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Predators of Asian Citrus Psyllid (*Diaphorina citri*) in Southern California

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

Doctor of Philosophy

in

Entomology

by

Aviva Goldmann

December 2017

Dissertation Committee:
Dr. Richard Stouthamer, Chairperson
Dr. Matt Daugherty
Dr. Jocelyn Millar
Dr. Erin Rankin

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The Dissertation of Aviva Goldmann is approved:

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Committee Chairperson

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ABSTRACT OF THE DISSERTATION

Predators of Asian Citrus Psyllid (*Diaphorina citri*) in southern California

by

Aviva Goldmann

Doctor of Philosophy, Graduate Program in Entomology
University of California, Riverside, December 2017
Dr. Richard Stouthamer, Chairperson

Asian citrus psyllid (ACP) threatens the viability of California citriculture because it spreads a bacterium, *Candidatus* Liberibacter asiaticus, that kills citrus trees. Preliminary observations suggested that predatory arthropods in southern California reduce the ACP population, but it was not known which predator species were important. The goal of this PhD project was to identify potentially effective natural enemies of ACP among southern California predatory arthropods for use as biological control agents. To accomplish this, we first measured the natural mortality of ACP in a southern California orange orchard by conducting a predator exclusion experiment monthly for seven months. Overall, ACP survival was nearly four times higher in cohorts protected from predators than in control cohorts. To detect which predator species contribute to ACP mortality, we conducted a year-long survey of predatory arthropods in two orange orchards. Among the predators collected, ten were abundant. We designed a

molecular assay to detect ACP DNA in predator gut contents and used it to test field-collected specimens of these predators for recent predation of ACP. Three insects and all five spiders were positive for ACP predation in over 10% of specimens tested. Older *C. comanche* larvae and afternoon-collected adult *D. pumilio* were significantly more likely to test positive for ACP predation than conspecifics which were younger (*C. comanche*) and collected before dawn (*D. pumilio*). The two insects that tested positive most frequently, *Diomus pumilio* (Coleoptera: Coccinellidae) and *Chrysoperla comanche* (Neuroptera: Chrysopidae) were investigated further because of their potential for use in augmentative and/or conservation biological control. For each species, we conducted timed feeding trials to calculate a half-life for the detectability of ACP remains following predation, and conducted prey choice assays with all life stages of ACP to determine which were most likely to be consumed after being probed by the predator. *D. pumilio* adults preferentially preyed upon ACP eggs and first-third instar nymphs, while *C. comanche* larvae most frequently consumed eggs and third-fourth instar nymphs. We recommend development of these insects as biological control agents of ACP, and suggest developing spider conservation tactics compatible with citrus IPM.

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Background

Asian citrus psyllid (ACP), *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae) represents a serious threat to the long-term viability of California's iconic, $1.5 billion citrus industry (USDA-NASS, 2012), because it is the vector of a bacterium that is lethal to citrus trees. This bacterium, *Candidatus* Liberibacter asiaticus (CLas), is a causative agent of the devastating citrus disease huanglongbing (HLB), or citrus greening disease, in which trees produce unpalatable, unmarketable fruit before succumbing to an early death (Bové, 2006). Because there is no known cure for HLB, management programs currently depend on vector reduction, removal of infected trees, and protection of nursery stock (Grafton-Cardwell et al., 2013; Hall et al., 2013).

In conventionally managed orchards, vector reduction is accomplished with insecticide treatments (Hall et al., 2013), but certified organic groves are at risk of developing high ACP populations because organic treatments of comparable effectiveness are not available (USDA-APHIS, 2010). Furthermore, citrus planted at private homes is extremely common in southern California, with 60% of houses having an average of two typically “un-managed” trees (Gottwald et al., 2013). This urban orchard, if left untreated, is expected to harbor a large population of ACP and undermine effective area-wide management (USDA-APHIS, 2010). Initially, the California Department of Food and Agriculture (CDFA) attempted to detect ACP-infested trees on private property in Southern California, and subsequently treat them with foliar and systemic insecticides, but

the invasion outpaced available funding and personnel. The spray program has since been scaled back across most of the region to focus resources on the leading edges of the invasion and stave off incursions into commercial growing regions (Hoddle, 2012). Meanwhile, insecticide resistance has been reported in Florida (Tiwari et al., 2013; Tiwari et al., 2011), where ACP has been established since 1998 (Halbert, 1998). New control options, including improved biological control, are urgently needed to address this deficiency (USDA-APHIS, 2009; Hall et al., 2013). A consensus of the USDA-APHIS Asian citrus psyllid Technical Working Group (USDA-APHIS, 2009) stated that "biological control would be a viable component of an area-wide control program with respect to psyllid control in urban settings, natural areas, certified organic production, and possibly abandoned groves." A recent review of CLas management measures and current research goals (Blaustein et al., 2017) similarly concluded that “there is an urgent need to augment and reduce the dependency on insecticides by identifying feasible measures to mitigate *Ca.* Liberibacter spp. and associated HLB symptoms (e.g., via applications of antimicrobials, applications of plant-beneficial compounds, biological control).”

Classical biological control of ACP utilizes two specialist parasitoids: *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligharensis* Shafee, Alam, & Agarwal (Hymenoptera: Encyrtidae) (Hoy and Nguyen, 2001), the only confirmed primary parasitoids of ACP. These were introduced to California in 2011 and 2014, respectively (Mendoza, 2011;

Warnert, 2015), but have not brought about hoped-for levels of reduction of ACP (Kistner et al., 2016; Vankosky and Hoddle, 2017).

A possible source of new biological control agents was suggested by the results of an ACP phenology survey conducted in 2011 on residential citrus in east Los Angeles (Goldmann, unpublished), in which large population reductions were consistently observed between ACP life stages. These reductions, which occurred prior to the release of specialist parasitoids, suggested that ACP was attacked by resident predators. This observation inspired the subsequent work described in this dissertation, which aimed to identify these natural enemies and to recommend potentially effective species as agents for improved biological control of ACP.

ACP and HLB

ACP was first detected in California in 2008 (Grafton-Cardwell and Lazaneo 2012), and is now widely established across residential and citrus cultivation areas in southern California. It has begun to spread north through Central Valley commercial citrus and has been detected as far north as Sacramento (University of California, 2017). ACP is a phloem feeder that attacks citrus and related rutaceous plants, including the common ornamental shrub *Murraya paniculata* (Halbert and Manjunath, 2004; Yang et al., 2006). Females oviposit exclusively on new growth of host plants. Hatching follows after two to four days, and nymphs develop through five nymphal instars within 11 to 15

days. Males and females become reproductively mature about 20 days after entering the adult stage, at which time the abdomen turns from tan or green to orange (Liu and Tsai, 2000; Skelley and Hoy, 2004). Oviposition begins one day after mating, and a female may produce up to 800 eggs during her lifespan (Grafton-Cardwell et al., 2013).

ACP was first linked with HLB in India in 1927 (Husain and Nath, 1927). ACP and CLas are both thought to originate from the Indian subcontinent (Halbert and Manjunath, 2004; Shivankar et al., 2000) and to have evolved in association with *Murraya* sp. (Rutaceae) and other non-citrus Rutaceae before the arrival of citrus plantings in the 19th–early 20th century (Beattie et al., 2008; Nelson et al., 2013). CLas is a gram-negative, phloem sieve-tube limited bacteria (Bové, 2006) that causes mottled leaf chlorosis, yellowed stems, blocked veins, stunted above-ground and below-ground growth, poor fruit growth and incomplete ripening, and the tree's eventual death (Fujikawa et al., 2013; Graham et al., 2013). Koch's postulates cannot be fulfilled because CLas cannot be cultured outside of plants, but sufficient evidence exists to justify the conclusion that it is a causative agent of HLB (Bové, 2006).

Reduction of ACP population is an important component of HLB management because CLas infection rate increases with the number of infected psyllids feeding on a plant (Pelz-Stelinski et al., 2010), while productivity of HLB-infected trees increases as ACP population is decreased, e.g. by insecticide application (Monzo and Stansly, 2017). In addition, smaller vector populations

are generally predicted to select for decreased pathogen virulence (Ewald, 1994). Therefore, a substantial reduction in psyllid population may lead to a decrease in the incidence of disease, as was the case with ACP on Réunion Island (Etienne and Aubert, 1980).

In 2012, CLas was detected from one tree in a residential area of Los Angeles County (Wang et al., 2013). Additional CLas infections detected in southern California in 2015 were of a different genetic type than the 2012 detection, and are thought to have originated from a separate introduction (Yan et al., 2016). As of fall 2017, more than 100 CLas-infected trees had been detected in southern California (Citrus Pest and Disease Prevention Program, 2017), with the majority of these located from San Gabriel Valley in the north to Anaheim in the south (University of California, 2017). Climatic suitability research predicts different geographic distributions for ACP and CLas, with lower CLas establishment in California than in the Gulf Coast states, limited to coastal areas, including Los Angeles and San Diego (Narouei-Khandan et al., 2016). However, because this hypothesized limitation is due to low rainfall, Narouei-Khandan et al. (2016) speculate that irrigation might allow CLas to spread beyond this predicted range.

Biological control of ACP: current status and natural enemies

ACP is not controlled by natural enemies in India (Shivankar et al., 2000), its putative area of origin (Beattie et al., 2008), in an example of a native insect

becoming a pest on an introduced plant. ACP continues to constrain the range of citrus cultivars which can be grown in India (Shivankar et al., 2000) and the adjoining Punjab region of Pakistan (Khan et al., 2014). Because adequate population reduction has not yet been achieved through classical biological control using the two most promising natural enemies known from ACP's area of origin, predators or parasitoids from outside ACP's native range should be considered for their potential in biological control if they show sufficient promise. Dozens of predatory arthropod species have been reported as potential natural enemies of ACP on multiple continents (Supplementary tables 1 and 2, Appendix).

Arthropod predators of ACP in North America

The potential for predatory arthropods to play a role in ACP biological control was first formally investigated in two Florida predator exclusion studies (Michaud, 2004; Qureshi and Stansly, 2009). The studies each demonstrated substantial ACP mortality due to natural enemy attacks, with most of the observed mortality originating from predation by resident predatory arthropods, even in the presence of *T. radiata* (Michaud, 2004; Qureshi and Stansly, 2009). In the first of these studies, Michaud (2004) reported that ACP survival to adulthood was 120 times higher when predators were excluded and *T. radiata* was allowed access than in cohorts which allowed access by all natural enemies (predators and parasitoids). *Tamarixia radiata*'s success was reduced by other

orchard arthropods, which consumed 95% of *T. radiata* larvae and 64% of mummies in predator access cohorts, while even in predator-exclusion cohorts, 28% of mummies were lost to apparent ant predation.

Qureshi and Stansly (2009) reported that when predators were excluded, ACP R_o was 5 to 27 times higher, calculated as the survival rate multiplied by the expected ACP fecundity rates published by Liu and Tsai (2000) for the ambient temperatures. Parasitism by *T. radiata,* easily quantified by the presence of mummies that persist on foliage, killed only 1-3% of ACP nymphs, and 2-24% of field-collected, laboratory-reared 4^{th} and 5^{th} instar nymphs. Intraguild predation was observed indirectly, in that the rate of parasitism increased when predators were partially excluded. Host-feeding by *T. radiata*, which causes additional mortality (Chu and Chien, 1990; Skelley and Hoy, 2004), was not recorded in this study.

Chong et al. (2010) conducted a survey of ACP natural enemies on Florida residential orange jasmine, *Murraya paniculata*. This report estimated a reduction between ACP egg and adult emergence of 96.1% due to both biotic and abiotic factors. This included a reduction of 23.6% in the egg stage, 65.4% for early nymphal instars, and 85.1% for late nymphal instars. These numbers included 18.5% parasitism by *T. radiata* (calculated as “the number of emerged adult parasitoids divided by the sum of the late instars and the mummies without emergence holes”). While natural enemies cannot be assumed to behave

identically on citrus and *Murraya* sp., it is useful to know that natural enemies can reduce ACP on residential plants other than citrus.

Identifying candidate biological control agents among resident predators

Selection of biological control agents

At the outset of this research, a great deal of information had become available on the development of methods for successful identification of trophic relationships in agricultural systems using molecular gut analysis of field-collected predators (Greenstone et al., 2010; King et al., 2008; Pompanon et al., 2012). This approach appeared extremely promising for identifying predators of ACP, for several reasons. First, ACP in southern California is subject to substantial biotic mortality; second, little to nothing was known about the predators involved; third, improved biological control options are expressly needed for this pest. We therefore undertook to identify key trophic relationships between ACP and members of its resident natural enemy community in southern California using molecular analyses of potential predators' gut contents, in coordination with field surveys and laboratory studies. We also hoped to contribute to a growing body of work that specifically addresses the question of how to select the most promising candidate natural enemies from a large natural enemy community. This body of research has recently begun to zero in on ways of combining molecular analysis of gut contents with well-established field

methods to identify the most potentially effective biological control agents (Birkhofer et al., 2017; Furlong, 2015; Unruh et al., 2016; Yang et al., 2017).

Dissertation objectives

The overarching goal of the work presented in this dissertation was to identify new biological control agents for ACP in California, while also contributing to the development of tools and protocols for identifying good biological control candidates from a large natural enemy community. The studies reported herein address the following four objectives:

1) Confirm that ACP is subject to natural biotic mortality in southern California citrus.
2) Identify abundant predators that are found with ACP during a yearlong survey, and describe their seasonal abundance.
3) Develop a molecular assay to detect ACP remains in predator gut contents, and use it on field-collected specimens to obtain an estimate of ACP predation frequency.
4) Calibrate these values with digestion time analysis.
5) Describe predation habits of the most promising candidate natural enemy species identified by these studies.

In Chapter 2, predator exclusion cage experiments were used to confirm that ACP is subject to natural biotic mortality in southern California citrus. The experiment was repeated monthly for seven months, and relationships between

ACP survival to adulthood, time of year, and predator exclusion treatment were analyzed.

Chapter 3 discusses the results of a year-long survey of predatory insects and spiders in two southern California citrus orchards. The identity, abundance, and seasonality of predators collected in this survey are reported.

Chapter 4 describes the design and use of a DNA-based analysis of predator gut contents for use in detecting predation of ACP by field-collected predators obtained from the survey described above. The chapter reports ACP detection rates for each predator tested and analyzes detection rates by study site, predator life stage, and time of day the specimen was collected.

Finally, Chapter 5 reports the results of digestion time analysis experiments for the top two insect predators of ACP identified in Chapter 4, *Chrysoperla comanche* and *Diomus pumilio,* and discusses the implications of each species' detectability half-life on our understanding of their predation rates. ACP life stage preferences were determined for each predator species in a prey choice assay.

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Chapter 2:

Natural mortality of ACP in Southern California

Abstract

Improvements in biological control of the Asian citrus psyllid (ACP), *Diaphorina citri*, have been called for by both industry and government to better manage ACP in organic citrus groves and to reduce uncontrolled infestations of this pest on southern California residential and abandoned citrus. To assess whether resident natural enemies in southern California include species with potential for use in ACP biological control, a predator exclusion experiment was conducted in an organic orange grove. In trials repeated monthly over the course of seven months, overall survival of ACP in complete predator-exclusion cohorts was ~4 times higher than in unprotected cohorts, with this difference varying by month from 1.5 times as high to 51.7 times as high. These results show that natural enemies in southern California play an important role in reducing ACP population growth, and that resident species may be exploited for ACP biological control.

Introduction

The Enemy Release Hypothesis (ERH), which proposes that an invasive species' "escape" from co-evolved natural enemies contributes to its increased abundance in a new region, is often cited as an explanation for the pest status of arthropods which are benign in their native ranges (Hufbauer and Torchin, 2007; Torchin and Mitchell, 2004). Successful cases of classical biological control, in which an invasive pest's abundance and economic impact are dramatically

reduced after its reunion with a key natural enemy from its area of origin, provide support for the ERH (Bale et al., 2008; Dreistadt and Hagen, 1994; Groenteman et al., 2011). However, not all invasive pests are controlled by natural enemies in their native range (Bellows and Fisher, 1999, p. 90), and meta-analyses comparing natural enemy pressure in native ranges vs. areas of origin have not provided unequivocal support for the Enemy Release Hypothesis (Hawkins et al., 1997; Keane and Crawley, 2002; Liu and Stiling, 2006). Cases have been reported in which novel predator-prey (Hawkins et al., 1997; Hogg et al., 2013; Symondson et al., 2002) and/or parasitoid-host relationships (Bürgi and Mills, 2014) form as a pest spreads across new territory that contribute significantly to biological control. In the absence of “silver bullet” natural enemies from a pest’s area of origin, this phenomenon may hold the key to establishing the best possible biological control program for an invasive pest.

Such may be the case with Asian citrus psyllid (ACP), *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae). ACP was first detected in California in 2008 (Grafton-Cardwell and Lazaneo, 2012), and is now found throughout southern California residential and commercial citrus, with substantial establishment on commercial citrus in the south Central Valley as well as continued incursions to the north (University of California, 2017). ACP poses a serious threat to citriculture because it is the vector of a pathogen that causes a lethal disease in citrus trees. This pathogen, the bacterium *Candidatus* Liberibacter asiaticus (CLas), causes trees to develop the disease huanglongbing (HLB), or citrus

greening disease. Symptoms of this disease include leaf and shoot chlorosis, stunted growth above and below-ground, failure of fruit to ripen completely, and premature death of infected trees (Bové, 2006; Fujikawa et al., 2013; Graham et al., 2013). The three main components of HLB management are typically psyllid control, infected tree replacement, and planting with uninfected young trees (Grafton-Cardwell et al., 2013; Hall et al., 2013).

The first detection of HLB in California occurred in 2012, involving a residential tree in the city of Hacienda Heights, Los Angeles County (Kumagai et al., 2012). The source population for this bacterial strain was likely of Asian origin (Wang et al., 2013). No additional CLas infections were detected in southern California until 2015. As the bacteria involved in these infections were of a different genetic type than those detected in 2012, the later infections are thought to have originated from a separate introduction of infected material (Yan et al., 2016; Zheng et al., 2017). At the time of writing, CLas infection has been detected in over 100 residential trees in California, all of which have been removed and destroyed (Citrus Pest and Disease Prevention Program, 2017). The eventual geographic range of this pathogen in California is unknown: while widespread establishment throughout California citrus has generally been considered inevitable (Hirsch, 2009; Lawrence, 2012), climate factors such as low rainfall, may limit its prevalence in some areas of California (Narouei-Khandan et al., 2016).

ACP control is considered an important component of HLB management for several reasons. First, the HLB infection rate of previously uninfected plants increases with the number of infected psyllids allowed to feed on them (Pelz-Stelinski et al., 2010), while large psyllid populations decrease the productivity of already infected trees (Monzo and Stansly, 2017). If the psyllid population can be substantially decreased, the expected result is a decrease in HLB infection rate, as was observed on Réunion Island (Etienne and Aubert, 1980).

Insecticide treatments are the primary method for achieving vector reduction in conventionally managed commercial orchards (Hall et al., 2013), however, biological control is considered an important component of ACP control as well (USDA-APHIS, 2009). First, similarly effective treatments are not yet available that are compatible with organic certification (USDA-APHIS, 2010). Second, citrus is a very common residential yard plant in southern California: ~60% of houses have citrus trees, with an average of approximately two citrus trees each, which are typically not under pest management (Gottwald et al., 2013). This presents what has been called a "huge problem" for area-wide ACP management in southern California (USDA-APHIS, 2010). Furthermore, it is not unlikely that insectide resistance will begin to appear in California, as it already has among ACP in Florida (Tiwari et al., 2013; Tiwari et al., 2011). Improved biological control would begin to address these deficiencies in management options (USDA-APHIS, 2009; Hall et al., 2013). A consensus of the USDA-APHIS Asian citrus psyllid Technical Working Group (USDA-APHIS, 2009) stated

that "biological control would be a viable component of an area-wide control program with respect to psyllid control in urban settings, natural areas, certified organic production, and possibly abandoned groves."

Classical biological control of ACP has been implemented in California with the introduction of two specialist parasitoids from ACP's native range (Mendoza, 2011; Warnert, 2015), *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligharensis* (Shafee, Alam, & Agarwal) (Hymenoptera: Encyrtidae) (Hoy and Nguyen, 2001), which are the only confirmed primary parasitoids of ACP. Ideally, a classical biological control exploration program will find a natural enemy species or community in the pest's area of origin which substantially reduces the level of damage done by a pest. However, ACP is not controlled by these wasps or by other natural enemies in India (Shivankar et al., 2000), its putative area of origin (Beattie et al., 2008). ACP's pest status and ability to vector HLB, first reported in India in 1927 (Husain and Nath, 1927), continues to constrain the range of citrus cultivars which can be grown there (Shivankar et al., 2000) and in the adjoining Punjab region of Pakistan (Khan et al., 2014). *Tamarixia radiata* and *D. aligharensis* were introduced to California in 2011 and 2014, respectively (Mendoza, 2011; Warnert, 2015), and have been continually reared and released ever since (Hornbaker and Kumagai, 2016; Mohammadi, 2016). Neither has yet been reported as an important contributor to ACP population reduction, either in field studies or the ongoing monitoring program in California (Kistner et al., 2016a;

Vankosky and Hoddle, 2017). *Tamarixia radiata* had little impact on ACP survival in a Florida predator exclusion study which accounted for both parasitism and host-feeding (Michaud, 2004). In a similar study conducted in California, eight to forty-eight times more ACP survived to adulthood in *T. radiata*-access cages than in unprotected cohorts (Kistner et al., 2016b). A second predator exclusion study conducted in Florida reported similar results, although it tracked only parasitism and did not account for host-feeding by *T. radiata* (Qureshi and Stansly, 2009). However, these studies reported substantial reduction of ACP by resident predatory arthropods. Michaud (2004), measuring the effect of predator exclusion in terms of psyllid survival from 1st instar to adult, reported that ACP survival to adulthood was 120 times higher when predators were excluded and *T. radiata* was allowed access, vs. cohorts which allowed access by all natural enemies. Qureshi and Stansly (2009) reported a 5 to 27 times higher ACP R_o when predators were excluded, calculated as ACP survival multiplied by ACP fecundity rates given by Liu and Tsai (2000) for the recorded field temperatures. *Tamarixia radiata* killed 1-3% of ACP nymphs overall, and 2-24% of 4^{th} and 5^{th} instar nymphs that were collected in the field and observed for parasitoid emergece. *Tamarixia radiata* host-feeding causes additional mortality (Chu and Chien, 1990; Skelley and Hoy, 2004), but was not recorded in this study.

The potential of predatory arthropods to play an important role in ACP control in California was initially suggested by an ACP phenology survey conducted in Los Angeles residential citrus in east Los Angeles communities, La

Puente and El Monte, by A. Goldmann in 2011-2012 (Goldmann, unpublished). In this survey, large population reductions were consistently observed between *D. citri* life stages. The specialist parasitoid wasp *T. radiata* was not a contributing factor to this mortality, as it was not present in the study area during the survey period.

