, the end result of multi-season *L. humile* control was considerable suppression of populations of *D. citri* and other economically important hemipteran pest species. Consequently, *L. humile* control should be considered a critical constituent of integrated pest management programs targeting these pests in commercial citrus.

Currently available management options for control of *L. humile* in commercial citrus are insufficient. Sprays of broad-spectrum insecticide are readily applied but carry significant environmental risks and provide little to no control of subterranean ant colonies. In addition, the only registered toxicant for use in this format (i.e., chlorpyrifos) was banned by California in early 2019. In chapter 3, a liquid bait-and-dispenser program was shown to provide excellent control of *L. humile,* but the treatment was labor-intensive to maintain on a commercial scale. In chapter 4, a broadcastable, biodegradable delivery system for insecticide-laced liquid bait (alginate hydrogels) was assessed for control of *L. humile* in commercial citrus orchards (McCalla et al. in press). This novel baiting format combines the area-wide, colony-level suppression and biological control synergy of liquid baiting programs with the ease of application and lower cost of broad-spectrum insecticide barrier sprays. Hydrogel treatments strongly reduced *L. humile* activity, with the level of control achieved rising incrementally across three successive applications. A 91% reduction in ant activity from baseline estimates was observed 48 hours after the third and final hydrogel disbursement. Activity was significantly lower (up to 17 times less) in treated trees than control trees. Hydrogel treatments provided considerable residual activity, with a ~70% reduction in ant activity from pre-treatment levels present three weeks after the final bait dispersement. These results suggest that hydrogel baits can provide control of *L. humile* on par with or exceeding that of commercially available treatments while remaining cost-effective and deploying a fraction of the insecticide into orchards (i.e., 15,000 times less than a comparable chlorpyrifos barrier spray). Consequently, this treatment has excellent potential for commercial production and adoption. In conclusion, the alginate hydrogel bait delivery system is a promising new treatment option for controlling infestations of sugar-feeding ants in vulnerable cropping systems (e.g., citrus, wine grapes, nuts) and may help minimize losses sustained directly through ant damage and indirectly through hemipteran pest mutualists.

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