These observations suggested that resident predatory arthropods in the natural enemy community of ACP are worthy of further study, with the potential for presenting new biological control options as sought by the USDA (USDA-APHIS, 2009). A logical first step in this process is to quantify the impact of resident predatory arthropods on ACP survival.

The most frequently used method for evaluating the impact of natural enemies in a field environment is exclusion by means of cages or another type of barrier (Luck et al., 1988), a technique that was first reported in 1942 for evaluation of citrus black scale control by natural enemies (Smith and DeBach, 1942). Advantages of predator exclusion cages, which are typically made of fabric and secured around all or part of a plant, include low cost and ease of installation on replicates that are branch-sized or smaller. Fabric mesh size can be selected to exclude specific predators of interest, and controls can be easily conducted by omitting one or more components of the exclusion design. Owing to these advantages, predator exclusion cages were selected for use in this study. The goal of this work was to determine whether predatory arthropods decrease ACP density sufficiently in southern California citrus orchards to justify

evaluation of local species as natural enemies of ACP for use in conservation or augmentative biological control.

Materials and methods

Location and timing of experiments

The predator exclusion study was conducted in an organic Valencia orange grove in Yorba Linda, CA, with an established ACP population. The grove is located within the floodplain of the Santa Ana River, which runs immediately south of the orchard, with a residential area located directly to the north. This site was desirable because it was one of few groves of standardized trees which at the time harbored sufficiently high ACP population density to detect the effects of experimental manipulations. A grove under organic management was ideal because few ACP are found in insecticide-treated groves. Trials were conducted monthly from March 2013 to September 2013, the seven months of that year during which ACP laid sufficient eggs to conduct the experiment on branches pruned to spur the growth of leaf flush. The biological control agent *Tamarixia radiata*, although released in southern California at the end of 2011, was not yet established at our field site, and remained absent throughout the experimental period. Furthermore, imidacloprid and other conventional pesticides were not applied during the course of the study (J. Barcinas, personal communication).

Diaphorina citri cohorts used in experiments

Experimental cohorts consisted of ACP eggs on newly grown leaf flush of tree branch terminals. Branch terminals were selected that were on separate trees, at a practical height for taking field counts, and easy to isolate from contact by minimal clipping of neighboring branches. If necessary, branches were pruned 2 weeks prior to the experiment to induce leaf flushing. Branches were not re-used, so each month's results were independent of all other months. Pruned branches were monitored for the appearance of new flush and the presence of ACP eggs. Eggs were laid *in situ* by ACP females present in the local population. Observations were initiated after the appearance of sufficient ACP eggs on flush. Eggs were counted visually with the aid of an OptiVISOR binocular headband magnifier (Donegan Optical Company, Inc., Lenexa, KS). All other visible arthropods were removed manually, and surrounding branch terminals were pruned away to prevent other foliage from coming into contact with the experimental branch terminal. Each branch terminal was randomly assigned to one of the four predator exclusion treatments described below. Six to nine replicates of each treatment were conducted every month, depending on the availability of ACP-colonized flush.

Starting egg counts varied because of natural variation in oviposition, and ranged from seven to fifty-nine eggs per trial. Although this variable could not be standardized, starting egg count did not differ significantly between months or between treatments within months. Each month's starting egg count distribution

was checked for normality using the Shapiro test function (shapiro.test) in the R package “stats” (R Core Team, 2015). Starting egg counts were non-normally distributed within all months, likely due to the nature of count data and the parameters of the experiment requiring a minimum number of eggs, which caused egg count to be subject to a lower bound. Because egg count was non-normally distributed, the Kruskal-Wallis test, which does not assume a normal distribution, was used to compare starting egg counts.

Exclusion treatments

1) No predator exclusion (control): This treatment used no exclusion mechanisms, leaving ACP accessible to all predators.

2) Tanglefoot barrier: Tanglefoot (Scotts Miracle-Gro Company, Marysville, OH) was applied around the circumference of the basal branch of the cohort. No cage was used. This treatment was intended to allow access to ACP by flying predators, while excluding ants and walking predators.

3) Open cage: A sleeve cage (4 1/4" x 8" cone shape white sheer synthetic organza bags, www.papermart.com, #09-947-01) was placed over the experimental cohort but not tied shut, while Tanglefoot was applied around the circumference of the basal branch. The organza of the sleeve cages was of a weave sufficiently tight to exclude passage of all flying and crawling arthropods. This treatment was designed to exclude predators to the same extent as a Tanglefoot barrier, while creating a microclimate similar to that of a closed cage.

The intention of this treatment was to separate the effects of microclimate from those of predator exclusion (Bellows and Fisher, 1999).

4) Closed cage: This treatment provided full predator exclusion, using a sleeve cage that was tied shut to completely enclose the experimental cohort, with Tanglefoot applied around the circumference of the basal branch. This treatment was designed to exclude both walking and flying arthropods.

Each replicate branch was monitored and adult ACP emergence was counted three times per week. For closed sleeve cage treatments, adult emergence was measured by counting and removing adult ACP from the sleeve cage. For treatments with open sleeve cages or no sleeve cages, ACP 5th instar exuviae, which typically remain attached to the plant substrate, were used to estimate adult emergence. Adults were also removed from these treatments, but were not counted as additional emergences. Any additional ACP eggs laid on an experimental branch during a trial were removed. Observation continued until all members of the cohort disappeared or emerged as adults.

Average monthly temperatures were obtained from two weather stations located near the study site (0.75 km NW and 2 km WSW) (The Weather Underground, 2013).

Statistical analysis

All analyses were carried out in the R computing environment (R Core Team, 2015) using the RStudio graphical user interface (RStudio Team, 2015).

Graphics were generated using the R package ggplot2 (Wickham, 2009). Differences by treatment and month were analyzed by fitting a model of ACP survival for all seven months of the experiment. The model also tested for treatment by month interactions. A generalized linear mixed model was fitted using the R function glmer in package 'lme4' version 1.1-12 (Bates et al., 2015). Logistic regression was chosen to model the data because the response variable, survival rate, is a proportion bounded by zero and one, calculated as the number of adult emergences in a cohort divided by the starting count of eggs in that cohort (Crawley, 2013). The model was weighted with the initial number of eggs for each cohort, which varied as the result of free oviposition by resident ACP females. Weighting by this value mitigates the loss of information about population size that occurs when egg count and adult emergence data are used to calculate survival rates bounded by zero and one. The model was fitted with the fixed variables "month," "treatment," and the interaction term treatment:month. Binomial error was assumed. “Month" refers to the month in which a replicate was conducted, and was included because abiotic factors were expected to substantially influence ACP survival (Kistner et al., 2016b). Because the data were overdispersed, a random effect of replicate was included in the model (Crawley, 2013; Korner-Nievergelt et al., 2015) to account for correlation in the outcomes of eggs within a replicate. Processing was optimized using bound optimization by quadratic approximation (BOBYQA) in order to increase the likelihood of model convergence (Powell, 2009).

A significant effect of treatment was followed up with pairwise comparisons among the treatments using the R function testInteractions in package phia (De Rosario-Martinez, 2015), using the Bonferroni correction for multiple comparisons. testInteractions uses a method developed by Boik (1979) which performs a two-step analysis of multiple combinations of two factors, by making each comparison of multiple levels within a factor separately, estimating contrasts between these, and then testing the hypothesis that the contrast between two pairs of interactions is equal to zero.

Results

ACP maturation success by treatment

Evaluation of the logistic regression model for ACP survival showed that effects of month, treatment, and the treatment by month interaction were all significant (Table 2.1).

Table 2.1: Analysis of variance table for ACP survival model
Model predicts ACP survival by month, treatment and the interaction term treatment:month.

	Df	F value	p
treatment	3	33.2602	< 0.01
month	6	18.094	< 0.01
treatment:month	18	41.6	< 0.01

Over all seven months of the experiment, the ACP adult emergence rate for closed-cage replicates was 39.7% This rate was significantly higher than the adult emergence rates for all other treatments, including the adult emergence rate for open cages (24%), Tanglefoot, (10.0%), and control (10.4%) (Fig. 2.1). ACP adult emergence was significantly higher in open cage replicates than in Tanglefoot and control replicates, which did not differ significantly from each other.

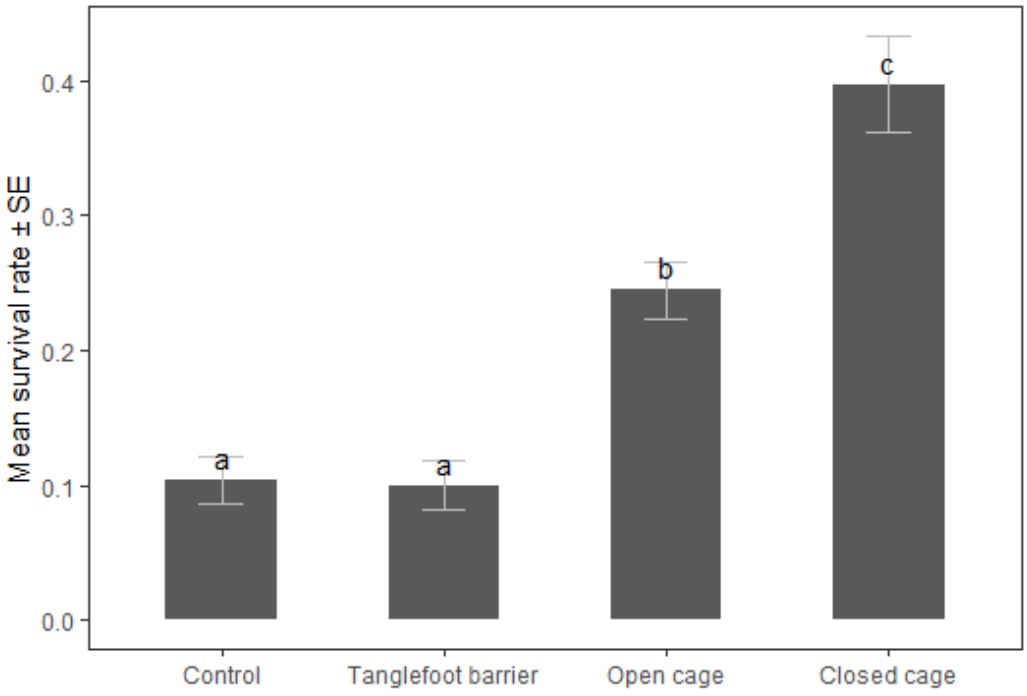

Figure 2.1: Mean +/- SE ACP survival from egg to adulthood by treatment.
Means in the same column followed by the same letter are not significantly different at $p < 0.05$.

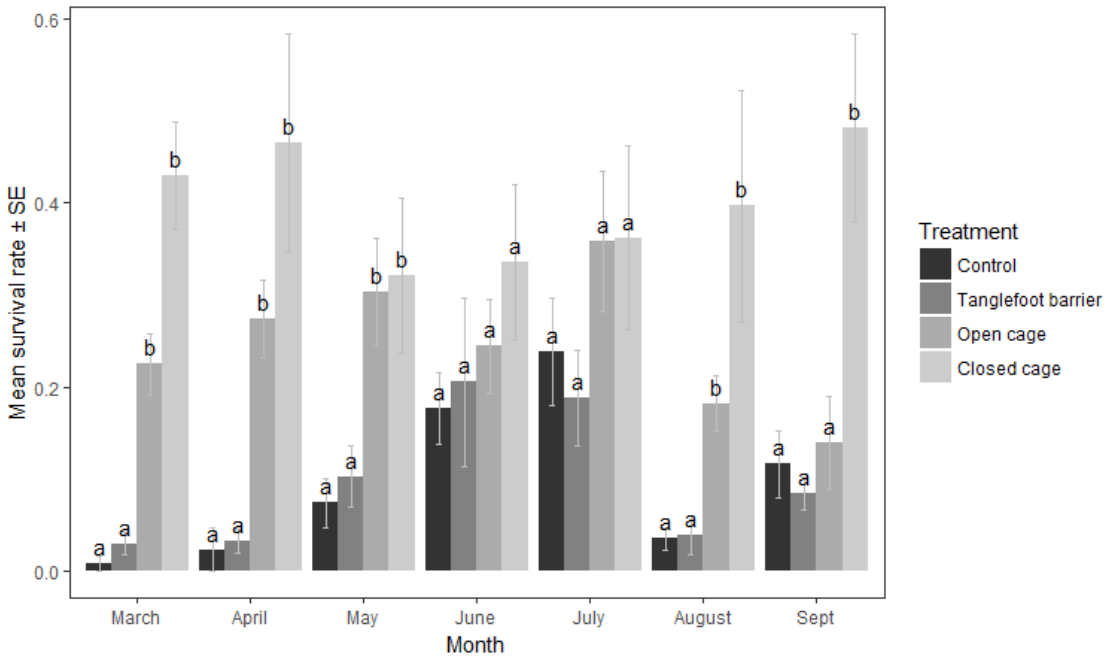

Figure 2.2: Mean +/- SE survival rate per month of ACP eggs to adulthood
Survival is depicted for ACP cohorts exposed to differing levels of predator access. Within each month, bars denoted with the same letter did not differ significantly.

For all seven months, the closed-cage treatment resulted in the highest rate of adult emergence (Fig. 2.2), significantly higher than that of all other treatments in September and significantly higher than the control and Tanglefoot adult emergence rates in March, April, May, and August. Closed-cage and open cage adult emergence rates did not differ significantly in any month except September, likely due to high variability between replicates. In June and July, emergence rate did not differ significantly between treatments, perhaps because the emergence rate in no-cage treatments was highest during those months. Adult emergence rates in the two no-cage treatments, Tanglefoot and control, did not differ significantly from each other in any month.

The control treatment produced the lowest ACP survival rate for five out of the seven months of the study. Mean emergence rate in control replicates for individual months ranged from a low of < 1% in March to a high of 24% in July. The Tanglefoot barrier treatment produced the lowest adult emergence rate in two months, July and September. Tanglefoot emergence rates ranged from 1% in May to 21% in June. The closed-cage treatment produced the highest rate of ACP survival to adulthood for all months, with a maturation rate ranging from 32% in May to 48% in September. Although the overall open-cage survival rate was significantly lower than the overall closed-cage survival rate, open-cage and closed-cage survival rates were not significantly different from each other in any month except September.

Average monthly temperature ranged from a low of 15.2°C in March to 24.0°C in July (Table 2.2) (The Weather Underground, 2013).

Table 2.2: Average monthly temperature and rainfall
Average monthly temperature and rainfall at two weather stations located near the site, 0.75 km NW (Bryant Ranch, KCAYORBA6, 33.876 -117.717) and 2 km WSW (Morningstar Drive, KCAANAHE19, 33.865 -117.729) (The Weather Underground, 2013).

Table 2.2 Month	March	April	May	June	July	August	September
Dates of experiment	2/20-3/25	3/22-4/15	4/29-5/22	6/7-6/26	7/7-7/21	7/17-8/16	9/11-10/18
Bryant Avg. temp. (°C)	15.2	16.4	20.2	n/a	23.3	21.8	21.1
Total precip. (cm)	1.9	0	1.1	n/a	0.1	0.1	0.8
Morningstar Avg. temp. (°C)	15.2	16.7	21.3	21.7	24.0	22.5	21.4
Total precip. (cm)	3.1	0	0.8	0.1	0.1	0.2	0.5

Discussion

While co-evolved natural enemies are a standard starting point in the search for biological control agents, they need not be the end point, especially if they do not sufficiently reduce a pest's abundance. Biological control practitioners should consider all other possible natural enemies, which may include locally established predators and/or parasitoids (Bellows and Fisher, 1999, p. 91). Augmentation and/or conservation of selected local natural enemy species should be considered for inclusion in a biological control program if the community causes substantial mortality to the pest, after a thorough survey is conducted and experimental evaluations are made to indicate which species likely have the largest impact (see Ch.'s 3-4). Consideration of resident natural

enemies may greatly expand the range of available control options (Michaud, 2002). This study, by establishing that ACP survival to adulthood is subject to significant natural mortality by resident predators in southern California, begins this process. The findings presented here justify evaluation of the southern California predator community to determine which local natural enemy species might be most effective in ACP biological control.

If predation is an important contributor to mortality, complete natural enemy exclusion should result in substantially higher survival of a prey species than no-cage treatments, which allow entry by predators. This was the case in this experiment. The complete natural enemy exclusion (closed-cage) treatment produced the highest rate of ACP survival from egg to adulthood in every month, while overall, the survival rate of ACP was significantly higher for complete predator exclusion than for any other treatment. As no parasitoids were present during this study, these results establish predation as an important contributing factor to the observed reduction in ACP population between life stages. Similar results were reported for ACP predator exclusion experiments in both Florida and California, in which high mortality due to predation was observed compared to mortality due to parasitism (Kistner et al., 2016b; Michaud, 2004; Qureshi and Stansly, 2009).

ACP survival to adulthood in open cages was overall significantly lower than survival to adulthood in complete predator exclusion cages. Within individual months, this difference was statistically significant during September, when ACP

survival in the closed cage treatment (48%) was 3.4 times as high as in the open cage treatment (14%). The difference between open and closed-cage survival was large but not statistically significant in other months, including August (with closed-cage survival 2.2 as high as open cage survival), March (1.9 times as high), and April (1.7 times as high). The lack of statistical significance in these comparisons was likely due to low sample size and variability between replicates.

ACP survival rates in the two no-cage treatments, control and Tanglefoot barrier, were significantly lower than for the two cage treatments in all months except June and July. Survival was at its highest level in both no-cage treatments during June and July, while there was no such increase in survival for either of the two cage treatments. Warm weather in June and July could have contributed to higher survival of ACP exposed to predators by shortening developmental time, and thereby reducing the overall risk of predation (Culler et al., 2015). The optimal temperature range for ACP development is 25-28°C, with low-temperature developmental thresholds for each nymphal instar between 10-11°C, and a high-temperature developmental threshold of 33°C (Liu and Tsai, 2000). The highest average temperature of this study occurred during the July experiment, at 23.3°C (The Weather Underground, 2013), which coincided with the highest control survival rate, as well as the highest survival rate over all treatments.

Although average temperature during the August trials was second only to July's, ACP survival in the no-cage treatments was quite low (< 4% for both),

while the closed cage survival rate was slightly higher than the previous month's. A possible contributing factor to lower control survival in the August (7/17-8/16) experiment vs. the July (7/7-7/21) experiment is the higher abundance of predatory insects and spiders recorded in late vs. mid-July (see Chapter 3).

Open cage treatments are used in predator exclusion experiments as a control for the climate effects of closed sleeve cage treatments (Boavida et al., 1995; Furlong et al., 2014; Way and Banks, 1968). According to this method, the difference between closed and open cages is assumed to indicate the effect of predation, while the difference between open cage and no-cage replicates is assumed to indicate the effect of microclimate. However, these assumptions have not been validated. While intended as a control for climate effects, a cage can also contribute to higher survival rates than in no-cage controls by inhibiting the activity of natural enemies (Bellows and Fisher, 1999). Visual and tactile obscuration of branches by sleeve cages could contribute to such an effect. Visual stimuli are important host-finding cues for insects, and visual obscuration of leaf morphology may interfere with insects' ability to identify appropriate habitat (Lev-Yadun, 2014; Prokopy and Owens, 1983; Reeves, 2011). For example, *Chysoperla* lacewings, which are common predators in California citrus (Dreistadt, 2012), choose oviposition sites based on visual and tactile cues (Koczor et al., 2017; Sattar and Abro, 2011). Their spectral sensitivity is highest in the range representing fresh green leaves, where nutritional resources and oviposition habitat are found (Kral and Stelzl, 1998). Other natural enemies,

including lady beetles and parasitoid wasps, use visual cues (Fischer et al., 2004; Hattingh and Samways, 1995; Hénaut et al., 1999; Maredia et al., 1992), as do ACP (Wenninger et al., 2009).

Thus, a plausible interpretation of our results is that open cages provided ACP nymphs with some protection against predation, rather than providing full predator access as in the classic interpretation. Taking into consideration results of previously reported ACP predator exclusion experiments, it is doubtful that the increased survival of ACP in predator exclusion treatments was due to microclimate in all cases. First, both Qureshi and Stansly (2009) and Kistner et al. (2016b) found that nymphal survival on branches treated with a sticky barrier was intermediate between survival on caged and uncaged branches, a result which indicated the effect of predation un-confounded by microclimate. Secondly, Kistner et al. (2016b) reported equivalent ACP development rates between fine-mesh (total exclusion) and coarse mesh (partial predator exclusion) cages, but significantly higher ACP nymph survival in fine-mesh cages. Because ACP development rate is tied to temperature (Hall et al., 2011; Kistner et al., 2016b; Liu and Tsai, 2000), any biologically relevant differences in microclimate between these two treatments would likely have influenced development rate, suggesting that Kistner's reported difference in survival outcome was due to natural enemy activity. Finally, Michaud (2004), reported that in 105 of 273 exposed branch terminals, the last remaining nymphs in a replicate were found mummified by parasitoids, observed being actively killed by predation, or were gone with a

predator having appeared in their place. Michaud (2004) also observed that nymphs were seldom dislodged by rainfall, which argues against rainfall as an important cause of abiotic mortality.

Due to potential confounding effects of the open cage treatment, a comparison of development rate according to treatment may be a better control for microclimate effects than the open cage treatment in future ACP predator exclusion studies. More generally in predator exclusion studies, validation experiments could be conducted to compare natural enemy colonization of open sleeve and no-cage branches in the system of interest. If natural enemies colonize open cage treatments differently, open cages should not be used as a control for microclimate effects.

High mortality of immature ACP was expected for the no-cage control treatment because of exposure to predators, as was observed in other ACP predator exclusion studies (Kistner et al., 2016b; Michaud, 2004; Qureshi and Stansly, 2009). Survival to adulthood was not significantly different between the Tanglefoot barrier and control treatments, indicating that exclusion of walking arthropods does not necessarily increase ACP survival. There are several possible explanations for the failure of a Tanglefoot barrier to increase survival of immature ACP. First, a Tanglefoot treatment cannot be assumed to truly exclude so-called “walking” predators, due to some wingless predators’ ability to colonize branches by aerial means. Spiders may reach a new site without walking across the plant surface by extending silk bridge lines or drop lines, while spiderlings

may arrive by ballooning. Oviposition by brachypterous adults of predatory species, such as lacewings, lady beetles, and syrphid flies, may result in colonization of sites not directly accessible by their flightless larvae. For example, one predator exclusion treatment used by Qureshi and Stansly (Qureshi and Stansly, 2009) consisted solely of a 5-cm Tanglefoot barrier on the supporting twig of ACP-infested branch terminals. Spiders were seen in the same numbers on Tanglefoot-protected and control colonies, indicating that spiders were able to bypass the sticky barrier in various ways, as described above. However, the Tanglefoot barrier successfully excluded ants.

Ant exclusion complicates the effect of excluding walking arthropods because ACP is an ant-tended honeydew producer (Tena et al., 2013). Ant tenders of honeydew-producers can protect these sugar resources by harassing or removing predators (Way, 1963). The relationship of ACP with the resident Argentine ant (*Linepithema humile*) is complicated, because Argentine ants tend ACP in some cases, and prey upon them in others (Tena et al., 2013). Furthermore, Argentine ants have unequal effects on natural enemy species (Daane et al., 2007). Argentine ants were present in large numbers and were the only ant species collected on site during the study period in a separately conducted predator survey (Chapter 3). The overall effect on ACP survival of excluding walking arthropods in an Argentine-ant tended system thus depends on several factors. These include the amount of aggression by ants towards predators (Daane et al., 2007; Tena et al., 2017), potentially varying levels of

aggression towards different predators (Daane et al., 2007), protective vs. predatory behavior of ants towards ACP, the ability of resident “walking” predators to make incursions into Tanglefoot-protected colonies, and potential increases in colonization of ACP cohorts by predators in the absence of ants. In our case, the protective effect of a Tanglefoot barrier to immature ACP may have been offset by the loss of protection by Argentine ants. Effective control of Argentine ants is required to determine the effects of these ants on natural biological mortality of ACP in orchard systems.

Conclusion

This study has demonstrated that predators in southern California can significantly decrease the survival rate of ACP from oviposition to adult emergence. Overall, ACP survival in predator exclusion replicates (39.7%) was 3.8 times as high as ACP survival in control replicates (10.4%). Within individual months, ACP survival in predator exclusion replicates vs. control replicates ranged from 1.5 times as high in July to 51.7 times as high in March.

These results are comparable to those reported in Florida. Qureshi and Stansly (2009) observed a five to twenty-seven times higher net reproductive rate in predator exclusion treatments compared to control treatments. Net reproductive rate was calculated as ACP survival to adulthood multiplied by ACP fecundity in the relevant temperature range, with fecundity values drawn from Liu and Tsai (2000) (representing the average number of eggs laid per ACP female

at six temperatures ranging from 15C to 33C). Michaud (2004) reported 120 times higher ACP survival from first instar to adulthood in predator exclusion cages compared to unprotected branch terminals, using first instar counts to calculate pre-predation abundance, due to the difficulty of non-destructively obtaining an accurate count of ACP eggs. Starting the observation with first instar nymphs likely allowed a more accurate starting count than could have been obtained with eggs, but the study would not have been able to consider egg predation, which probably accounted for some of the mortality observed in our study, as well as in Qureshi and Stansly's Florida study (2009).

Considered along with previously reported studies, these results provide a strong indication of the importance of predators as natural enemies of ACP in southern California and of their potential use in improved biological control of this pest. Identification of species which frequently or preferentially feed on ACP can therefore add to the list of natural enemies that can be included in IPM programs through augmentative or conservation biological control (Chapter 3).

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Chapter 3:

Survey of predatory arthropods in southern California commercial citrus

Abstract

A survey of the predator community of *Diaphorina citri,* Asian citrus psyllid (ACP), in southern California was conducted to determine which predators were abundant enough to contribute significantly to ACP mortality, and to select candidate species for evaluation as ACP biological control agents. Over the course of a year, bimonthly vacuum samples were taken in two orchards before dawn and in late afternoon. The most common predators included five insects and five spiders. The most frequently collected predatory insects were *Stethorus* spp., *Chrysoperla comanche* Banks, *Diomus pumilio* Weise, *Cryptolaemus montrouzieri* Mulsant, and *Rhyzobius lophanthae* Blaisdell. This is the first report of *Diomus pumilio* on a non-*Acacia* sp. host plant. Common spiders included *Sassacus vitis*, *Anyphaena pacifica*, *Hibana incursa*, *Trachelas pacificus*, and *Theridion* spp. 90% of collected spider specimens were juvenile. More predatory insects and adult ACP were caught in late afternoon, while more Argentine ants and nocturnal hunting spiders were caught before dawn.

Introduction

A substantial body of literature evaluates the effectiveness of natural enemies used in biological control programs and reviews methods for conducting these evaluations (Gurr and Wratten, 2000; Luck et al., 1988). Techniques for discovering and selecting specialist natural enemies for inoculative (“classical”) biocontrol through foreign exploration are also well-established (Bellows and

Fisher, 1999; van Lenteren and Manzaroli, 1999). However, formal protocols for selecting and evaluating resident natural enemies for use in conservation and augmentative biological control programs are not extensively documented. The methods by which candidate species for use in biological control programs are chosen from among a sometimes very large natural enemy community has even been described as “often highly arbitrary” (van Lenteren, 2000), or a predator’s assumed importance may be based on anecdotal observations (Greenstone and Morgan, 1989). Development of tools and protocols for experimentally supported selection of natural enemies from among a large number of species could improve the effectiveness and reliability of biological control and help to “change biological control from an art into science” (van Lenteren, 2000).

Some practitioners have recommended conducting a thorough survey and evaluation of a natural enemy community prior to selecting candidate natural enemies, advising colleagues to weigh the potential usefulness of local species against importation of a pest’s co-evolved natural enemies (Michaud, 2002b; van Lenteren, 2000). Such a project can begin by quantifying the reduction of a pest by the local natural enemy community, as in a phenology survey and/or exclusion experiments (see Ch. 2). If mortality of the pest is sufficient to warrant further exploration of the natural enemy community, it is useful to detect which natural enemies are attacking the pest in the field. Careful observation of all susceptible life stages will uncover any parasitoids due to their obligate association with the host pest. However, predation is difficult to observe and quantify due to the

ephemeral and easily disturbed nature of the interaction (Greenstone and Morgan, 1989; Greenstone et al., 2010). Predator-prey relationships may be suggested by a predator's numerical response to a pest, or by literature reporting a predator's host range. However, the existence of a natural predator-prey relationship can only be confirmed by extensive field observation or by gut content analysis (see Ch. 4). This confirmation of a predator-prey relationship may be expanded upon by laboratory feeding experiments (Ch. 5), and/or experimental field trials. Additional practical knowledge can be gained during this process, including information on the appropriateness of a natural enemy for conservation vs. augmentative biological control, colony rearing requirements, seasonality, and pest life stages attacked.

Survey and evaluation of the California natural enemy community has the potential to provide additional biological control options for Asian citrus psyllid (*Diaphorina citri*), ACP. ACP is a serious and economically harmful invasive pest that is not adequately controlled by co-evolved natural enemies in its native range (Khan et al., 2014; Shivankar et al., 2000), or in California, where two of its specialized parasitoid wasps have been imported (Kistner et al., 2016a; Vankosky and Hoddle, 2017). ACP is an existential threat to California's citrus industry because it transmits *Candidatus* Liberibacter asiaticus, a causative agent of huanglongbing (Bové, 2006). This disease causes trees to produce inedible, unmarketable fruit, and to die an early death. Although insecticide application is the primary means of reducing ACP populations in commercial

citrus, many residential citrus plantings in southern California are inaccessible or impractical for chemical control (Gottwald et al., 2013). Thus, industry and regulatory agencies have called for improved biological control options to target these areas (USDA-APHIS, 2009).

Substantial biotic mortality of ACP in southern California, reported in Chapter 2 and by Kistner et al. (2016b), indicates that there is potential for the improvement of augmentative or conservation biological control in this region. The predator exclusion experiment described in Chapter 2 demonstrated the importance of resident predators as contributors to biotic mortality of ACP in southern California, with four times higher ACP survival in predator exclusion replicates. Kistner et al. (2016b) reported eight to forty-eight times more ACP surviving to adulthood in parasitoid-access cages than in unprotected ACP sentinel colonies placed in residential areas and a citrus research orchard in Riverside, CA.

Predator exclusion studies conducted in Florida support the conclusion that predators reduce ACP populations in that region as well (Michaud, 2004; Qureshi and Stansly, 2009). This naturally occurring predation may represent significant potential for the improvement of ACP biological control, if the species primarily responsible can be identified and their ecology and predation habits better understood.

A literature review of predatory arthropods reported as potential natural enemies for ACP was conducted in preparation for this study. Forty-one

publications that identified potential predators of ACP to genus or below were included. The resulting list of proposed predators worldwide includes ~80 predatory arthropod species. About half of these are Coccinellidae, and the remainder are primarily Araneae, Chrysopidae, and Syrphidae (Supplementary tables 1 and 2, Appendix). In these studies, over forty species were observed feeding on ACP in the field, an additional seven were observed feeding on ACP solely in the laboratory, and many were proposed as potential predators of ACP based solely on co-occurrence.

Studies of frequency of predation or impact on ACP by particular predator species with the goal of identifying promising biological control agents have generally not been undertaken. However, Kistner et al. (2017), analyzed 24-hr video recordings of arthropod predators on ACP colonies on potted sentinel *Citrus volkameriana* placed in residential areas and a research citrus orchard in Riverside, California. Of visiting arthropods large enough for predatory activity to be recorded, syrphid larvae (likely *Allograpta*) and chrysopid larvae (likely *Chrysoperla*) were the groups most frequently seen feeding on ACP, while the most frequent predatory visitors included Araneae, Thripidae, and Phytoseiidae. Taxonomic resolution was not possible within these groups due to the limitations of video data collection. Additional taxa observed feeding on ACP included the coccinellids *Harmonia axyridis* and *Cycloneda* spp.

In Florida, the two primary surveys of potential ACP predators, both conducted by daytime visual inspection, reported that lady beetles

(Coccinellidae) were the most commonly collected predators. The most abundant of these were *Olla v-nigrum*, *Harmonia axyridis*, *Cycloneda sanguinea*, *Exochomus childreni*, and, to a lesser degree, *Curinus coeruleus* (Michaud, 2004; Qureshi and Stansly, 2008; Qureshi and Stansly, 2009). In laboratory feeding experiments, all but one of these lady beetle species were able to develop and reproduce on a diet of ACP nymphs. The exception was *Cycloneda sanguinea*, which completed development half as often and reproduced poorly compared to conspecifics raised on *Ephestia kuehniella* eggs. (Michaud and Olsen, 2004). Of the above species, *Olla v-nigrum* was the most promising as a natural enemy of ACP because it exhibited a numerical response to the appearance of ACP in Florida (Michaud, 2001).

Spiders been have implicated as ACP predators in Florida (2002a; 2004; Qureshi and Stansly, 2009) and Iran (Rakhshani and Saeedifar, 2013). In California agro-ecosystems, spiders are often the most abundant predator (Fournier et al., 2008), thus it is important to investigate their contribution to ACP mortality locally. Costello and Daane (1999) note that few studies have compared the abundance of spiders with that of predatory insects in agricultural systems, and of the studies that did so, some presented the spiders collected as an undistinguished group. The ability of previous ACP predator surveys to assess spider predation may have been hampered by the chosen method of visual daytime inspection, which can bias sampling against smaller, more cryptic species (Coddington et al., 1996). Numerous ecological and agricultural spider

surveys highlight the importance of conducting both diurnal and nocturnal collections to adequately describe the spider community (Cardoso et al., 2008; Coddington et al., 1996; Costello and Daane, 2005; Dobyns, 1997; Green, 1999; LeSar and Unzicker, 1978; Pinto-Leite and Rocha, 2012; Sørensen et al., 2002). However, no optimal time for collecting could be deduced from these surveys, which varied substantially in sampling time-of-day, with sampling typically conducted during one or two blocks of several hours in length for each phase of the day.

Insect surveys benefit from the inclusion of nocturnal sampling as well, because different relative abundances of diurnal and nocturnal predators may be collected during different phases of the day. For example, some nocturnal predators remain hidden in arboreal refuges during the day, while others are ground-dwelling and only enter trees at night to hunt, such as the cockroach *Blattella asahinai* (Qureshi and Stansly, 2009).

We conducted a year-long survey of arthropod predators in two southern California orange groves, with the goal of revealing the diversity, relative abundance, and seasonality of the most prevalent predatory arthropods in these orchards, and to obtain specimens for molecular gut content analysis. Because the literature is inconclusive with regards to optimal collection times for many arthropods, especially spiders, we compared collection rate by time of sampling to determine whether time of day influenced the overall number of predatory insects, spiders, ants, and ACP caught. These aims support the overall goal of

this dissertaton: to identify and recommend promising novel biological control agents for ACP from the resident predator community in southern California. This work is also intended to contribute to the development of tools and protocols for quantitative selection of the best biological control agents from a large community of potential natural enemies.

Materials and methods

Predator collection

Predators were surveyed at an organic Valencia orange grove located in Yorba Linda, California (Orange County) and a conventionally managed navel orange grove located in Mentone, CA (San Bernardino County). ACP was established at both sites, but *Tamarixia radiata* and CLas were not present at any time during the survey period. Imidacloprid and other conventional pesticides were not used on either site during the course of this study (J. Barcinas, personal communication).

The survey was conducted during a twelve-month period from March 2013 to April 2014. Collections were made twice per month from April to October and once per month from November to March (with a second collection taken in February). Collections were divided into pre-dawn and late afternoon sampling periods to obtain both nocturnal and diurnal predators which had recently fed. We conducted nocturnal in addition to diurnal vacuum collection to ensure collection of nocturnal predators, and to analyze whether nocturnality/diurnality of

predators influenced the time of day at which they were likely to be collected. It was also desirable to collect predators with fresh gut contents during both phases of the day for the purpose of conducting molecular analyses on gut contents of collected specimens (see Ch. 4). Powered vacuum collecting was chosen to maximize specimen collection, to eliminate collection bias towards easily seen and slow-moving species, and to equalize the collection abilities of novice and experienced workers.

Each collection consisted of two sampling trips at each site, including a pre-dawn visit begun at ~05:00 during summer and ~05:30 during winter, and an afternoon visit begun at ~16:00 during summer and ~15:00 during winter. Morning and afternoon trips for a given collection site were conducted on the same or subsequent days, with one exception: the December collection for Mentone, in which the pre-dawn sample was collected on December 19 and the afternoon sample on December 23. For each collection, the four sampling trips taken at the two sites were made during a two-day period, except for the November collection, which was made during a three-day period, and the December collection, as mentioned above. Overall, forty sampling trips were conducted in Yorba Linda over twenty-one dates, and thirty-nine sampling trips were conducted in Mentone over twenty-two dates, with one Mentone sampling trip omitted due to staff unavailability.

Four trees were sampled by vacuum collection during each visit to a site. Arthropods were collected from foliage using a gas-powered leaf-blower vacuum

(2-Cycle Gas Blower-Vac, Ryobi Ltd., Fuchu, Hiroshima Prefecture, Japan), with a mesh paint strainer inserted as a collection bag in the mouth of the blower tube to trap material. Interior and exterior tree branches were vacuumed for five minutes around the circumference of each tree at all reachable heights (0 to 10 feet). Trees were randomly selected and were resampled no more than once every two months. At the end of each five-minute collection period, the mesh collection bag was tied off and placed in a cooler filled with dry ice, so that arthropods were quickly euthanized by freezing. The mesh bag was then placed in a Ziploc bag filled with dry ice-chilled 95% EtOH. This procedure was designed to prevent regurgitation and to quickly halt digestion to preserve DNA for later experiments (Ch. 4). Upon return from field collection, specimens were stored at -20°C.

Predator identification

Specimen sorting for identification was conducted on ice trays to minimize temperature fluctuation and preserve DNA. Initial species counts were obtained by assigning specimens to morphotypes. Commonly collected species were identified using field guides, dichotomous keys, and “barcoding” by mitochondrial CO1 gene DNA sequence. Expert identification was obtained for spiders and the insect species *Diomus pumilio* and *Chrysoperla comanche* (Tauber, 2015; Vandenberg, 2016), for which morphological identification was difficult and published mitochondrial DNA barcodes were unreliable or unavailable. Immature

arthropods, if not obviously identifiable by morphology, were linked to adults by rearing out subsequently field-collected immatures and/or by DNA barcoding. Because juvenile spiders are not identifiable by standard dichotomous keys, juveniles of common species were identified by sorting into morphotype categories which, examined in series, formed a morphological progression from the youngest spiderlings to adult specimens. Adults collected for four of the five most common spider morphotypes were identified by R. Vetter, UC Riverside, Dept. of Entomology (Vetter, 2017b). The common identity of single-species age series was confirmed by DNA barcoding when unclear.

CO1 barcoding utilized DNA sequences amplified by polymerase chain reaction using the “universal” primer pair LCO1490/HCO2198, which amplifies a region of the mitochondrial cytochrome oxidase I (COI) gene across a wide range of invertebrate taxa (Folmer et al., 1994; Hebert et al., 2003). PCR product was cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and sequenced in both directions using the Big-Dye version 3.1 kit with the Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sequencing was performed by the University of California Riverside Genomics Institute Core Instrumentation Facility. Sequences were aligned using BioEdit (multiple versions) (Hall, 1999) and Sequencher version 5.1 (Gene Codes Corporation, 2012), and deposited in GenBank (Benson et al., 2017) with accession numbers MF924577- MF924618.

Statistical analysis

We compared effectiveness of vacuum collection for major orchard arthropod groups by time of day using one-sided Wilcoxon rank-sum testing, which allows for non-normally distributed count data and unequal variances. Correlation of Argentine ant abundance with that of other arthropod groups was tested using linear modeling with post-hoc ANOVA. All analyses were carried out in the R computing environment (R Core Team, 2015) using the RStudio graphical user interface (RStudio Team, 2015).

Results

Collection summary

Vacuum collection yielded 25,059 adult ACP, 2,015 spiders, and 2,595 predatory insects (Table 3.1). During the six months when ants were counted (May-Oct. 2013), 50,060 Argentine ants were collected. Argentine ants (*Linepithema humile*) were the only ant species collected, and outnumbered all other insects in nearly all samples over the course of the survey. Adult ACP, spiders, predatory insects, and Argentine ants were consistently more abundant at Yorba Linda than at Mentone (Table 3.1, Fig. 3.2).

Table 3.1: Summary of survey totals
Total ACP, predatory insects, spiders, and Argentine ants collected in Mentone, CA and Yorba Linda, CA from May 2013 to April 2014.

	Mentone, CA		Yorba Linda, CA	
	AM	PM	AM	PM
ACP	4,581	5,129	6,678	8,671
Predatory insects	278	484	736	1,098
Spiders	399	354	783	484
Argentine ants (5/13-10/13)	9,077	5,725	22,116	13,142

Major predator taxa

Five predatory insect groups predominated in the survey collection, with at least ~90 specimens collected: *Stethorus* spp., *Chrysoperla comanche* Banks, *Diomus pumilio* Weise, *Cryptolaemus montrouzieri* Mulsant, and *Rhyzobius lophanthae* Blaisdell. At least 70 other morphotypes of predatory insects were identified (Table 3.2). The most abundant insect families were Coccinellidae and Chrysopidae.

Spiders were collected during all 79 sampling trips, in numbers ranging from 3 to 146 specimens collected per trip. By far the most frequently collected spiders were *Sassacus vitis* (Salticidae) Cockerell (375 specimens), *Anyphaena pacifica* (Anyphaenidae) Banks (327 specimens), *Hibana incursa* (Anyphaenidae) Chamberlin 1919 (269 specimens), *Trachelas pacificus* (Trachelidae) Chamberlin & Ivie (254 specimens), and *Theridion* spp., a mixture

of *T. submissum* and *T. dilutum* (Theridiidae) (150 specimens). Identified specimens represented fifteen families (Table 3.2). The most frequently collected families were Anyphaenidae (596 specimens), Salticidae (557 specimens), Araneidae (272 specimens), Trachelidae (254 specimens), and Theridiidae (170 specimens). Araneidae was the third-most commonly collected family, and included twenty-two individual morphotypes, with no single apparent species represented by more than forty-six specimens.

Of over 2,000 spiders collected, ~90% were juveniles, ~5% were adult females, and ~5% were adult males (Figure 3.3). Spiderlings which cannot be linked to adult specimens are frequently not identifiable to genus, but in most cases our juvenile spider specimens were identifiable to family. A total of thirty-two specimens representing thirteen morphotypes could not be identified to family. Thirty-one of these specimens were immature spiderlings, and one was a partial specimen of an adult male.

Differences between nocturnal and diurnal collections

Predatory insects and adult ACP were collected at a higher rate by afternoon sampling than by pre-dawn sampling (predators: $W = 14591$, $p = 0.005$; ACP: $W = 14367$, $p = 0.01$), while Argentine ants were collected at a higher rate by pre-dawn sampling ($W = 14367$, $p = 0.003$) (Table 3.1). Spiders were collected in statistically equal numbers in predawn and afternoon samples ($W = 13708$, $p = 0.065$). However, spiders' circadian activity varied by taxon. Two

of the five most abundant spiders, the cobweb spider genus *Theridion* and the jumping spider *Sassacus vitis,* are considered diurnal, whereas the hunting spiders *Anyphaena* sp.*, T. pacificus,* and *H. incursa* are considered nocturnal. The nocturnal spiders were caught at a higher rate before dawn (W = 22011, p = 0.017), whereas there was no significant difference in the capture rate of the diurnal spiders between pre-dawn and afternoon collections (W = 22394, p-value = 0.22).

Ants

For the six months when ants were counted in vacuum samples, mean predatory insect count per sampling trip was positively correlated with mean ant count per sampling trip ($F_{1,22} = 7.0$, $p = 0.014$), as was mean spider count per sampling trip ($F_{1,22} = 6.8$, $p = 0.016$) and mean ACP count per sampling trip. However, mean adult ACP count was not correlated with mean ant count ($F_{1,22} = 0.6$, $p = 0.46$).

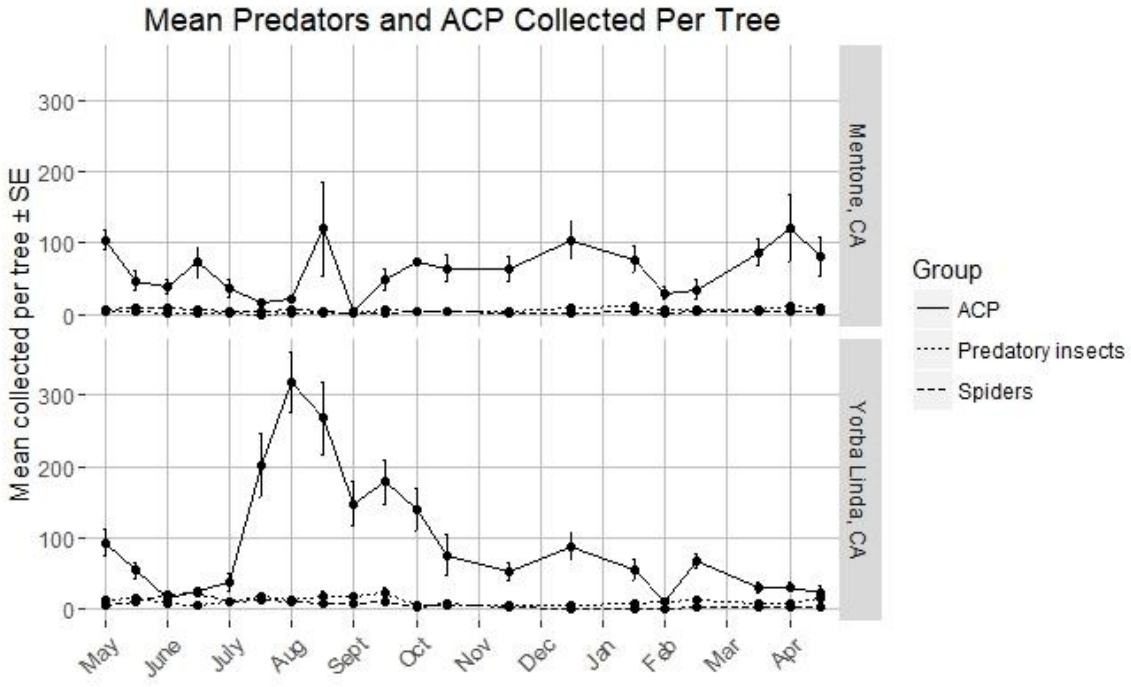

Figure 3.1: Mean predators and ACP collected per tree
Mean ±SE ACP, predatory insects, and spiders collected in Mentone, CA and Yorba Linda, CA from May 2013 – April 2014.

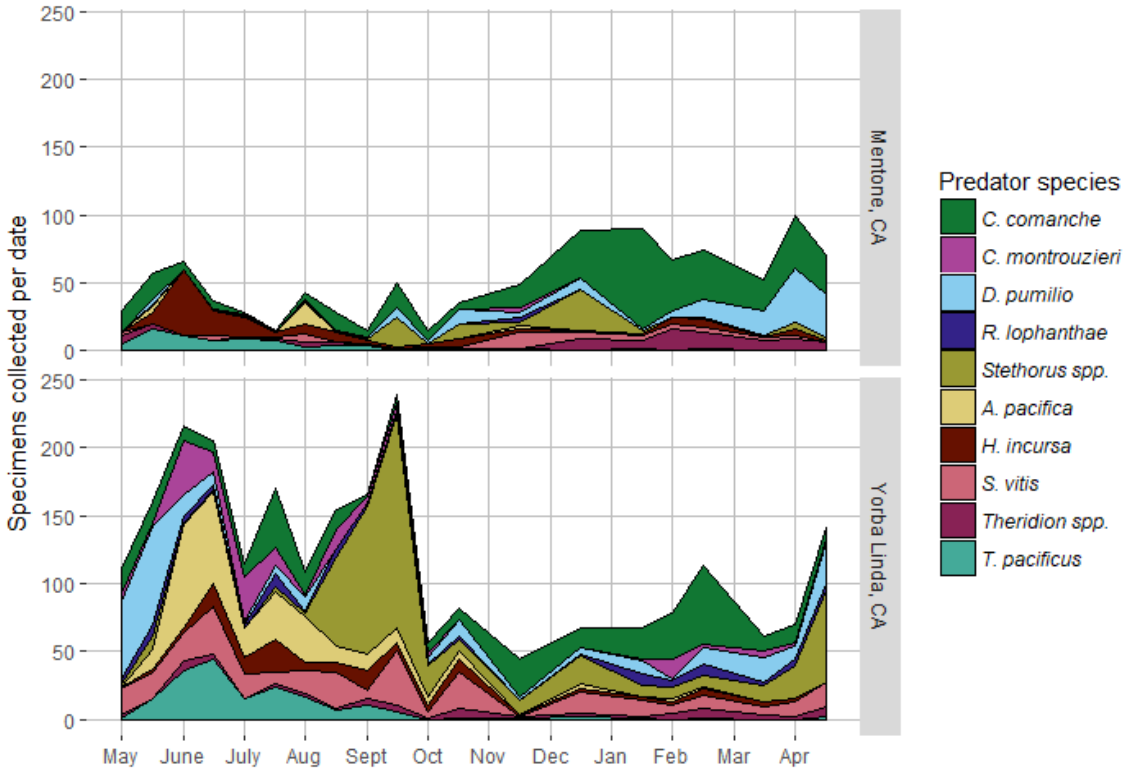

Figure 3.2: Total abundance of common predators
Total abundance of the most common spiders and predatory insects in survey collections, May 2013-Apr 2014.

Table 3.2: Predatory insects collected during survey, sorted by abundance

		Yorba Linda		Mentone		
Taxon	**Life stage**	**Pre-dawn**	**Afternoon**	**Pre-dawn**	**Afternoon**	**Total collected**
Stethorus punctum	adult	142	246	15	63	466
Diomus pumilio	adult	121	172	54	99	446
Chrysoperla comanche	adult	76	113	78	151	418
Chrysoperla comanche	larva	63	100	71	91	325
Stethorus punctum	larva	33	121	0	4	158
Coccinellidae spp.	larvae	50	48	3	5	105
Cryptolaeumus montrouzieri	larva	61	40	1	1	103
Rhyzobius lophanthae	adult	22	49	1	4	76
Cryptolaeumus montrouzieri	adult	26	38	1	1	66
Cybocephalus sp.	adult	6	7	23	8	44
Coccinellidae spp.	adults	12	26	4	1	43
Staphylinidae spp.	adult	17	22	1	1	41
Hemerobiidae spp.	adult	7	14	3	9	33
Carabidae spp.	adult	5	13	4	6	28
Harmonia axyridis	larva	21	3	0	0	24
Harmonia axyridis	adult	10	10	0	2	22
Cycloneda sanguinea	adult	9	8	1	2	20
Coniopterygidae spp.	larva	2	6	1	7	16
Rodolia cardinalis	adult	7	5	0	3	15
Psyllobora vigintimaculata	adult	3	7	2	2	14
Diomus pumilio	larva	6	6	0	1	13
Rhyzobius lophanthae	larva	5	6	1	1	13
Syrphidae spp.	larva	4	7	1	0	12
Coccinella septempunctata	adult	5	1	1	4	11
Coleoptera spp.	adult	3	3	1	1	8
Coniopterygidae spp.	adult	3	1	1	3	8
Cycloneda polita	adult	3	4	1	0	8
Hemerobiidae spp.	larva	1	2	1	4	8
Olla v-nigrum	larva	1	1	3	2	7
Chrysopidae spp.	all	1	3	1	1	6
Hippodamia convergens	adult	1	2	1	1	5
Leptothrips mali	adult	3	1	0	1	5
Olla v-nigrum	adult	2	2	0	0	4
Rodolia cardinalis	larva	1	3	0	0	4
Brachiacantha sp.	adult	1	0	1	1	3
Cicindelinae	adult	0	3	0	0	3
Forficulidae spp.	adult	2	1	0	0	3
Mantodea spp.	nymph	0	0	0	3	3
Vespidae spp.	adult	0	2	0	0	2
Asilidae	adult	1	0	0	0	1
Cleridae	adult	0	0	0	1	1
Franklinothrips sp.	larva	0	0	1	0	1
Raphidioptera sp.	adult	0	1	0	0	1
Reduviidae sp.	adult	0	1	0	0	1
Zelus renardii	adult	0	0	1	0	1
		736	1098	278	484	2595

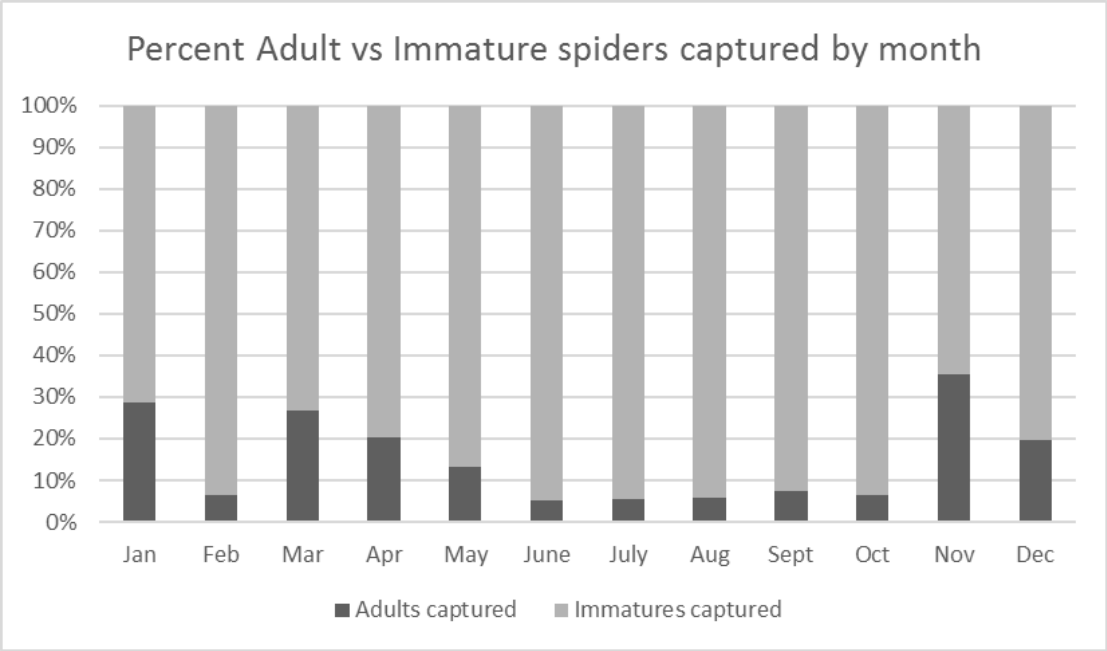

Figure 3.3: Percent adult vs. immature spiders captured by month

Table 3.3: Spiders collected by site and time, sorted by family.

		Yorba Linda		Mentone		
Family	**Taxon**	**Pre-dawn**	**Afternoon**	**Pre-dawn**	**Afternoon**	**Total collected**
Agelenidae	*Hololena* sp.	0	0	0	1	1
Anyphaenidae	*Anyphaena pacifica*	222	83	6	17	328
Anyphaenidae	*Hibana incursa*	90	38	76	66	270
Araneidae	Araneidae spp.	22	29	50	51	152
Araneidae	*Araniella displicata*	7	1	5	14	27
Araneidae	*Araniella displicata*	0	0	0	1	1
Araneidae	*Cyclosa sp.*	29	24	0	1	54
Araneidae	*Larinia* sp.	1	1	1	0	3
Araneidae	*Larinioides sclopetarius*	6	1	1	3	11
Araneidae	*Mastophora cornigera*	1	0	0	0	1
Araneidae	*Metepeira* sp.	1	3	4	6	14
Araneidae	*Neoscona sp.*	1	1	6	1	9
Clubionidae	Clubionidae spp.	0	1	0	0	1
Dictynidae	*Dictyna calcarata*	1	0	0	0	1
Dictynidae	Dictynidae spp.	11	9	1	1	22
Eutichuridae	*Cheiracanthium inclusum*	21	4	9	3	37
Gnaphosidae	Gnaphosidae spp.	0	0	0	1	1
Linyphiidae	Linyphiidae spp.	1	2	2	2	7
Oxyopidae	*Hamataliwa grisea*	6	0	4	1	11
Oxyopidae	Oxyopidae sp.	0	0	1	0	1
Oxyopidae	*Peucetia viridans*	0	0	1	0	1
Philodromidae	Philodromidae spp.	1	1	2	0	4
Philodromidae	*Philodromus* sp.	0	1	3	0	4
Philodromidae	*Tibellus* sp.	0	1	1	1	3
Pholcidae	*Psilochorus* sp.	0	0	0	1	1
Salticidae	*hiddiups/Pelegrina* species c	4	6	22	31	63
Salticidae	*Phiddipus audax*	1	0	0	0	1
Salticidae	Salticidae spp.	11	14	13	5	43
Salticidae	*Sassacus vitis*	150	175	23	27	375
Salticidae	*Synageles* sp.	1	1	0	0	2
Salticidae	*Thiodina hespera*	4	4	35	30	73
Theridiidae	*Anelosimus* sp.	0	0	0	1	1
Theridiidae	*Latrodectus* sp.	0	1	1	0	2
Theridiidae	Theridiidae spp.	5	1	6	5	17
Theridiidae	*Theridion sp.*	31	33	44	42	150
Thomisidae	*Mecaphesa* sp.	0	0	9	5	14
Thomisidae	*Misumenoides formosipes*	0	1	0	0	1
Thomisidae	Thomisidae spp.	2	4	6	4	16
Thomisidae	*Tmarus* sp.	0	0	1	0	1
Trachelidae	*Trachelas pacificus*	144	40	49	22	255
Philodromidae	Philodromidae spp.	0	0	3	1	4
unknown	unknown	9	4	9	10	32
		783	484	394	354	2015

Discussion

Major predator taxa

This survey identifies and describes the seasonal phenology of the predatory insects and spiders found over the course of a year in two orchards in southern California that were heavily infested with ACP. This is an important step towards understanding the trophic relationships between ACP and resident predators, and towards selecting natural enemy species as candidate biological control agents. Five predatory insects predominated over the sampling period, including one green lacewing species and four coccinellids, as well as five groups of spiders, which represent the families Anyphaenidae, Salticidae, Trachelidae, and Theridiidae. These taxa and their possible relationships with ACP are discussed below.

Our survey suggests that the citrus predatory arthropod community in southern California consists largely of lady beetles, lacewings, and spiders. Worldwide, previously published ACP predator studies (Supplementary tables 1 and 2, Appendix) from Florida, Mexico, Puerto Rico, India, Iran, and Cuba suggest that the predator community of ACP worldwide typically consists of lady beetles, lacewings, spiders, and syrphid flies. Also implicated are ants, a predatory mite, a vespid wasp, predatory bugs, and a cockroach. Results and citations of ACP predator surveys in which predators were identified to genus or below are summarized in supplementary tables 1 and 2 (Appendix).

The small number of syrphid larvae (n = 12) in our samples was surprising, because they were commonly observed feeding on experimental ACP cohorts in Riverside County (Kistner et al., 2016b), and appeared in greater abundance at the Yorba Linda study site during follow-up visits in 2016 (Goldmann, personal observation). Vacuum sampling has been successfully used for sampling syrphid larvae sampling in the past, so our choice of collection method does not explain the low numbers of syrphids collected in this survey (Vickerman and Sunderland, 1975).

Site differences

Arthropods, including predators, ACP, and Argentine ants, were much more abundant at the Yorba Linda site, with several factors likely contributing to this. First, trees sampled at the Yorba Linda site were spaced apart from each other, such that each tree produced new growth over its entire canopy when flushing. This new flush was more heavily colonized by arthropods than was mature foliage. In contrast, the Mentone site was composed of both widely and closely spaced trees. The majority of these trees had closely spaced canopies, and did not produce new flush on sections where their canopies joined. Second, the Yorba Linda orchard is located within a naturally vegetated riparian area where water flows nearly year-round, which likely contributes to greater background arthropod diversity and abundance. The Mentone site is located near a dry riverbed that did not contain water during 2013-2014, a period of severe

drought. Third, the entire Yorba Linda orchard is certified organic, while the study site at Mentone is located at the outer edge of a large, conventionally farmed orchard. The portion of the Mentone orchard that contains our survey trees is not sprayed with insecticides because it abuts a farmstand and a residence. However, it may be subject to pesticide drift, and therefore less likely to maintain large and diverse populations of arthropods.

Time of sampling

Insects

More predatory insects and adult ACP were collected during afternoon sampling than during pre-dawn sampling, whereas more Argentine ants were collected during pre-dawn sampling than during afternoon sampling. It is unclear why adult ACP, which presumably spend the night in citrus, would be caught at a lower rate before dawn, unless they shelter in refugia which shield them from vacuum collection. The higher pre-dawn collection of Argentine ants, which are ground-nesting and do not rest in trees, is likely to be a reflection of higher activity during that time. The fact that different groups were caught in greater abundance at each time of day suggests that these differences reflect activity patterns rather than the simple effect of ambient temperature.

Spiders

Spiders are generally considered nocturnal, but there was no significant difference between overall spiders caught pre-dawn vs. afternoon for the five

most abundant spider groups. Of these, the three specifically known to be nocturnal, *Anyphaena pacifica*, *Hibana incursa*, and *Trachelas pacificus*, were collected in higher numbers before dawn than during afternoon sampling. All three of these are cursorial nocturnal hunters (Adams and Manolis, 2014; Costello and Daane, 2005; Ubick and Richman, 2005). The other two most abundant spiders were collected at similar rates during both collection times. Of these, *Theridion* spp. are diurnal web-builders, while *Sassacus vitis* are diurnal cursorial hunters (Adams and Manolis, 2014; Suana et al., 2009).

We were interested in optimal time periods to collect spiders because these are not established in the literature, especially for agricultural systems. Molecular analyses of gut contents (see Ch. 4) depend on obtaining specimens which have recently fed, so it is important to collect soon after each taxon of interest's peak activity time. If predators of interest include both diurnally and nocturnally active taxa, late night and/or early morning sampling is needed in addition to daytime sampling. This represents a substantial increase in time and expense of sampling, thus the necessity of the approach should be validated. In this case, due to higher collection of nocturnal spiders during pre-dawn sampling, this approach was shown to be valid.

In previous studies, as in this one, time of day did not necessarily affect the overall quantity of spider specimens collected. Costello and Daane (2005) and Coddington et al. (1996) reported no overall significant difference between spider abundance for daytime and night-time collections. Using visual collection

methods, McCaffrey et al. (1983) collected significantly more spiders during daytime. On the other hand, as this study found, presence and relative abundance of individual spider taxa typically varies between day and night collections (Coddington et al., 1996; Costello and Daane, 2005; Dobyns, 1997; McCaffrey et al., 1983; Sørensen et al., 2002), or by the interaction between collection method and time of day (Coddington et al., 1996). Sørensen et al. (2002) found that unique species were collected by each combination of method and time of day, and that significantly more specimens were collected during the day, whereas there was no significant difference between the number of species collected during the day vs. at night. McCaffrey et al. (1983) collected significantly more salticids and total spiders during day sampling, significantly more clubionids at 03:00, and equivalent numbers of web-builders and thomisids during each phase. Dobyns (1997) compared collection techniques using both daytime sampling (09:00 - 18:00), and night-time sampling (20:00 - 04:00), and reported that non-repetitive plot sampling collected more adult spiders during the daytime, repetitive sampling collected equivalent numbers of adult spiders during both time periods, and that each of the two time periods yielded unique species.

The interaction of time of day and collection technique with respect to collection success may be related to the types of retreats used by various taxa. Costello and Daane (2005) speculated that more salticids were collected during daytime sampling because their nighttime retreats are less accessible to vegetation beating, and that nocturnal spiders may be collected at equivalent

rates during both phases of the day if they have easily-disturbed bivouacs in leaves.

Optimal collection methods for spider survey work are not fully established in the literature. Amalin et al. (2001) found that vacuum sampling collected fewer hunting spiders than the shake-cloth method or visual sampling in Florida lime orchards, while web-building spiders were found with similar frequency by all three methods. However, only adult spiders—which made up just 10% of our collection—were included in this analysis. Costello and Daane (1997) found that vacuum collection collected a larger proportion of smaller, more mobile spiders than did vegetation beating, and stated that vacuum collection underestimated adult spider population. However, expected field proportions of juvenile and adult spiders are not established in the literature. Without this information, it is difficult to determine whether a collection method accurately captures the demographics of a population. This topic was addressed by Coddington et al. (1996), who reported collecting 76% juvenile specimens across four collection methods, which included aerial hand collecting, ground hand collecting, beating, and leaf litter extraction. Two of these methods, aerial and ground, involved deliberate selection of apparent adult specimens, which likely resulted in an underestimation of the juvenile spider population. Aerial collection, which involved species with greater sexual dimorphism, produced a highly sex-biased sample with a 13.5:1 female to male ratio, while ground collection, which involved species with lesser sexual dimorphism, produced a 1.5:1 sex ratio (Coddington et

al., 1996). This suggests that our survey method, vacuum collection, which produced an even sex ratio, was not biased toward larger or smaller spiders, although this would be difficult to prove without a more comprehensive survey method, such as whole-tree fumigation. For a study such as this one, which focuses on the impact of predators on an ecosystem or a target species, it is important to represent the relative contributions of all spider life stages as accurately as possible. Because spiders are predatory at all life stages, and a large majority of individuals in a population are immature, juveniles are likely responsible for a substantial part of spiders' ecological impact as predators and should be studied accordingly.

Ants

We did not detect a correlation between abundance of Argentine ants and ACP adults. Argentine ants tend ACP in California, collecting their honeydew and defending colonies from predators, but their presence does not produce a uniform increase in ACP population, because the relationship is complicated by the presence and abundance of other honeydew producers (Tena et al., 2013). ACP are less preferred hosts for ant tending than several other hemipteran honeydew producers in citrus which were present at our study sites, including citrus mealybug (*Planococcus citri*), cottony cushion scale (*Icerya purchasi*), and brown soft scale (*Coccus hesperidum*) (Tena et al., 2013).

Mean Argentine ant abundance was positively correlated with mean predatory insect abundance and mean spider abundance. However, it has been established that high, uncontrolled Argentine ant populations result in both decreased predator abundance and decreased parasitism of ACP in southern California citrus (Schall and Hoddle, 2016). The positive correlation between Argentine ant abundance and arthropod predators in this study may reflect other factors that increase both Argentine ant and predator abundance, such as temperature and/or density of honeydew producers. Furthermore, Argentine ants affect predator species unequally, resulting in reductions of some species and increases of others. For example, *C. montrouzieri* larvae are able to co-exist with Argentine ants due to their mimicry of mealybugs (Daane et al., 2007). Because nearly every replicate in our study was heavily infested with Argentine ants, it is not possible to ascertain from this dataset which predators were negatively affected by the presence of ants, or whether any of the more abundant predators were made more so by a resulting lack of competition or intraguild predation.

Abundant predators collected:

Insects

Of the four most abundant insects collected in our survey, two appear to hold promise as ACP predators based on what is known about their host range and feeding habits: the lady beetle *Diomus pumilio* and the green lacewing *Chrysoperla comanche.* The other two most abundant insects, *Stethorus* spp.

and *Cryptolaemus montrouzieri*, are known to specialize on other citrus pests present at our sites, thus we consider them less likely to be important predators of ACP.

Diomus pumilio is not native to the Americas, and its establishment in California citrus is a new and surprising development in a story that spans more than a century of citrus biocontrol. This lady beetle was first brought to California in 1892 as an intended biocontrol agent of scale insects, under the synonym *Scymnus flavifrons.* In 1928, Compere released this species in the hope that it would attack citrus mealybug (Essig, 1931). This was followed by a string of additional releases between 1958 and the 1970s (Gordon, 1985), all of which failed to establish until the early 1970s, when *Diomus pumilio* was brought to California for biological control of acacia psyllid, *Acizzia uncatoides*. By 1977, *D. pumilio* was confirmed to be established in California and was contributing substantially to the successful biological control of acacia psyllid (Pinnock et al., 1978). Acacia psyllid was subsequently declared under control, and the beetle has not been monitored since. This was the first historical case of successful classical biological control of a psyllid (Dreistadt and Hagen, 1994). Prior to this survey, acacia psyllid was the only recorded natural prey of *D. pumilio* (Leeper and Beardsley, 1976), and acacia the only recorded host plant. *Diomus pumilio* has not previously been found in citrus or associated with psyllids in any system other than acacia, although two unsuccessful attempts were made to establish it

as a natural enemy of pear psylla, first in California in 1975, and again in Oregon in 1977 (Unruh et al., 1995).

Diomus pumilio is < 2 mm long and sexually dimorphic. Morphological identification of male *D. pumilio* was made by Dr. Natalia Vandenberg, Coleopterist Emeritus, USDA-Systematic Entomology Laboratory, Agricultural Research Service, US Department of Agriculture (Vandenberg, 2016). One male specimen from each site was dissected, and genitalic features agreed with *D. pumilio* as illustrated by Pang and Slipinski (2009). Conspecificity of male and female *D. pumilio* was confirmed by matching the CO1 mitochondrial "barcode" sequence (GenBank accession numbers MF924593- MF924597). Identification of fourth (final) instar *Diomus* larvae to genus may be aided by the larval key found in Slipinski (2007). As this species does not currently appear in citrus biocontrol guides, males might be mistaken for *Delphastus pusillus,* a whitefly predator, while females may be mistaken for *Scymnus* spp.

Chrysoperla comanche is a green lacewing native to the American Southwest that has previously been available for sale from commercial insectaries (Hunter, 1997), but is not currently on the market. It represented >99% of green lacewings collected at both study sites. *Chrysoperla comanche* adult specimens require genital dissection for positive morphological ID (Adams, 1962), but larvae are easily identified by head markings (Tauber, 1974). *Chrysoperla comanche* is a member of the *Chrysoperla carnea* species group. Morphological identification of adult specimens was carried out by Dr. Catherine

Tauber (Tauber, 2015). Species identity was verified by matching the CO1 mitochondrial barcode sequence for adult *C. comanche* with that of all three larval instars (GenBank accession numbers MF924577-MF924592).

Chrysoperla comanche was observed feeding on ACP on potted sentinel *Citrus volkameriana* in Riverside, CA (Kistner et al., 2016b). Other *Chrysoperla* spp. have been reported feeding on ACP in Mexico, Florida, Cuba, and Iran (Contreras and Argumedo, 2012; González et al., 2000; Michaud, 2002a; Michaud, 2004; Miranda-Salcedo and López-Arroyo, 2010; Qureshi and Stansly, 2008; Rakhshani and Saeedifar, 2013). Chrysopid larvae, including *C. comanche,* produce anal defensive secretions that repel ants (Lamunyon and Adams, 1987), a potentially useful adaptation in citrus heavily trafficked by Argentine ants. Interestingly, the first specimens of *C. comanche* recorded on Hawaii were collected on acacia during a vacuum collection survey of Acacia psyllid predators (Leeper and Beardsley, 1976).

***Stethorus* spp.** are coccinellid predators of tetranychid (spider) mites (Gordon and Chapin, 1983). A native species, *Stethorus punctum*, is commercially available and has the common name “spider mite destroyer” (Entomological Society of America, 2016). *Stethorus punctum picipes* is native to the west coast of the United States, while another subspecies, *S. punctum punctum* LeConte, spans the east coast from Maine to Virginia and extends west to the northern Great Plains states (Gordon, 1985). It is considered an important biological control agent of spider mites in multiple crop systems, including citrus

(Dreistadt, 2012), as well as others, such as grapes and hops (Maeda et al., 2015). Specimens identified by morphology according to Gordon and Chapin (1983) were *S. punctum*; however, CO1 sequences of barcoded specimens were sufficiently different to suggest the presence of multiple species (GenBank accession numbers MF924611- MF924618). Gordon (1985) cites ten introductions of *Stethorus* spp. from Asia, Australia, and Africa to California, with some of these introduced populations not identified to species. All of these introductions were reported as failed. However, the failure of an unidentified insect to establish could potentially be difficult to confirm.

Cryptolaemus montrouzieri is a commercially available beetle with the common name “mealybug destroyer” (Dreistadt, 2012). It is considered to be the most important predator of mealybugs in citrus. Larvae and adults feed on mealybugs, preferring eggs and young nymphs. *Cryptolaemus montrouzieri* larvae mimic mealybugs, producing wax extrusions (Dreistadt, 2012). This mimicry allows them to avoid disturbance while feeding on ant-tended colonies of mealybugs (Daane et al., 2007). Adult and larval *Cryptolaemus montrouzieri* are distinctive in appearance, but larval specimens lose their characteristic wax extrusions when stored in ethanol. For this reason, we confirmed larval species identity through mitochondrial CO1 “barcode” sequencing (GenBank accession numbers MF924617- MF924618), followed by a BLAST search (Benson et al., 2017) that showed multiple matches between our sequences and others published for *C. montrouzieri.*

Spiders

Five spider species predominated in the survey collection: nocturnal hunting spiders *Hibana incursa* Chamberlin*, Anyphaena pacifica* Banks*, Trachelas pacificus* Chamberlin & Ivie*,* diurnal hunter *Sassacus vitis* Cockerell 1894*,* and diurnal cobweb-weaver *Theridion* spp. Little information is available to indicate which of these species or their congeners may be important predators of ACP, however, several members of these genera have been reported as predators in California agricultural ecosystems or as predators on African citrus psyllid, *Trioza erytreae*.

Anyphaenidae, the Ghost Spiders, were the most common spider family in our collection. Members of Anyphaenidae are wandering nocturnal hunters (Adams and Manolis, 2014). All Anyphaenidae identified in this survey were either *Anyphaena pacifica* or *Hibana incursa.* Trachelidae, the Ground Sac Spiders (formerly placed in Corinnidae), are also wandering nocturnal hunters (Costello and Daane, 2005; Ubick and Richman, 2005). All Trachelidae collected were *Trachelas pacificus.* Abundant diurnal spiders in our survey included *Sassacus vitis* and *Theridion* spp. *Sassacus vitis* is a member of the family Salticidae, the jumping spiders (Adams and Manolis, 2014). *Theridion* is a diurnal genus of the family Theridiidae, the cobweb-weavers (Suana et al., 2009).

***Anyphaena pacifica*:** *Anyphaena pacifica* was the second most abundant spider collected in our survey, and was observed by Kistner et al. consuming ACP nymphs in Riverside, CA (Kistner et al., 2016b). This species yielded no

adult specimens for morphological identification during the survey period. Species identity was suspected based on morphology of older juvenile specimens and geographic collection records (Platnick, 1974; Vetter, 2017a) and confirmed by a BLAST search (Benson et al., 2017) which matched the mitochondrial CO1 “barcode” sequences obtained from our specimens (GenBank accession numbers MF924604 - MF924605) with one published by Blagoev et al. (2016).

***Hibana incursa*:** Formerly placed in the genus *Aysha* (Brescovit, 1991), *H. incursa* was reported in 1980 to have been collected at University of California Riverside citrus groves, but not at Lindcove Field station, or in Tulare and Fresno counties, by Carroll (1980). A congener of *H. incursa, Hibana velox,* was observed by Michaud (Michaud, 2002a; Michaud, 2004) consuming ACP in Florida in both the laboratory and the field. *H. velox* was able to complete a portion of immature development on a diet of ACP nymphs (Michaud, 2002a). Amalin et al. (2001) reported that *Hibana velox* was among the most abundant hunting spiders in three Florida lime orchards. Adult *H. incursa* were identified morphologically by R. Vetter, and juveniles followed a clear morphological age progression. Mitochondrial CO1 “barcode” sequences for two immature specimens (GenBank accession numbers MF924604-MF924605) most closely matched those of a congener, *H. gracilis*, in a BLAST search (no *H. incursa* sequences were available in the database).

***Trachelas pacificus*:** In a survey of spiders in central and southern California citrus, Carroll (1980) found this species present year-round in arboreal collections, with the highest abundance in summer and fall. Likewise, our survey recorded the highest abundance of *T. pacificus* from May to September. Carroll reported (1980) that ground litter hosts a population reservoir of *T. pacificus,* and states that abundant litter may therefore increase populations of this species. *Trachelas pacificus* fed nocturnally on slow or sessile soft-bodied prey, including thrips, mites, insect eggs, and lepidopteran larvae (Carroll, 1980), promising feeding habits with respect to predation on immature ACP. A congener of *T. pacificus, Trachelas volutus*, was among the most abundant hunting spiders in three Florida lime orchards (Amalin et al., 2001). Adult *T. pacificus* were identified morphologically by R. Vetter, and juveniles followed a clear morphological age progression. In a BLAST search (Benson et al., 2017), the mitochondrial CO1 “barcode” sequence for an immature specimen from this series (GenBank accession number MF924606) closely matched that of a congener, *T. tranquilas* in multiple records (no *T. pacificus* sequences were available). However, one adult specimen submitted for identification as *T. pacificus* was in fact *Clubiona pomoa,* raising the possibility that specimens recorded as *T. pacificus* in this survey may include individuals of other species.

***Sassacus vitis*:** *Sassacus vitis*, a jumping spider (Salticidae), was the most abundant spider species collected in this survey. Vetter et al. (2013) suggested that immature Salticidae were likely predators of ACP in Faisalabad,

Pakistan based on abundance, co-occurrence with immature ACP, and predation habits, but noted that molecular diet analysis and/or observational studies are needed to evaluate this hypothesis. Van den Berg et al. (1992) observed unidentified Salticidae preying on adult African citrus psyllid, *T. erytreae* in South Africa, and noted that spiders of the the salticid genus *Mymarachne* sp. trapped adult and nymph *T. erytreae* in their retreats. Salticidae was the most abundant family in the South African study, making up 34.4% of spider fauna in a citrus orchard; however, there was no delayed density dependence between weekly spider abundance and weekly psyllid abundance, suggesting that higher spider population did not cause a decrease in *T. erytreae* population. Adult *S. vitis* were identified morphologically by R. Vetter. Coloration of the youngest *S. vitis* juveniles is dramatically different from that of older juveniles, which in turn vary in color between each other. For this reason, we compared mitochondrial CO1 barcode sequences of adults and juveniles to confirm common species identity (GenBank accession numbers MF924607- MF924610).

***Theridion* spp.:** *Theridion* is a diurnal genus of the family Theridiidae, the cobweb-weavers (Suana et al., 2009). Unlike typical theridiid spiders, which use breakable gumfoot lines as snares, *Theridion* weaves sticky threads into its webs to catch small flying insects (Adams and Manolis, 2014). They build a small tree canopy web that stretches across a single leaf, and were observed by Carroll (1980) to catch a variety of small hard-bodied and soft-bodied insects in citrus, feeding habits which are promising with respect to ACP predation. Moreover,

Theridion sp. trapped both adults and nymphs of *T. erytreae* in South African citrus (Van den Berg et al., 1992).

Carroll (1980) found that *Theridion leechi* and other *Theridion* spp. were most abundant in southern California citrus March-May. Mansour and Whitecomb (1986) likewise recorded the highest abundance of adult *Theridion* sp. during the month of April in an Israeli orchard, in which *Theridion* sp. (including juveniles) made up 34% of grapefruit spider fauna over the course of a year in daytime tap samples. Most (83%) of our adult *Theridion* spp. were collected March-May as well, with the highest number of immatures collected in February. Adult *Theridion* spp. collected in our study were identified by genital morphology as belonging to *T. dilutum* and *T. submissum* (Vetter, 2017b). *Theridion submissum* and *T. dilutum* are both native to the southwestern United States and northern Mexico, but *T. submissum* has not previously been recorded in California.

Conclusion

This survey described the diversity and seasonality of the most abundant predatory insects and spiders found in two southern California orange groves. Because of relatively high abundance of certain species, it is possible that they contributed significantly to the concurrently measured biotic mortality of ACP as reported in Chapter 2, and should be investigated further as potential natural enemies of ACP by gut content analysis, as reported in Chapter 4. By following this protocol, we hope to select promising predators of ACP for consideration and

use in augmentative and conservation biocontrol programs. Molecular gut content analysis and field testing are required to help estimate the relative impact of each species on ACP. Moreover, we believe that careful, evidence-based selection of natural enemies for augmentative and conservation biocontrol can improve the quality, reliability, and investment value of biological control programs. This study aims to contribute to a body of literature exploring techniques and protocols for doing so.

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Chapter 4:

Molecular analyses of gut contents to identify predators of *Diaphorina citri*, Asian citrus psyllid

Abstract

Molecular techniques for detecting trophic relationships between predators and an agricultural pest species that may be difficult to observe otherwise can improve understanding of food webs and potentially suggest novel biological control options. To enable efficient and accurate detection of predation upon *Diaphorina citri,* Asian citrus psyllid (ACP), we designed a qPCR bioassay that detects ACP residues in predator gut contents with a high degree of specificity and sensitivity. This test was applied to samples of the ten most abundant predator taxa collected over the course of a year in two ACP-infested orange groves in southern California. The test detected ACP predation in three out of the five most abundant predatory insects: *Chrysoperla comanche, Diomus pumilio*, and *Rhyzobius lophanthae.* In addition, all five of the most abundant spiders, *Anyphaena pacifica*, *Hibana incursa, Sassacus vitis, Theridion* spp., and *Trachelas pacificus*, tested positive for ACP DNA in at least 10% of specimens.

Introduction

Trophic relationships of invasive pests

During the design phase of a biological control program for a recently arrived invasive pest, practitioners study the ecology of the pest in its invaded landscape and evaluate both local and foreign natural enemies for use against it. It is widely accepted and required that to avoid harm to non-target species, host specialization is a necessity in any foreign natural enemies that are to be

imported (Ehlers, 2011; Hunt et al., 2008; Nowell and Maynard, 2005; van Lenteren et al., 2006). However, there is no generally accepted protocol for selecting and ranking natural enemies from the totality of predatory and parasitic species available (Shea et al., 2002), and it cannot be predicted in any specific case whether co-evolved or newly associated natural enemies are more likely to effectively control the target pest. While both co-evolved and newly associated natural enemies have played major roles in biological control programs (Legner and Bellows, 1999), advantages have been argued for each. Co-evolved natural enemies or natural enemy complexes may be preferred if they posess adaptations for efficiently attacking the target pest (Legner and Bellows, 1999). On the other hand, attack strategies of newly associated natural enemies may be more effective if they differ from any a pest has previously been exposed to (Hokkanen and Pimentel, 1989).

Many pests are not controlled below an acceptable level of economic damage by co-evolved natural enemies in their native range (Legner and Bellows, 1999), so it is encouraging that biological control programs using newly associated natural enemies are reportedly as successful as those using co-evolved natural enemies (Waage and Greathead, 1988), or even more successful (Hokkanen and Pimentel, 1984). A risk of importing newly associated natural enemies is that a natural enemy predisposed to attack a new prey species is considered likely to attack non-target species as well (Simberloff and Stiling, 1996). However, new predator-prey relationships of this type can form

without human intervention when a newly invasive pest encounters locally established natural enemies that are predisposed to attack it. Inclusion of such locally established species in a biological control program is likely to be far less controversial than would be the importation of exotic natural enemies capable of forming a new predator-prey relationship with an invasive pest.

If it is confirmed that a pest is reduced by resident natural enemies, the predator and parasitoid communities should be investigated for the possibility of enhancing biological control of the pest by augmentation or conservation. This goal carries with it the challenge of detecting and evaluating a potentially large number of trophic relationships which involve the target pest.

Arthropod trophic relationships involved in natural resistance or biological control may be either parasitoid-host or predator-prey relationships. Of the two, parasitoid-host relationships are simpler to detect and quantify, due to an obligate, long-lasting physical association which often leaves evidence in the form of a mummy, cocoon, or exit holes. Arthropod predator-prey relationships, on the other hand, are by their nature more difficult to detect, possibly “the most difficult interspecific interaction to study in the field (Greenstone et al., 2010),” as they largely consist of ephemeral interactions that leave no trace (Furlong and Zalucki, 2010). However, predation fundamentally influences the workings and outcomes of natural and agricultural ecosystems. Decoding predator-prey interactions of a target species is essential to understanding its natural history and ecology, and, for pest species, for determining the best means of control. In

the case of biological control, predator-prey relationships have economic significance, because management of key predators can lead to decreased pest control costs and/or improved crop yields (Symondson et al., 2002).

Ideally, key predators would be identified among the natural enemy community by screening of the whole arthropod predator complex for interactions with a species of interest (Furlong and Zalucki, 2010). However, this is not a simple prospect. The pool of potential predators for any one pest can be quite large, and may include small, nocturnal, and/or cryptic species, while predation interactions are fleeting and often easily disrupted by the act of observation (Furlong and Zalucki, 2010; Greenstone and Morgan, 1989). Extensive field observation may reveal true predator-prey relationships, but a thorough, season-long or year-round predator survey conducted by observation is prohibitively labor-intensive. Furthermore, visual observation may overlook nocturnal or otherwise cryptic predators (Breene et al., 1989). Without good information, natural enemies may be, and have been, selected for biological control programs based on anecdotal observations (Greenstone and Morgan, 1989), arbitrarily (van Lenteren, 2000), or, historically, by trial and error (Mills and Kean, 2010). Such protocols may hinder the effectiveness of biological control programs, because only experimental observations can confirm the effectiveness of biological control agents (Luck et al., 1988), while the release of extraneous biological control agents may reduce the overall effectiveness of the program (Briggs, 1993; Brodeur and Rosenheim, 2000; Rosenheim et al., 1995).

Therefore, efficient protocols for detecting trophic interactions between members of a pest-natural enemy complex are needed for optimization of biological control.

Predator gut analysis

A streamlined approach for discovering likely trophic relationships between predators and prey of many taxa, including arthropods, is offered by molecular analysis of gut contents. This method is based on the analysis of partially digested prey remnants that remain detectable in the guts of field-collected predators after they are captured and preserved. Molecular tests of predator specimens for the presence of these prey remnants may identify individuals which recently fed on the target pest, and the resulting data is used to characterize and rank relative importance of species in a natural enemy community (Agustí et al., 2003a; Chen et al., 2000; Greenstone et al., 2010; Weber and Lundgren, 2009; Zaidi et al., 1999).

Predator gut content analysis was developed before DNA-based methods became available and utilized a variety of methods, including releases of radiolabeled prey insects, serological methods such as ELISA and the precipitin assay, and electrophoretic detection of prey-specific proteins (Breene et al., 1989; Grant and Shepard, 1985; Greenstone and Morgan, 1989; Luck et al., 1988). In recent years, DNA-based methods such as prey-specific DNA amplification by polymerase chain reaction (PCR) or quantitative PCR (qPCR)

have become prevalent, along with continuing use of monoclonal antibody-based assays (Greenstone et al., 2010; Symondson, 2002). To our knowledge, DNA-based analyses of predator gut contents were first used to detect field predation within an unmanipulated predator-prey system in 2003 (Agustí et al., 2003a).

Analysis of predator gut contents is a useful means of identifying predators of a target prey species when natural predation is known to occur, there are a large number of potential predators, and identification of key predators is a desirable goal. These conditions are applicable in the case of Asian citrus psyllid (ACP), *Diaphorina citri,* a serious pest of citrus in California and most of the world's other citrus-growing regions. ACP is a pest of great concern because it is the vector of a bacterium, *Candidatus* Liberibacter asiaticus, a causative agent of the fatal citrus disease huanglongbing (HLB), also known as citrus greening disease (Bové, 2006). Vector control is a significant component of HLB management because transmission rate increases with the number of infected psyllids feeding on a plant (Pelz-Stelinski et al., 2010), and because HLB-infected trees can be more productive when psyllid pressure is reduced (Monzo and Stansly, 2017). In addition, smaller vector populations are predicted to select for decreased pathogen virulence (Ewald, 1994). Therefore, a substantial reduction in psyllid population may lead to a decrease in the incidence and/or effects of the disease, as was the case on Réunion Island (Etienne and Aubert, 1980). In conventionally managed orchards, vector reduction is primarily accomplished with insecticide treatments (Hall et al., 2012),

but certified organic groves are at risk of developing high ACP populations because organic treatments of comparable effectiveness are not available (USDA-APHIS, 2010).

ACP appeared in southern California in 2008 and established a beachhead in Los Angeles' abundant residential citrus (Grafton-Cardwell and Lazaneo, 2012). Citrus planted at private homes is extremely common in southern California, with 60% of houses having an average of two typically "un-managed" trees (Gottwald et al., 2013). This urban orchard, if left untreated, is expected to harbor a large population of ACP and undermine effective area-wide management (USDA-APHIS, 2010). Initially, the California Department of Food and Agriculture (CDFA) attempted to detect ACP-infested trees on private property in Southern California, and subsequently treat them with foliar and systemic insecticides, but the invasion outpaced available funding and personnel. The spray program has since been scaled back across most of the region to focus resources on the leading edges of the invasion and stave off incursions into commercial growing regions (Hoddle, 2012). Meanwhile, insecticide resistance has been reported in Florida (Tiwari et al., 2013; Tiwari et al., 2011), where ACP has been established since 1998 (Halbert, 1998). New control options, including improved biological control, are urgently needed to address these gaps in management (2009; Hall et al., 2012). A consensus of the USDA-APHIS Asian citrus psyllid Technical Working Group (USDA-APHIS, 2009) stated that "biological control would be a viable component of an area-wide control

program with respect to psyllid control in urban settings, natural areas, certified organic production, and possibly abandoned groves."

This situation points to biological control as a uniquely attractive strategy to reduce ACP in residential citrus, if effective biological control agents can be found and exploited. Unfortunately, finding effective biological control agents for ACP has been a challenge because ACP is not adequately controlled by co-evolved natural enemies in its area of origin (Khan et al., 2014; Shivankar et al., 2000) or in California, where two of its specialist parasitoids have been introduced (Kistner et al., 2016a; Vankosky and Hoddle, 2017). However, local predators kill a large number of ACP in southern California, as described in Chapter 2 (also, see Kistner et al. (2016b)). It follows that the resident predator community has the potential to yield novel biological control agents for use against ACP, which may be capable of causing greater mortality to ACP under optimal management than they do at present. Identification of key ACP predators in southern California may also contribute to the field of invasion biology, for example, by leading to a better understanding of ACP's case in the context of the Enemy Release Hypothesis (Chapter 2).

To identify resident species that have trophic relationships with ACP, we collected predator specimens in two ACP-infested citrus orchards for one year, designed a qPCR-based diagnostic test for ACP remains in predator guts, and tested specimens of the most abundantly collected predatory insects and spiders for evidence of ACP predation. These tests provide an initial look at ACP's

predator community in southern California, with percent testing positive for ACP serving as a preliminary metric of predation frequency, subject to later adjustment by the results of digestion time analysis (Greenstone et al., 2010). We further examine the data for indications about predator habits and ecology by examining within-species differences in rates of positive detections.

Materials and Methods

Specimen collection

Predator specimen collections were taken bimonthly over the course of a year in two southern California citrus orchards, with each collection divided into pre-dawn and late afternoon sampling periods. Study sites consisted of an organic Valencia orange grove located in Yorba Linda, CA (Orange County) and an untreated portion of a conventional navel orange grove located in Mentone, CA (San Bernardino County). ACP was established at both sites, but *Tamarixia radiata* and CLas were not present at any time during the survey period. Imidacloprid and other conventional pesticides were not used on these sites during the course of this study (J. Barcinas, personal communication).

The survey was conducted during a twelve-month period from April 2013 to March 2014. Collections were made twice per month from April to October and once per month from November to March. Collections were divided into pre-dawn and late afternoon sampling trips to ensure the collection of fresh gut contents from both nocturnal and diurnal predators, and of nocturnal predators which may

retreat to inaccessible refugia during the day, or vice versa. Pre-dawn sampling began at ~05:00 during summer and ~05:30 during winter, while afternoon sampling began at ~16:00 during summer and ~15:00 during winter. Morning and afternoon trips for a given collection event were conducted on the same or subsequent days, with the exception of the December collection in Mentone, for which the pre-dawn sample was collected on December 19 and the afternoon sample on December 23. For each bimonthly collection period, the two sites were sampled on two adjoining days, except for the November collection, which was made during a three-day period, and the December collection, as mentioned above. Overall, forty sampling trips were conducted in Yorba Linda over twenty-one dates, and thirty-nine sampling trips were conducted in Mentone over twenty-two dates. One Mentone sampling trip was omitted due to staff unavailability.

Arthropods were collected from foliage using a gas-powered leaf-blower vacuum (2-Cycle Gas Blower-Vac Ryobi Ltd., Fuchu, Hiroshima Prefecture, Japan), with a mesh paint strainer inserted as a collection bag in the mouth of the blower tube to trap material. This method was chosen to maximize the number of specimens collected, to eliminate collection bias towards slow-moving and easily sighted species, and to equalize collection capabilities between novice and experienced workers. Four trees were sampled by vacuum collection during each site visit. Interior and exterior tree branches were vacuumed for five minutes around the circumference of the trees at all reachable heights (0 to 10 feet).

Trees were randomly selected and re-sampled no more than once every two months. At the end of each five-minute collection period, the mesh collection bag was tied off and placed in a cooler filled with dry ice, so that arthropods were quickly euthanized and preserved by freezing. The mesh bag was then placed in a Ziploc bag filled with dry ice-chilled 95% EtOH. This procedure was designed to prevent regurgitation and to preserve prey DNA for gut analysis by quickly halting digestion. It is necessary to stop movement as well as digestion of arthropods immediately after turning off the vacuum; otherwise, some predators will attack and begin to consume nearby prey as soon as the vacuum is turned off, leading to false positives (King et al., 2008).

Upon return from field collection, specimens were placed in -20°C storage. Sample sorting was conducted on ice trays. Molecular testing of gut contents for ACP remains was performed for each of the ten most common predators, on up to half the collected specimens, with a maximum of ~100 per morphotype (Table 4.1). Specimens chosen for testing were selected with preference for 1) fully intact specimens, 2) sampling dates with multiple specimens (to preserve vouchers), and 3) sampling as evenly across sites, seasons, and time of day as possible. Multiple life stages of a species were tested if specimens were available in sufficient quantity. Several taxon-specific criteria also applied to specimen selection. *Diomus pumilio* specimens selected for testing included a complement of each sex, including 100 females and 88 males. All spiders tested were juvenile because very few adults were collected (Chapter 3), and these

were preserved as voucher specimens. *Theridion* spp. consisted of a mixture of immature *T. submissum* and *T. dilutum* (Theridiidae), which cannot be morphologically distinguished until they reach sexual maturity. *Chrysoperla comanche* adults were abundant, but were not tested because they are herbivorous. *Chyrsoperla comanche* larval stage was recorded before testing as 1st or 2nd-3rd instar. Predatory mites were collected in vacuum samples in low numbers compared to their abundance in concurrently taken beat samples, which suggests that many passed through the mesh of the vacuum collection bag. Predatory mites hold promise for ACP biological control (Juan-Blasco et al., 2012); however, analysis of these specimens was outside the scope of this study.

Table 4.1: Quantities collected and tested for each morphotype

				Yorba Linda		Mentone		Total	Total
Order	Family	Genus/species	Life stage	Pre-dawn	Afternoon	Pre-dawn	Afternoon	collected	tested
Araneae	Anyphaenidae	*Anyphaena pacifica*	juvenile	222	83	6	17	328	100
Araneae	Anyphaenidae	*Hibana incursa*	juvenile	90	38	76	66	270	100
Araneae	Salticidae	*Sassacus vitis*	juvenile	150	175	23	27	375	100
Araneae	Theridiidae	*Theridion spp.*	juvenile	31	33	44	42	150	60
Araneae	Trachelidae	*Trachelas pacificus*	juvenile	144	40	49	22	255	100
Coleoptera	Coccinellidae	*Cryptolaeumus montrouzieri*	adult	26	38	1	1	66	32
Coleoptera	Coccinellidae	*Cryptolaeumus montrouzieri*	larva	61	40	1	1	103	58
Coleoptera	Coccinellidae	*Diomus pumilio*	adult	121	172	54	99	446	188
Coleoptera	Coccinellidae	*Rhyzobius lophanthae*	adult	22	49	1	4	76	39
Coleoptera	Coccinellidae	*Stethorus punctum*	adult	142	246	15	63	466	74
Coleoptera	Coccinellidae	*Stethorus punctum*	larva	33	121	0	4	158	99
Neuroptera	Chrysopidae	*Chrysoperla comanche*	larva	63	100	71	91	325	105

Specimen sterilization

Predators taken by vacuum collection can become externally contaminated with prey DNA as insects are thrown together in the vacuum bag,

potentially leading to false positives (King et al., 2008). We prevented this by surface-sterilizing predator specimens with a dilute bleach extraction to destroy exterior DNA contaminants following the method of Greenstone et al. (2012). Thus, specimens were individually placed in 1 mL vials containing a solution of 2.5% commercial bleach (5.25% sodium hypochlorite) and 97.5% double distilled H_2O, which were placed in a tube rotator and rotated end over end for 45 minutes. Each specimen was then rinsed three times with 1 mL 95% EtOH on a Büchner funnel with suction to remove bleach residue before DNA extraction. The funnel's perforations were covered with filter paper to prevent specimens from being drawn down with the rinsate. After rinsing, each specimen was moved with individually flame-sterilized and cooled forceps into a vial containing 95% EtOH.

DNA extraction

Total DNA was extracted from individual predator specimens with either a Chelex-based method (Walsh et al., 1991) or the Qiagen Dneasy Blood & Tissue Kit, as detailed below. The Chelex method was used for all insects except *C. comanche*. Chelex failed to reliably extract DNA from *C. comanche* larvae and spiders; total DNA was extracted from these groups using the Qiagen DNeasy Blood & Tissue Kit.

Chelex extraction

Specimens were weighed and assigned to size classes to determine quantities of reagents to be used (Table 4.1). *Cryptolaemus montrouzieri* larvae weighed between 0.1 - 11.1 mg, while adults ranged from 4.8 - 11.5 mg. *Rhyzobius lophanthae, S. punctum*, *D. pumilio,* and ACP specimens all weighed less than 2 mg.

Each specimen was ground with a micropestle in the specified amount of proteinase-K (> 600 mAU ml^{-1}; Qiagen, Valencia, CA, USA), after which the corresponding amount of autoclaved 5% (w/v) Chelex® 100 resin suspension in ultrapure water (Bio-Rad Laboratories, Hercules, CA, USA) was added. Samples were incubated for one hour at 55ºC followed by 10 minutes at 99ºC, then centrifuged for four minutes at 14,000 RPM. The supernatant, containing extracted total DNA, was removed and stored at -20º C. Chelex extractions were centrifuged for 4 min at 14,000 rpm before use in PCR or qPCR to prevent inhibition by remaining residues.

Table 4.2: Size classes for Chelex extraction reagents
Predator specimens extracted with Chelex resin were weighed and assigned to size classes to determine the quantity of reagents used.

Wet weight of specimen (mg)	Proteinase K (µL)	5% Chelex suspension (µL)
0 - 2	2	60
2.1 - 3	3	100
3.1 – 5	4	100
5.1 – 10	6	150
> 10.1	8	200

Qiagen DNeasy kit extraction

Total DNA extraction using the Qiagen DNeasy® Blood & Tissue kit followed the manufacturer's supplementary protocol for purification of total DNA from insects using the DNeasy® Blood & Tissue Kit (QIAGEN, 2006), with the following modifications and specifications: 1) specimens were ground in 60 µl of Buffer ATL. 2) Lysis incubation at 56ºC was carried out for three hours for all specimens. 3) The final elution with Buffer AE was performed twice, using 50 µl of Buffer AE each time.

PCR test for quality of DNA extraction

Predator DNA extractions were individually tested for quality of total DNA extraction prior to the test for remains of ACP predation. DNA quality was confirmed with amplification by polymerase chain reaction (PCR) (Saiki et al.,

1988) using the “universal” primer pair LCO1490/HCO2198, which amplifies a region of the mitochondrial cytochrome oxidase I (COI) gene across a wide range of invertebrate taxa (Folmer et al., 1994).

Each 25 µl reaction was made up of 2 µl DNA template and 23 µl reaction mixture containing 1x ThermoPol PCR buffer (New England BioLabs, Ipswich, MA, USA), 0.2 mM each dATP, dCTP, and dGTP, and 0.4 mM dUTP (substituted for dTTP as a laboratory precautionary measure against PCR product contamination (Longo et al., 1990)), 2 mM MgCl, 12 µg BSA (NEB), 0.2 µM each LCO1490 primer and HCO2198 primer, and 1 Unit Taq polymerase (NEB) in ultrapure water. Thermocycling followed a program of initial denaturation at 94ºC for 120s, six cycles of 94ºC/30s, 45ºC/90s, 72ºC/70s, thirty-six cycles of 94ºC/30s, 51ºC/90s, 72ºC/70s, and one final extension period of 300s, using a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA). PCR product was visualized by electrophoresis on 0.7% agarose gels stained with ethidium bromide to confirm the presence of amplicons of the appropriate length.

Detection of ACP DNA:

Primer design

In designing a test for prey-specifc molecules in predator gut contents, the goals are sensitivity and specificity. To prevent false negatives, the test must detect very small amounts of prey material, while to prevent false positives, the

test must be specific to the target prey. With these goals in mind, we designed a test to target the ITS regions of ACP's nuclear ribosomal DNA. The advantages of targeting ITS regions in this protocol are, first, that there are many copies per cell, with an estimated 40 to 250 copies of ribosomal DNA per haploid genome in insects (Long and Dawid, 1980), and, second, that the ITS regions are conserved within species, but not between species (Gerbi, 1986).

To maximize species specificity, we designed a test to amplify and detect the target sequence using quantitative PCR (qPCR) with a TaqMan™ probe, which is complementary to part of the target sequence between the two primer regions. The probe binds during the annealing stage of the PCR cycle, then breaks apart and begins to fluoresce during the extension stage. Test results depend on the amount of fluorescence produced, and are highly target-specific because the probe and both primers must match the target sequence for fluorescence to occur. TaqMan™ probes are ideally used with amplicons of 50-150 bp, so we aimed to design primers for a ~100 bp fragment. qPCR amplification efficiency and specificity both benefit from use of the smallest amplicon that meets the experimental criteria (Bustin and Nolan, 2004). An additional reason for selecting a short DNA sequence for predator gut analysis was that short fragments were hypothesized to remain detectable longer after feeding (Agustí et al., 1999; Agustí et al., 2003b; Chen et al., 2000; Hoogendoorn and Heimpel, 2001; Zaidi et al., 1999).

To choose a target amplicon, we amplified ~1100 bp of ribosomal DNA using the general arthropod primer CS249 (5'-TCGTAACAAGGTTTCCG-3') coupled with CS250 (5'-GTTRGTTTCTTTTCCTC-3')(Moritz et al., 2000) from five adult ACP originating from four regions on two continents, including two specimens from California and one each from Mexico, China, and Pakistan. PCR product was cleaned using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and sequenced in both directions using the Big-Dye version 3.1 kit with the Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA). Sequencing was performed by the University of California Riverside Genomics Institute Core Instrumentation Facility. Sequences were aligned using BioEdit (multiple versions) (Hall, 1999) and Sequencher version 5.1 (Gene Codes Corporation, 2012), and deposited in GenBank (Benson et al., 2017) with accession numbers MF924619-MF924623.

ACP gene regions amplified between these primers included 18S (partial), internal transcribed region 1 (ITS-1), 5.8S, ITS-2 and part of 28S domain 1. ACP's ITS-1 region lacked good candidate sequences for target amplicons due to its small size (~200 bp) and the presence of microsatellite repeats. Of the remaining regions, a BLAST search showed that parts of the 5.8S and 28S sequences of *Bactericera cockerelli*, potato psyllid, are highly similar to those of ACP; thus, they are insufficiently species-specific for use in a diagnostic test. However, as predicted, ITS-2, a transcribed non-coding region, differed substantially between ACP and related species, while remaining highly

conserved between ACP individuals. This implicated ITS-2 as a promising region from which to select a species-specific diagnostic amplicon. Primer and probe sequences for ITS-2 were subsequently selected using Primer3 (Koressaar and Remm, 2007; Untergasser et al., 2012), with the following sequences: Dcit-its2-F1 (forward primer): 5'-TGC TCT TGT GTT TGA ACC G-3'; Dcit-its2-R1 (reverse primer): 5'-AAC TCA CTC GAC ACT CTC CC-3'; Dcit-its2-P1 (fluorescent internal probe): 5'-ACGCGAACCAAGCACCAGCA-3' (PrimeTime® 5' 6-FAM/ZEN/3' IB®FQ, Integrated DNA Technologies, Coralville, IA).

qPCR protocol

Predator DNA extractions were tested for ACP DNA using quantitative PCR (qPCR) carried out in a 72-well Qiagen Rotor-Gene® Q machine with Qiagen Rotor-Gene Q software (QIAGEN, 2009). Each 20 µL reaction was made up of 2 µL DNA template or NTC (no-template control) added to 18 µL reaction mixture, which contained 0.2 mM each dATP, dCTP, and dGTP, 0.4 mM dUTP, 1x ThermoPol PCR buffer (NEB), 0.8 mM each forward and reverse primers, 1 Unit Taq polymerase (NEB), and 0.2 µM fluorescent probe in ultrapure water. The probe was stored at -20°C in single day-use aliquots, kept wrapped in aluminum foil unless open, and added to the reaction master mix last to minimize light exposure. Wells were loaded on a cold block to minimize premature activity of reagents.

The thermocycler program began with a 90s ramp from 72-95^{O}C which rose 0.2^{O}C at each step, followed by 40 cycles at 95^{O}C for 10s, 55^{O}C for 15s, and 72^{O}C for 20s. Fluorescence was measured after each 72^{O}C (extension) step. Each 72-well qPCR run included three technical replicates (i.e., the same sample tested multiple times) of each of the following substrates: sterile distilled water as a no-template control, three serial dilutions of a pooled ACP DNA extraction of known concentration, and twenty DNA extractions of field-collected samples. The three serial dilutions of ACP extraction served a dual function as positive controls and standard dilutions of known DNA concentration. The pooled extraction from which these were drawn was made up of 54 combined total DNA Chelex extractions of adult ACP collected at the Mentone, CA field site. These extractions were combined, homogenized, and re-divided into single-use aliquots to reduce repeated thawing and freezing. Before each qPCR run, an aliquot was thawed and separated into three 10x dilutions of 100% (one adult ACP equivalent), 10% (5ul 100% template + 45 uL ddH_2O), and 1% (5 ul of 10% dilution + 45 uL ddH_2O). Following each run, the Rotor-Gene Q software determined a threshold of amplicon detection based on the amplification curve formed by these standard dilutions, preventing observer bias in threshold placement. Positive detection was recorded for each sample whose amplification curve passed the detection threshold in fewer than 37 cycles for two out of its three technical replicates.

Software settings:

Run data was collected on the qPCR machine's Cycling A.Green channel. Auto-Gain Optimization was performed between the first amplification cycle and the first fluorescence measurement on well #4, which always contained a template of undiluted ACP DNA extraction. Post-PCR quantitation analysis was run with activated Dynamic Tube Normalisation starting from cycle 1, which accounts for background fluorescence up to the amplification take-off point for individual wells, and Noise Slope Correction, which accounts for changing background fluorescence throughout a run (personal correspondence, Qiagen technical support). Outlier Removal was set at 2%, causing the software to ignore any changes in fluorescence less than 2% that of the tube with the largest change in fluorescence.

Preliminary experiments

Optimal software settings to eliminate false positives and false negatives were determined by preliminary experiments and consultation with Qiagen technical support.

Software settings

In a preliminary run to confirm the functionality of the software settings and detection criteria described above, Chelex DNA extractions of adult *Sympherobius barberi* (brown lacewing) (n = 1) and *Hippodamia convergens* (convergent lady beetle) (n = 2) were tested alone and combined with five serial

dilutions of ACP DNA. Predator DNA samples were diluted with an equal part sterile distilled water for use in this test to prevent PCR inhibition. The samples containing only predator DNA served as negative controls, while samples containing predator DNA and ACP DNA were used to confirm that the target material is detected by the test at biologically relevant concentrations. ACP DNA was obtained from a Chelex extraction of one adult ACP (one adult ACP equivalent) and serially diluted with ddH_2O to five concentrations ranging from 0.1 to 0.00001 adult ACP equivalent per 60 µL. To create mixed predator/ACP DNA samples, each of these dilutions was added to 18 µL of 1:1 predator extraction:ddH2O, for final ACP concentrations ranging from 0.01 to 0.000001 adult ACP equivalent per 60 µL. This was repeated for all three predator extractions, for a total of fifteen experimental samples. All fifteen experimental samples, a negative control for each predator, and a no-template control (NTC) were run in triplicate.

Every sample which contained at least 0.00001 adult ACP equivalent (10 ppm ACP extraction) tested positive, with at least two out of three wells passing the positive detection threshold in fewer than 37 cycles. Two of the three samples containing only 1 ppm ACP template tested positive by the same criteria. None of the negative controls or NTCs tested positive.

Prey detection in ACP-fed predators:

Twelve commercially procured adult *H. convergens* were allowed to feed on ACP nymphs for up to two hours and consumed 1-5 ACP nymphs apiece. They were then frozen in 95% EtOH which had been chilled at -20ºC. Total DNA was extracted from six of the beetles with Chelex, and from the other six with Qiagen DNeasy, as described above. Total DNA was also extracted from two unfed beetles for negative controls. Two adult ACP Chelex extractions were used as positive controls. These extractions were checked for DNA quality using standard PCR with LCO/HCO primers, then tested for ACP DNA using the qPCR assay. In this assay, each sample was replicated in three adjacent wells as 1) undiluted DNA extraction, 2) 50% DNA extraction and 50% sterile distilled water, and 3) 10% DNA extraction and 90% sterile distilled water. Diluting two of the replicates allowed monitoring for PCR inhibition, which can be caused by overly concentrated template. No-template control (NTC) containing sterile distilled water was run in two wells. Standards of known DNA concentration were in development, so the detection threshold was manually set within the upper third of the exponential portion of the positive controls' amplification curves, during which the experimental results were hidden from view to avoid bias.

Positive detections were produced for all six of the ACP-fed beetles extracted with Qiagen Dneasy, and five out of six of the ACP-fed beetles extracted with Chelex. Although only two out of three wells amplifying past the detection threshold are required for a positive detection, all three wells amplified

for the eleven ACP-fed beetles which tested positive. There was no amplification in wells containing unfed control beetle template or NTC. These results demonstrated that the qPCR assay could detect ACP predation and distinguish between ACP-fed and unfed predators.

External bleach sterilization

We tested the success of a protocol for preventing false positives by treating collected specimens with bleach prior to DNA extraction. This protocol was designed to prevent of false positives caused by contamination during powered-vacuum field collecting, which tumbles collected material together in a vacuum bag for up to five minutes. Sixteen pairs of non-predatory insects, each having no plausible trophic relationship with ACP, were selected from a vacuum-collected field sample taken at the Yorba Linda field site on May 3, 2013 (Table 4.3). The sample contained 195 adult ACP, and therefore presented a risk for external contamination of specimens. Each matched pair of insects was divided into untreated and treatment groups. The treated group was processed as described in Materials and Methods, following the protocols for external bleach washing, individual Chelex extraction, amplification with standard PCR using LCO/HCO primers, and the qPCR test for ACP DNA. The untreated group omitted the external bleach wash step but was otherwise treated identically.

Standard PCR using CO1 primers and visualization with gel electrophoresis confirmed that all specimens in both groups retained intact DNA,

regardless of whether they were bleach-treated first or not. DNA extracts were then tested for the presence of ACP DNA using the qPCR protocol. Every bleach-treated specimen was negative for ACP DNA, while three of the untreated counterparts (all pooled extractions) tested positive. A fourth untreated specimen amplified ACP DNA in a single well. These results demonstrated that external bleach treatment supports the reliability of our positive results by reducing the chance of false positives. Additionally, the resulting negative qPCR results for a taxonomically wide array of specimens provides support for the species-specificity of our test of gut contents for ACP predation.

Table 4.3: Insect specimens used to test bleach treatment protocol
Table shows field-collected insect specimens used to test bleach treatment protocol in preliminary experiment and results of experiment.

Order	Taxon	Tissue used	ACP DNA detection	
			untreated	treated
Coleoptera	Mordellidae sp.	whole	no	no
Coleoptera	Scolytinae sp.	whole	no	no
Hemiptera	Coccidae sp. #1	20 specmens	yes	no
Hemiptera	Coccidae sp. #2	whole	no	no
Hemiptera	Diaspididae sp.	whole	no	no
Hymenoptera	Chalcidoidea sp. #1	5 specimens	yes	no
Hymenoptera	Chalcidoidea sp. #2	3 specimens	yes	no
Hymenoptera	Chalcidoidea sp. #3	whole	no	no
Hymenoptera	Chalcidoidea sp. #4	whole	no	no
Hymenoptera	Chalcidoidea sp. #5	whole	1/3 wells	no
Hymenoptera	Ichneumonoidea sp. #1	whole	no	no
Hymenoptera	Ichneumonoidea sp. #1	whole	no	no
Hymenoptera	Ichneumonoidea sp. #2	whole	no	no
Hymenoptera	Ichneumonoidea sp. #2	whole	no	no
Hymenoptera/Hemiptera	Diaspididae #2 (parasitized)/ Chalcidoidea sp. larva	whole	no	no
Hymenoptera/Hemiptera	Diaspididae #2 (parasitized)/ Chalcidoidea sp. larva	whole	no	no

Statistical analysis

Positive ACP detection rates were compared within predator species to determine whether they differed between collection time of day and/or predator life stages. This was carried out using binomial tests to compare two proportions with Chi-squared approximation, or with Fisher's exact test for comparisons in which one or more expected result included five or fewer data points. Analyses were carried out in the R computing environment (R Core Team, 2015) using the RStudio graphical user interface (RStudio Team, 2015).

A generalized linear model was fitted to the ACP detection data to evaluate whether the number of adult ACP or Argentine ants in a specimen's vacuum sample influenced the likelihood of a specimen testing positive for ACP DNA. Binomial error structure and the logit link function were used because of the binary nature of the dependent variable (i.e. positive or negative). The influence of ants and the interaction term of ants and ACP was evaluated for specimens collected in the first six months of sampling, during which Argentine ants per sample were counted.

Results

Overall results

Three insect species and all five spider species tested positive for ACP predation at rates greater than 10% (Table 4.4). *Chrysoperla comanche* tested positive more often than any other group, with ~43% of samples indicating

evidence of ACP predation. Two lady beetle species, *D. pumilio* and *R. lophanthae,* tested positive at 17.5% and 12.8%, respectively. There was no difference in positive detections between *D. pumilio* sexes ($X^2 = 0.16374$, df = 1, p = 0.69). The other two lady beetles, *S. punctum* and *C. montrouzieri,* rarely tested positive for ACP predation. 1% of *S. punctum* larvae and none of the adults tested positive, while 3% of *C. montrouzieri* adults and none of the larvae tested positive. Of the spiders, *A. pacifica* tested positive most often (33%), followed by *T. pacificus* (18%), *Theridion* spp. (15%), *S. vitis* (12%), and *H. incursa* (11%). Rate of positive detection was not influenced by quantity of adult ACP or Argentine ants in a sample, or by the interaction term of ants and ACP (p > 0.1 for all three).

Table 4.4: Summary of ACP predation test results for all groups tested.

				ACP predation test	
Order	**Family**	**Genus/species**	**Life stage**	*n*	**% positive**
Araneae	Anyphaenidae	*Anyphaena pacifica*	juvenile	100	**33.0**
Araneae	Anyphaenidae	*Hibana incursa*	juvenile	100	**11.0**
Araneae	Salticidae	*Sassacus vitis*	juvenile	100	**12.0**
Araneae	Theridiidae	*Theridion spp.*	juvenile	60	**15.0**
Araneae	Trachelidae	*Trachelas pacificus*	juvenile	100	**18.0**
Coleoptera	Coccinellidae	*Cryptolaemus montrouzieri*	adult	32	**3.1**
Coleoptera	Coccinellidae	*Cryptolaemus montrouzieri*	larva	58	**0**
Coleoptera	Coccinellidae	*Diomus pumilio*	adult	188	**17.6**
Coleoptera	Coccinellidae	*Rhyzobius lophanthae*	adult	39	**12.8**
Coleoptera	Coccinellidae	*Stethorus* spp.	adult	74	**0**
Coleoptera	Coccinellidae	*Stethorus* spp.	larva	99	**1.0**
Neuroptera	Chrysopidae	*Chrysoperla comanche*	larva	105	**42.9**

Comparisons of detection rates within species

2nd-3rd instar *C. comanche* larvae tested positive for ACP predation three times as often as 1st instar larvae. 54% of 2nd-3rd instar larvae (n=72) tested positive, while ACP predation was detected in only 18% of 1st instar larvae (n=33) (X^2 = 10.54, df = 1, p = 0.001).

Diomus pumilio detection rate differed significantly by time of day, with specimens collected during late afternoon testing positive more often than specimens collected before dawn (24% vs. 7%) (X^2 = 8.28, df = 1, p = 0.004). There was no difference between pre-dawn and afternoon detection rate for any other group (*C. comanche*: X^2 < 0.01, df = 1, p = 0.98; *R. lophanthae*: odds ratio = 0.53, p ≈ 1; *A. pacifica*: X^2 = 1.58, df = 1, p = 0.2; *H. incursa*: odds ratio = 1.80, p = 0.52; *S. vitis*: odds ratio = 0.76, p = 0.76; *Theridion* spp.: odds ratio 0.45, p = 0.46; *T. pacificus*: odds ratio = 1.9, p = 0.5).

Detection rates did not differ between sample sites for any species with enough specimens from each location to allow comparisons. These include *D. pumilio* (X^2 = 1.37, df = 1, p-value = 0.24), *C. comanche* (X^2 = 1.3, df = 1, p = 0.25), *H. incursa* (X^2 = 6.01e-32, df = 1, p-value ≈ 1), *Theridion* spp. (odds ratio = 1.03, p ≈ 1), and *T. pacificus* (odds ratio = 2.21, p = 0.20). Species which were too sparsely collected at one of the study sites to allow comparison were *A. pacifica, R. lophathae,* and *S. vitis.*

No within-species comparisons of detection rates were performed for *S. punctum* or *C. montrouzieri* because they had only one positive detection apiece.

Discussion

Detection rates

The goal of this study was to identify likely natural enemies of ACP among southern California's resident predator community that could potentially increase the effectiveness of ACP biological control programs if managed through augmentation and/or conservation. The results reported above show which of the ten most abundant predatory insects and spiders at our sites preyed on ACP, and which therefore may be contributing to the natural mortality of ACP in southern California described in Chapter 2.

Based on rates of ACP detections in predator gut contents, *C. comanche, D. pumilio,* and *R. lophanthae* are likely predators of ACP, while *Stethorus* spp. and *C. montrouzieri* are unlikely predators of ACP. All five spiders had ACP detection rates consistent with predation on ACP, with *A. pacifica* testing positive the most frequently. These findings support the results presented in Chapter 2, which showed that ACP is subject to substantial biotic mortality from predator attacks in southern California. Furthermore, this work identifies species within the predator community which are likely contributors to that mortality. These species, which have now been identified as natural enemies of ACP, have the potential to be useful for reduction of ACP as part of an integrated management program. Manipulative field experiments with the most promising insect species, *C. comanche* and *D. pumilio*, would yield useful information about their ability to reduce ACP populations in agricultural settings. Further study of the spider

community in citrus may reveal ways in which modified management practices could increase spider predation on ACP.

Within-species differences of ACP detection rates suggest patterns of predation habits for certain species. The high rate of *D. pumilio* positive test results for specimens collected in late afternoon (24%), three times that of specimens collected before dawn (7%), strongly suggests that *Diomus pumilio* adults are diurnal predators. This interpretation is supported by the temperature difference between night and day, because higher daytime temperatures are expected to lead to more rapid deterioration of detectable prey material in predator guts (Hoogendoorn and Heimpel, 2001; Hosseini et al., 2008; Von Berg et al., 2008), although Hosseini et al. (2008) found that detection time decreased with temperature for a lady beetle, but not a spider.

Detection rates were the same for pre-dawn and afternoon-collected specimens for all three species of nocturnal hunting spiders, although more individuals were collected before dawn, as described in Chapter 3. There are several possible interpretations of why more nocturnal spiders have equivalent positive detection rates in pre-dawn and afternoon collections, even though more are caught before dawn. First, these spiders may exhibit within-species variation in hunting time while maintaining the same likelihood of feeding on ACP regardless of what time of day hunting takes place. Alternately, these spiders may retain detectable ACP remnants in their gut contents long enough that no conclusions can be drawn about what time of day they fed. The latter hypothesis

applies to any species in this study, with the exceptions of *D. pumilio* and *C. comanche*. These species were confirmed to have short detectability intervals, with detectability half-lives of 1.9 hr and 0.33 hr, respectively (Chapter 5).

Differences in detection rates between life stages likely indicate differences in predation habits as well. The rate of 54% positive detections in older *C. comanche* larvae was the highest found in this study, and suggests a degree of preference for ACP by 2nd-3rd instar larvae rather than random opportunism, while the much lower rate of positive detections in 1st instar larvae could indicate either that a different food source is sought by the youngest *C. comanche* larvae, or that they feed less frequently than older larvae. Based on results described in Chapter 5, the latter interpretation is more likely to be correct: *C. comanche*'s preferences for different ACP life stages as prey do not differ between the three larval instars; however, first instar *C. comanche* larvae ate significantly fewer ACP individuals than second and third instar *C. comanche* larvae. (Chapter 5). This finding is highlighted by the unequal rates of positive ACP detection between 1st vs. 2nd-3rd instar larvae.

As stated in Chapter 3, all arthropods were much more abundant at the Yorba Linda site. Likely contributing factors are, 1) tree spacing: in Yorba Linda, tree canopies were sufficiently far apart that the entire canopy was able to produce flush, while many of the trees at Mentone were closely spaced and produced flush on only two sides; 2) the Yorba Linda orchard is in a riparian area with flowing water, which likely contributes to greater background diversity and

abundance of arthropods, while the Mentone site is near a dry riverbed and surrounded by other orchards; 3) the entire Yorba Linda orchard is certified organic, while the study site at Mentone is a small unsprayed plot at the outer edge of a large, conventionally farmed orchard, and thus may be subject to pesticide drift, which could decrease arthropod populations.

Despite these differences, detection rate did not differ between the two sites for all five predators which were sufficiently abundant at both sites to allow comparison: *D. pumilio, C. comanche*, *H. incursa*, *Theridion* spp., and *T. pacificus*. This suggests that the differences listed above do not have a strong influence on the rate of ACP predation by these groups; however, more research is needed to evaluate this possibility.

Detection results by month are inconclusive with regard to seasonal variation in ACP predation. Further study is needed to determine whether predation rates differ by time of year for each species, and which factors influence this. However, ACP predation detection rates for *D. pumilio* and *C. comanche* were highest during the months of their greatest abundance at the corresponding field sites (Figure 3.2).

Adult ACP and Argentine ants did not affect detection rates in our models. This suggests that adult ACP may not be commonly preyed upon, and that adult ACP abundance does not necessarily predict the availability of immature ACP, which are more sessile and soft-bodied than adults. Our survey data indicated that Argentine ant abundance and predator abundance were positively correlated

(Chapter 3). However, predator densities rise in controlled experiments when Argentine ant populations are reduced (Schall and Hoddle, 2016), suggesting that the positive correlation between Argentine ant abundance and predatory arthropods in the absence of ant control may reflect other factors that increase both Argentine ant and predator abundance, such as temperature, leaf flush, and/or overall density and species makeup of honeydew producers. None of our trees were ant-controlled, so low numbers of ants on any given tree or branch may have pointed to a lack of these shared resources. Further research comparing predation on trees with and without ant control is needed to determine whether ant control increases predation at the predator species level.

When abundance data (Chapter 3) is considered alongside ACP predation detections, *C. comanche, D. pumilio,* and *Theridion* spp. appear to have been the major predators in January, February, April, and May. The most promising insect predators, *C. comanche* and *D. pumilio,* peaked in both population and positive detections from January through May. These species declined at the end of spring, while *A. pacifica*., *H. incursa*, and *T. pacificus* sharply increased. These three species, all nocturnal hunting spiders, remained present over the summer months and slowly declined throughout fall. Fewer specimens were available for testing from samples collected from late fall to early winter, and those tested had low rates of positive detections. These may be related to low survival of ACP nymphs: as Kistner et al. (2016b) reported, survival of ACP to adulthood in

experimental predator exclusion cohorts dropped sharply when average temperatures fell below 10ºC.

The hunting spiders *A. pacifica* and *H. incursa* became abundant in mid-May and remained so through August, each one being the dominant species at a single site, *A. pacifica* at Yorba Linda and *H. incursa* at Mentone. Fewer specimens of these spiders were available to be tested from September through April, but the detection rate was not very different for those specimens which were tested. This pattern of seasonally dependent abundance without an observable decline in off-season detection rate held for *T. pacificus,* which was most abundant May-Sept., and *Theridion* spp., which peaked in late winter-early spring. *Sassacus vitis* did not display a strong seasonal pattern either in population or ACP detection, with multiple abundance peaks over the course of the year and varied positive detection rates.

Potential of candidate predators as biological control organisms

Based on abundance, positive detection rates of ACP predation, and life histories, *C. comanche* and *D. pumilio* are the most promising natural enemies of ACP of the ten predators tested. It is premature to judge between *C. comanche* and *D. pumilio* as biological control agents of ACP, because many factors influence their predation potential. For example, *C. comanche* are predatory only during larval development, while *D. pumilio* are predatory as both larvae and adults. Both have alternate host species, although only one, acacia psyllid, is

recorded for *D. pumilio* (Dreistadt and Hagen, 1994). In addition, *C. comanche* are subject to autointraguild predation due to larval cannibalism (Daane and Yokota, 1997), while *D. pumilio* larvae are cannibalistic only rarely and in starved conditions (personal observation). Further research is needed to determine to what degree and under which circumstances each species contributes to ACP control in field conditions. Mass rearing is possible for both species, as it has previously been accomplished for each one (Hunter, 1997; Pinnock et al., 1978).

The number of positive test results for spiders found in this study is promising with respect to their ability to prey on ACP. Little quantifiable data has been published regarding spider predation of ACP. However, field observations of spiders preying on ACP were reported in Florida for at least four spider species in four different families (Michaud, 2002; Michaud, 2004), while *A. pacifica* has been observed consuming ACP in California, as stated above. These reports confirm that primary predation of ACP occurs in spiders. However, further research is needed to confirm that additional spider species of interest feed on ACP directly, because molecular analysis of gut contents does not distinguish between primary predation and secondary predation (Foltan et al., 2005; Sheppard et al., 2005).

Limitations of molecular prey detection

Although standard molecular analyses of gut contents cannot typically distinguish between primary vs. secondary predation, or between predation and

scavenging, analyses can be designed to detect protein markers specific to live vs. dead prey (Mansfield and Hagler, 2016). Laboratory feeding experiments and field experiments can also help determine whether a true trophic relationship exists *in vivo.* It is important to note that the ranking of predators by positive detections, while a good first step, is a rough estimate of true predation, which must be refined by digestion time analysis in order to approach the true relative frequency with which species feed on a target prey in the field (Chapter 5) (Greenstone et al., 2010). Spiders generally have longer predation detectability intervals than do insects, although this varies widely between species (Anderson, 1970; Hosseini et al., 2008).

Under ideal circumstances, the cycle number of a qPCR assay at which a specimen's amplification curve crosses a threshold of detection, known as the the C_T value (QIAGEN, 2009), would indicate meal size or the number of prey items eaten. However, the amount of intact DNA decreases with time since feeding (Hosseini et al., 2008; King et al., 2008). C_T value could be used to indicate time since feeding if meal size is known, or vice versa, but neither of these values can be estimated from field-collected specimens.

Hotter temperatures in summer might have reduced detections for specimens relative to other times of the year (Hoogendoorn and Heimpel, 2001; Hosseini et al., 2008; King et al., 2008; Von Berg et al., 2008). Further study is required to determine whether different rates of detection over the course of a year reflect ambient temperature changes or true differences in feeding habits.

DNA-based molecular analysis of predator gut contents in citrus

The potential for DNA-based molecular analysis of predator gut contents to contribute to biological control in citrus has been sparsely explored. A qPCR-based technique to detect ACP in bulk insect samples taken from sticky traps was developed by Fujiwara et al. (2016) using ACP-specific CO1 primers which amplify an 821-bp fragment. Hagler et al. (2012) used both ELISA and PCR in a comparison of the two methods for detection of *Homalodisca vitripennis* in gut contents of predatory arthropods in citrus; this study was proof of concept, because it identified predators only to the family level. Monzó et al. (2009) developed a PCR-based method for detecting Mediterranean fruit fly predation by wolf spiders.

Conclusions

This is the first study to systematically survey and evaluate a citrus predator community for ACP predation over the course of a year, both diurnally and nocturnally. This work showed molecular analysis of gut contents to be a useful tool in distinguishing candidate natural enemies from other predators present in the system. Two of these predators, *Diomus pumilio* and *Chrysoperla comanche*, are especially promising as potential biological control agents of ACP. Because there has been substantial interest in developing new biological control agents for ACP, mass rearing protocols for these insects should be revived and tree-scale or orchard-scale augmentation experiments should be

conducted to determine the amount of ACP reduction that can be obtained per unit of natural enemies released. The spider community at our study sites also included natural enemies of ACP, with all five common spider species feeding on ACP. Tree-scale or orchard-scale field studies to determine the significance of spider predation on ACP would allow IPM practitioners to determine whether spider conservation practices are an economically viable component of ACP management.

Given that co-evolved natural enemies are essential to successful biological control in many cases (Legner and Bellows, 1999), it would be counterproductive to argue that co-evolved natural enemies should be disregarded or given lower priority during the design phase of a biological control program. Rather, studies of successful biological control based upon novel host associations serve as a reminder to examine all possible sources for candidate biological control agents, in the interest of not overlooking a species which might possibly be the best option. The ultimate challenge is not to determine whether co-evolved or newly associated natural enemies are more effective, but to enable practitioners to determine which natural enemies are the most effective and environmentally safe species available for biological control of a specific pest.

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Chapter 5:

Life stage preferences and digestion time analysis of two ACP predators, *Chrysoperla comanche* and *Diomus pumilio*.

Abstract

Chrysoperla comanche (Neuroptera: Chrysopidae) and *Diomus pumilio* (Coleoptera: Coccinellidae) were identified by molecular analyses of gut contents as frequent predators of *Diaphorina citri* (Asian citrus psyllid). Results of such analyses from field-collected specimens cannot be directly compared between species because digestion rates vary between species, with prey DNA becoming undetectable more quickly in some species than others. To properly rank predators according to frequency of predation, we performed digestion time analysis and fitted a logistic model for each species to predict the time point at which 50% of specimens fed upon ACP would test negative, a value analogous to LD_{50}, here referred to as the DT_{50} (for detection time). These were: 0.33 h for *C. comanche* and 1.9 h for *D. pumilio*. Field-collected *C. comanche* specimens tested positive for ACP predation 2.4 times as often as *D. pumilio* field specimens, so the ranking of these two predators by frequency of ACP detection is reinforced, not changed, by the DT_{50}s. Furthermore, these short detectability half-lives indicate that ACP predation is a frequent occurrence for both species. We performed a prey life stage choice assay to determine which of the seven ACP life stages each predator is likely to prey on. Eggs were the most preferred ACP life stage for all three *C. comanche* larval instars, nymphal stages were equally preferred, and ACP adults were consumed, but less frequently than all other life stages. *Diomus pumilio* preferred ACP eggs and first-third instar nymphs over the older ACP life stages.

Introduction

The previous chapter reported the results of molecular analyses of gut contents of field-collected predators for DNA of *Diaphorina citri,* Asian citrus psyllid (ACP). The predator species chosen for analyses were the ten most common predatory insect and spider species identified in a year-long foliar vacuum collection survey of two orange orchards located in Orange County and San Bernardino County, CA. Molecular analyses of gut contents revealed two predatory insects, *Chrysoperla comanche* (Neuroptera: Chrysopidae) and *Diomus pumilio* (Coleoptera: Coccinellidae), as the most frequent insect predators of ACP at these sites. 18% of first instar and 54% of second and third instar *C. comanche* larvae tested positive for ACP, with no difference between pre-dawn and late afternoon collected specimens, and 7% of pre-dawn and 24% of late afternoon *D. pumilio* adults tested positive for ACP. We selected these species for further study because, as insects, they have the potential to be used in biological control programs for either augmentative or conservation biological control. Two topics are addressed in this study: ACP life stage preferences and digestion time analysis (also known as detectability half-life analysis (Greenstone et al., 2014)) of *C. comanche* and *D. pumilio*.

Analyses of predator gut contents offer insight into trophic relationships which would be difficult or impossible to observe otherwise. However, the data produced by these techniques cannot be compared between species or mined for information about meal size or time since feeding. Positive detection rates of

field-collected specimens are insufficient to rank predators according to frequency of predation, because the rankings will be biased in favor of species which are slower to break down the target amplicons (Greenstone et al., 2014).

The length of time during which prey DNA can be detected within a predator's gut varies dramatically. Depending on predator species and life stage, possible factors influencing the period of detectability are temperature, subsequent meals, predator weight and sex, amount of prey consumed, characteristics of the diagnostic DNA fragment, primer quality, and DNA extraction method (Greenstone et al., 2010; Hoogendoorn and Heimpel, 2001; Hosseini et al., 2008; Von Berg et al., 2008). Determining gut persistence time for each predator under consideration is essential to understanding the significance of prey DNA presence/absence data (Hosseini et al. 2008, Greenstone et al. 2010).

Materials and methods

Insects

ACP

ACP nymphs were collected from a laboratory colony at UC Riverside, where they were reared on *Murraya koenigii* seedlings.

Chrysoperla comanche

Chrysoperla comanche larvae used in this study were reared in a laboratory colony founded from insects collected on orange trees at our study sites in Yorba Linda and Mentone, CA (Chapter 3), and from residential orange trees located near the UC Riverside campus. Rearing protocols were modified from those developed by Amarasekare and Shearer (2013) for *C. carnea* and *C. johnsoni*. Mating and oviposition cages for adult *C. comanche* consisted of open-topped glass terraria covered with nylon gauze. The gauze, which served as oviposition substrate, was held in place by wire mesh lids and changed twice weekly, or as often as eggs were needed. Eggs harvested for rearing were individually removed from the fabric by their silk stalks with forceps. When a large number of eggs was needed, the bottom halves of polypropylene microtube freezer storage boxes were placed inside the cages to provide extra oviposition substrate, while larvae that hatched inside the cage were removed to prevent cannibalism. Eggs were incubated individually in 12 x 75 mm glass culture tubes plugged with cotton and held at 24°C on a 16:8 hr light/dark schedule. Each was provisioned with 0.08 cm^3 (1/64 tsp) of irradiated *Ephestia kuehniella* eggs (Beneficial Insectary, Inc., Redding, CA, USA), which were replaced 2x/week with 0.15 cm^3 (1/32 tsp) of fresh *E. kuehniella* eggs until larvae pupated. After a larva pupated, remaining *E. kuehniella* eggs were removed. Each tube was then set upright in a rack and a narrow strip of card stock (~6 x 60 mm) was inserted, which allowed teneral adults to assume a vertical position to expand their wings.

All three *C. comanche* larval instars were used in prey life stage choice experiments. Each set of larvae to be used within a day's experimental trials were laid within a single 24-hour oviposition period. Eggs of known age were collected by laying a fresh sheet of gauze over oviposition cages and harvesting it 24 hr later. First instar larvae were used <24 hr after hatching (4 days post-oviposition), and were given only water soaked into each tube's cotton stopper before they were introduced into the experimental arena. Second and third instar larvae were likewise starved, given water, and used 24-48 hr after their first and second molts, respectively, which occurred at seven and ten days post-oviposition. To confirm the correct life stage, we checked each vial for exuviae the morning after molting was expected, and confirmed each larva's stage-specific morphology according to Tauber (1974). If the larva had molted we removed its remaining food, then used it in a prey choice trial the following day. Larvae were provided with water before use in prey choice trials so that dehydration would not bias behavior.

Digestion time analysis was conducted on *C. comanche* larvae 24-48 hr after they molted to the second instar. Eggs hatched four days after oviposition, and food was replaced 2x/week until seven days post-hatch, when first-instar larvae molted to the second instar. Larval stage was confirmed as described above. Larvae were then held without food for 24 hr and given only water before the experiment was conducted.

Diomus pumilio

Diomus pumilio adults used for the prey choice experiment were collected June 6-8, 2016 by beating vegetation in a navel orange grove in Mentone, CA. After collection, beetles were held individually in 12 x 75 mm glass culture tubes at 24°C on a 16:8 hr light/dark schedule and provided with 0.08 cm^3 (1/64 tsp) irradiated *E. kuehniella* eggs 2x/week. *Diomus pumilio* were starved 24 hr prior to use in the experiment, which was conducted July 21-27, 2016.

Diomus pumilio used in the digestion time analysis experiment were reared in laboratory culture from eggs laid by mated females collected between April and June 2016 at orange groves in Yorba Linda, CA and Mentone, CA. Adult *D. pumilio* were fed *E. kuehniella* eggs 2x/week. This diet maintained beetles for up to several months, but did not support oviposition by mated females, which very rarely laid eggs unless fed ACP eggs and nymphs. When *D. pumilio* eggs were needed, females were placed with males for 24 hr, then given ACP nymphs and eggs to feed upon. Narrow strips of longitudinally creased card stock (~6 x 60 mm) were provided as oviposition substrate (Meyerdirk, 1983).

Card stock strips were checked for eggs and collected daily. Eggs were typically laid in an overlapping mass on this substrate, so card stock strips were moved to *M. koenigii* seedlings infested with 1st-3rd instar ACP nymphs. *Diomus pumilio* larvae migrated onto the plants after hatching, and after feeding on ACP for 2-3 d were collected from the plant into glass culture tubes, where they were provided with 1st-5th instar ACP nymphs daily until pupation. Larvae were not

reared to maturity on the ACP-infested plants because a single mass of >20 eggs would depopulate the ACP infestation of a 15-20 cm *M. koenigii* seedling, resulting in starvation of the beetle larvae. After eclosion, adult beetles were provided with *E. kuehniella* eggs. Adult beetles were mated before they were used in the digestion time experiment.

Prey choice experiment

Arena preparation

Twenty ACP eggs and ten each of 1st-5th instar ACP nymphs and adults were placed in a 5.08 x 5.08 x 1.9 cm clear plastic arena (#60220, AMAC Plastic Products Corporation, Petaluma, CA) using fine paintbrushes. To reduce escape into the crevice between box and lid, the outside lip of the box and inside lip of the lid were painted with Fluon (BioQuip Products, Rancho Dominguez, CA). A five-channel counting device was used to track the number of 1st-5th instars placed in the arena. During the arena-loading process, which lasted approximately 30 min, the arena was kept on an ice plate to slow psyllid movement and prevent escapes. To avoid freezing the psyllids during this time, the arena was separated from the ice by several layers of paper towels inside a plastic bag. Adult ACP were added after all the immature stages were in place from a vial which was buried in ice for 2 min to temporarily immobilize the insects. Immobilized adults were transferred from the vial into the arena using a paintbrush, and the arena was covered with its lid. The arena lid was then slightly

lifted to transfer a predator with a paintbrush to the center of the arena. The experiment timer began immediately afterward, with the removal of the arena from the ice plate to a dissecting microscope stage for observation.

Experiment observation

Each predator was observed in this arena for 60 min, and all encounters, attacks, and feeding upon each ACP life stage were noted. An encounter was defined as contact between a predator and prey, regardless of which approached the other. Probing of the prey by the predator's mouthparts was classified as an attack. If a prey item was probed by the predator's mouthparts, the interaction was recorded as both "encounter" and "attack." If some or all of a prey item was consumed by the predator, the interaction was classified as all three types of interaction: "encounter," "attack," and "feeding." The experiment was replicated thirty times with each of the three *C. comanche* larval instars and thirty times with *D. pumilio* adults, which included fifteen females and fifteen males.

Digestion time analysis

Predators were starved for 24 hr prior to the experiment, and provided with water soaked into each vial's cotton plug the morning of the experiment. Predator starvation intervals were begun in a staggered fashion the previous day so that later replicates would not be biased by longer starvation periods.

Feeding took place in a 1.85 mL (½ dram) glass shell vial containing 10-20 second-instar ACP nymphs placed inside with a fine paintbrush. Second instar nymphs were chosen as the standard meal for this experiment following the results of the prey life stage choice experiment, which indicated that second instars would be the most easily accepted prey for both predator species. Each predator was singly introduced to the feeding vial and observed until it had completely consumed a single ACP nymph. The predator was then immediately moved to a 2-mL plastic screw cap vial, and the clock was started for the assigned digestion time interval.

Predators assigned to the 0-hr digestion time interval were immediately placed on dry ice (-78.5°C) or in a -80°C freezer for 2 min, after which 95% EtOH was added to the vial. This protocol was designed to simulate the procedure for vacuum-collected field specimens (Chapter 4). Predators assigned to longer digestion time treatments were moved to a 24°C incubator for the duration of the treatment period, after which the predator was frozen as described above. A 24-hr light schedule was used to avoid introducing circadian effects for the treatments which extended into night hours. Specimens were stored at -20°C pending external bleach treatment and DNA extraction.

Prior to DNA extraction, predator specimens were treated with a dilute bleach solution as in Greenstone et al. (2012) to remove external contamimation with ACP DNA. Specimens were placed individually in 1 mL vials containing a solution of 2.5% commercial bleach (5.25% sodium hypochlorite) and 97.5%

double distilled H_2O in a tube rotator and rotated end over end for 45 min. Each specimen was then placed on a Büchner funnel with suction and rinsed three times with 1 mL 95% EtOH to remove bleach residue. The funnel's surface was covered with filter paper to prevent specimens from being suctioned into the funnel's perforations. After the rinses, each specimen was moved with flame-sterilized and cooled forceps into a sterile vial containing 95% EtOH.

The range of post-feeding intervals used for each insect was chosen after obtaining approximate prey detectability limits for each predator species in preliminary experiments. *Chrysoperla comanche* were tested at 0 hr post-feeding (n=25), 1, 2, and 6 hr (n=20 each), 12 hr (n=21), and 24 hr (n=8). Twelve *D. pumilio* were tested for each of the following post-feeding intervals: 0 hr, 1 hr, 2 hr, 3 hr, and 4 hr, and six were tested at 6 hr.

DNA extractions of predators

Total DNA extraction was performed using the Qiagen DNeasy® Blood & Tissue kit according to the manufacturer's supplementary protocol for purification of total DNA from insects (QIAGEN, 2006), with the following modifications and specifications: 1) specimens were ground in 10 µL of Buffer ATL, followed by the addition of 170 µL of Buffer ATL. 2) Lysis incubation at 56ºC was carried out for 3 hr for all specimens. 3) The final elution with Buffer AE was performed twice, using 50 µL of Buffer AE each time.

PCR test for quality of DNA extraction

Each predator DNA extract was tested for DNA quality prior to testing for ACP gut contents. DNA quality was confirmed by amplification with polymerase chain reaction (PCR) (Saiki et al., 1988) using the “universal” primer pair LCO1490/HCO2198, which amplifies a region of the mitochondrial cytochrome oxidase I (COI) gene across a wide range of invertebrate taxa (Folmer et al., 1994).

Each 25 µl reaction was made up of 2 µl DNA template and 23 µl reaction mixture containing 1x ThermoPol PCR buffer (New England BioLabs, Ipswich, MA, USA), 0.2 mM each dATP, dCTP, and dGTP, 0.4 mM dUTP (substituted for dTTP as a laboratory precautionary measure against PCR product contamination (Longo et al., 1990)), 2 mM MgCl, 12 µg BSA (NEB), 0.2 µM each LCO1490 primer and HCO2198 primer, and 1 Unit Taq polymerase (NEB) in ultrapure water.

Thermocycling followed a program of initial denaturation at 94ºC for 120 s, six cycles of 94ºC/30 s, 45ºC/90 s, 72ºC/70 s, thirty-six cycles of 94ºC/30 s, 51ºC/90 s, 72ºC/70 s, and one final extension period of 300 s, using a Mastercycler® ep gradient S thermocycler (Eppendorf North America Inc., New York, NY, USA). PCR product was visualized by electrophoresis on 0.7% agarose gels stained with ethidium bromide to confirm the presence of amplicon of the appropriate length.

qPCR detection of *D. citri* DNA

Predator DNA extractions were tested for detectability of ACP DNA using quantitative PCR (qPCR) carried out in a 72-well Qiagen Rotor-Gene® Q machine with Qiagen Rotor-Gene Q software (QIAGEN, 2009).

Each 20 µL reaction was made up of 2 µL DNA template or NTC (no-template control) added to 18 µL reaction mixture, which contained 0.2 mM each dATP, dCTP, and dGTP, 0.4 mM dUTP, 1x ThermoPol PCR buffer (NEB), 0.8 mM each forward and reverse primers, 1 Unit Taq polymerase (NEB), and 0.2 µM fluorescent probe in ultrapure water. The probe was stored at -20°C in single day-use aliquots, kept wrapped in aluminum foil unless open, and added to the reaction master mix last to minimize light exposure. Wells were loaded on a cold block to minimize premature activity of reagents.

The thermocycler program started with a 90s ramp from 72-95^{O}C, rising 0.2^{O}C at each step, followed by 40 cycles at 95^{O}C for 10s, 55^{O}C for 15s, and 72^{O}C for 20s. Fluorescence was measured after each 72^{O}C (extension) step.

Run data were collected on the qPCR machine's Cycling A.Green channel. Auto-Gain Optimization was performed between the first amplification cycle and the first fluorescence measurement on well #4, which always contained undiluted ACP extraction template. Post-PCR quantitation analysis was run with activated Dynamic Tube Normalisation starting from cycle 1, which accounts for background fluorescence up to the amplification take-off point for individual wells, and Noise Slope Correction, which accounts for changing background

fluorescence throughout a run (Dobias, 2014). Outlier Removal was set at 2%, causing the software to ignore any changes in fluorescence less than 2% that of the tube with the largest change in fluorescence.

Each 72-well qPCR run included three technical replicates (multiple wells containing replicates of a single template) of each of the following substrates: sterile distilled water as a no-template control (NTC), three serial dilutions made from a single-use aliquot of pooled ACP DNA extraction of known concentration (origin and methods below), and twenty DNA extractions of experimental samples. The three serial dilutions of ACP extraction served dual functions as positive controls and standard dilutions of known DNA concentration.

Following each run, the Rotor-Gene Q software determined a threshold of amplicon detection based on the amplification curve formed by these standard dilutions, preventing observer-biased threshold placement. Positive detection was recorded for each sample whose amplification curve passed the detection threshold in fewer than 37 cycles for two out of its three technical replicates.

The ACP DNA used to generate the standard curve originated from the same pooled extraction used for that purpose during testing of field-collected predators for ACP predation (Chapter 4). This pool was made up by combining 54 individual Chelex® total DNA extractions of adult ACP collected on orange trees in Mentone, CA. Each adult ACP was ground with a micropestle in 2µL proteinase-K (> 600mAUml^{-1}; Qiagen, Valencia, CA, USA), after which 60 µL 5% Chelex® 100 resin suspension (w/v) (Bio-Rad Laboratories,Hercules, CA, USA)

was added. Samples were incubated for 1 hr at 55ºC followed by 10 min at 99ºC, then centrifuged for 4 min at 14,000 RPM. The supernatant, containing extracted total DNA, was removed from each tube, pooled in a single 2 mL tube, thoroughly vortexed, centrifuged again, then dispensed into single-use aliquots which were stored at -20º C. Before each qPCR run, an aliquot was thawed and separated into three 10x dilutions of 100% (one ACP adult equivalent), 10% (5 µL 100% template + 45 µL ddH_2O), and 1% (5 µL of 10% dilution + 45 µL ddH_2O).

Statistical analysis

Analyses were carried out in the R computing environment (R Core Team, 2015) using the RStudio graphical user interface (RStudio Team, 2015). Graphics were generated using the R package ggplot2 (Wickham, 2009).

Prey life stage choice

Prey life stage choice data were modeled to determine which ACP stages are preferred by each species and, for *C. comanche*, by each larval instar. Generally following the method used by Royer et al. (2008), relationships of encounters to attacks, encounters to feeding events, and attacks to feeding events for each life stage were used to compare preferences. To calculate mean rates of feeding/attack and consume/encounters for each combination of predator life stage and prey life stage, replicates with zero denominator values

(i.e., no attacks or encounters occurred for that life stage) were treated as zeroes.

Three models were fitted to prey choice data representing attacks as a function of encounters, feeding as a function of encounters, and feeding as a function of attacks. Predator group (*C. comanche* larval stage or *D. pumilio* sex) was included as a covariate. The models were fitted with a zero-inflated generalized linear model using function zeroinfl() from R package pscl (Jackman, 2015; Zeileis et al., 2008). This model distinguishes between two types of zero-count observations based on whether specified preconditions were met. When preconditions are not satisfied (e.g., there were no feeding events due to a lack of encounters), an observation is shunted to a binary model, and when preconditions are met (e.g., feeding events are ≥ 0 and encounters occurred), observations are included in a negative binomial model. Each type of interaction used as a predictor for a given model (either encounter or attack) was also coded as a precondition for that model.

Post-hoc pairwise comparisons were performed by least-squares means tests with Tukey p-value adjustment using R function lsmeans (Lenth, 2016). Mean total ACP consumed per replicate were compared between groups using a two-sample Wilcoxon test because it allows for unequal variances.

Digestion time analysis

qPCR detection of ACP DNA was recorded as positive or negative for each replicate and the data were processed using an R package, drc, for analysis of dose-response curves (Ritz et al., 2015). The probability of positive detection vs. digestion time interval was modeled for each species using function drm(). Models utilized the log-normal model for probit analysis (Finney, 1971) specified by the convenience function LN.2. Probit models are recommended for analysis of predator digestion time data (Greenstone et al., 2014; Payton et al., 2003). DT_{50} was predicted for each species by function ED.drc(), as were the 84% confidence intervals of each DT_{50}, also called fiducial limits (Payton et al., 2003), by the delta method (Bolker, 2008). Confidence level was set to 0.84 to produce $\alpha = 0.05$, following the recommendations of Payton et al. (2003).

Results

Prey life stage preferences

Mean ± SE encounters, attacks, and feeding per ACP life stage are shown in Figure 5.1 for *C. comanche* and Figure 5.2 for *D. pumilio*.

Second and third instar *C. comanche* larvae consumed significantly more individual ACP per trial than did first instar *C. comanche* larvae (1^{st} vs. 2^{nd}: $W = 25.5$, $p < 0.01$; 1^{st} vs. 3^{rd}: $W = 26.5$, $p < 0.01$). First instar *C. comanche* larvae consumed 2.67±0.24 (mean ±SE) individual ACP per trial across all ACP life stages, second instars consumed 13.67 ±1.39, and third instars consumed 13.93

±0.96. Second and third instar *C. comanche* did not differ significantly from each other in mean ACP individuals eaten per replicate ($W=461$, $p > 0.1$), but each group ate significantly more ACP per replicate than did first instars. *Diomus pumilio* adults consumed 13.73±1.42 ACP per trial.

To avoid biasing life stage preference results by the number of encounters with each prey stage, feeding preferences were analyzed as ratios of feeding events to encounters for each predator/ACP life stage combination (Royer et al., 2008). Attacks and feeding following encounters did not differ significantly between second and third instar *C. comanche* larvae for any ACP life stage. However, both second and third instar *C. comanche* were significantly more likely than first instar larvae to both attack and consume ACP after an encounter for every ACP life stage ($p < 0.001$ for all comparisons).

All three stages of *C. comanche* larvae displayed the same pattern of predation preferences. ACP eggs were consumed at the highest rates following attacks. Eggs were significantly more likely to be consumed following an attack than first, second, or fifth instar ACP nymphs by first instar *C. comanche* larvae (ACP eggs vs. first instar ACP nymphs: $z=3.2$, $p < 0.05$; vs. second instar nymphs: $z=3.3$, $p < 0.05$; vs. fifth instar nymphs, $z=3.17$, $p < 0.05$). The same pattern of preferences was displayed by second instar *C. comanche* larvae (ACP eggs vs. first instar ACP nymphs: $z=3.5$, $p < 0.01$; vs. second instar nymphs: $z=3.6$, $p < 0.01$; vs. fifth instar nymphs, $z=3.46$, $p < 0.01$) and by third instar *C. comanche* larvae (ACP eggs vs. first instar nymphs: $z=3.5$, $p < 0.01$; vs. second

instar nymphs: $z=3.5$, $p < 0.01$; vs. fifth instar nymphs, $z=3.4$, $p < 0.05$). Eggs were marginally more likely than third instar ACP nymphs to be eaten after an attack by second instar *C. comanche* larvae ($z=2.86$, $p=0.06$) and by third instar *C. comanche* larvae ($z=2.8$, $p=0.07$). 100% of eggs attacked by a second instar *C. comanche* larva were eaten. *C. comanche* larvae displayed no significant preference between any of the five ACP nymphal stages: rates of feeding following an attack were not significantly different for all comparisons across all ACP nymphal stages. Finally, all three larval instars of *C. comanche* were significantly less likely to feed on adults than to feed on any other life stage ($p <0.001$ for all comparisons).

Diomus pumilio feeding following encounters or attacks did not differ between the sexes for any ACP life stage ($z<1.5$ and $p> 0.05$ for all comparisons), so observations from both sexes were combined into one dataset for analysis. The beetles showed a distinct preference for younger ACP life stages, with 93% of attacks on ACP eggs leading to a feeding event. The percent of attacks that were followed by a feeding event decreased for each successive ACP life stage, with a major drop-off between third instar nymphs (49% of attacks leading to feeding) and fourth instar nymphs (6% of attacks leading to feeding). The percent of encounters that were followed by a feeding event generally decreased with life stage as well, with a drop-off between third instar (34%) and fourth instar nymphs (4%), but the percent of encounters followed by a feeding event on first instar nymphs (75%) was higher than for eggs (58%).

Pairwise comparisons confirmed the statistical significance of these observations. All four younger ACP life stages were significantly more likely to be fed upon following an attack by *D. pumilio* than were the three older ACP stages ($z>6$, $p<0.001$ for all comparisons). Frequency of feeding by *D. pumilio* following an attack did not differ significantly between the four youngest ACP life stages ($z<2$, $p>0.05$ for all comparisons). Fourth instar ACP nymphs were significantly more likely to be fed upon following an attack than were ACP adults ($z=3.1$, $p<0.05$), while frequency of feeding following an attack did not differ significantly between fifth instar nymphs and fourth instar nymphs, or between fifth instar nymphs and adults.

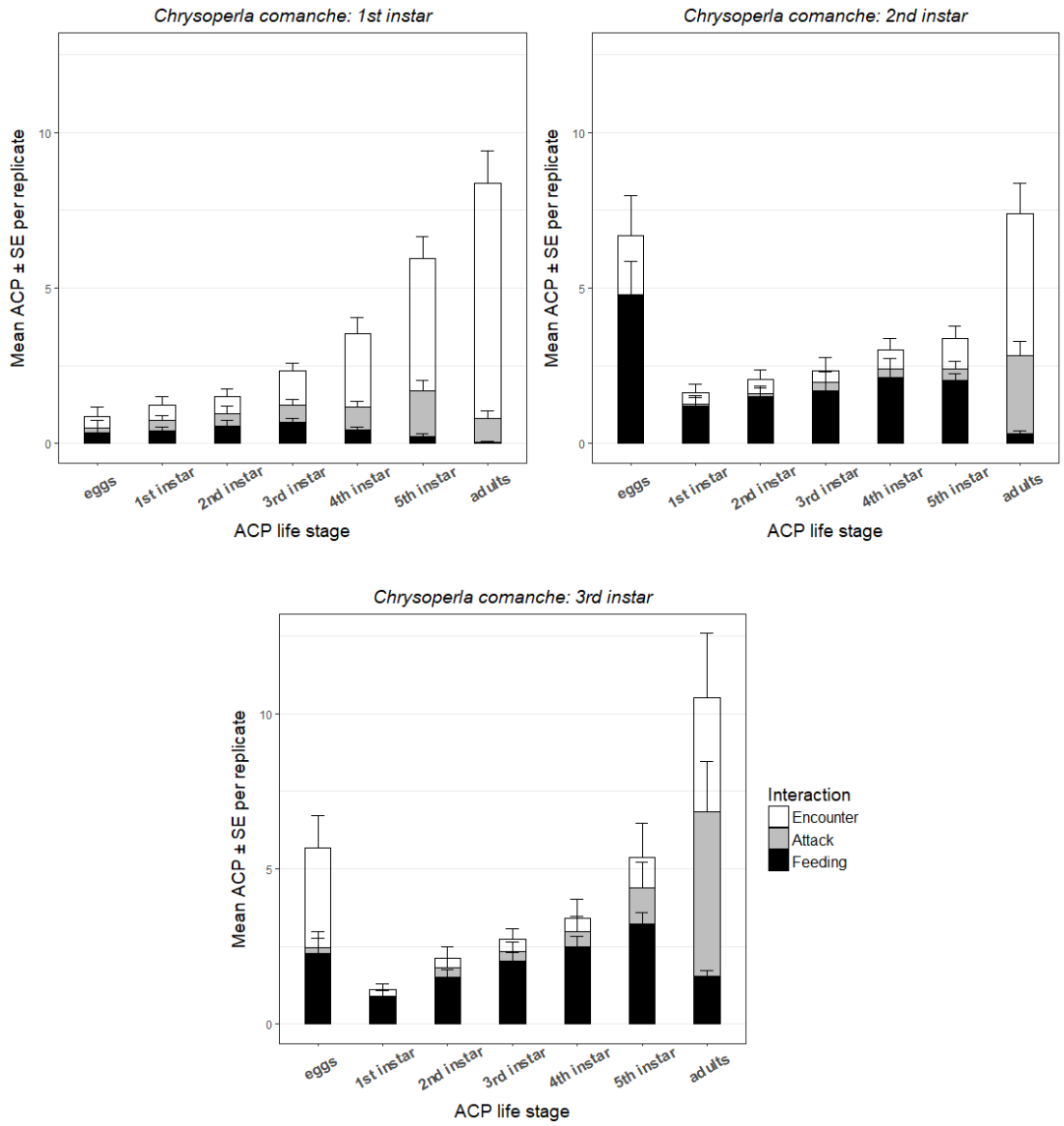

Figure 5.1: *Chrysoperla comanche* interactions with ACP by instar
Mean ± SE encounters (white bars), attacks (grey bars), and feeding (black bars) on each life stage of ACP by *Chrysoperla comanch*e larvae. An encounter is defined as contact between predator and prey for any body part, an attack is defined as a predator probing prey with mouthparts, and feeding is defined as partial or complete consumption of the prey. Bars are superimposed (not stacked) because feeding events are nested in attacks, which are nested in encounters. The top of each white, grey, and black bar represents the mean number of events for that predator/prey stage combination.

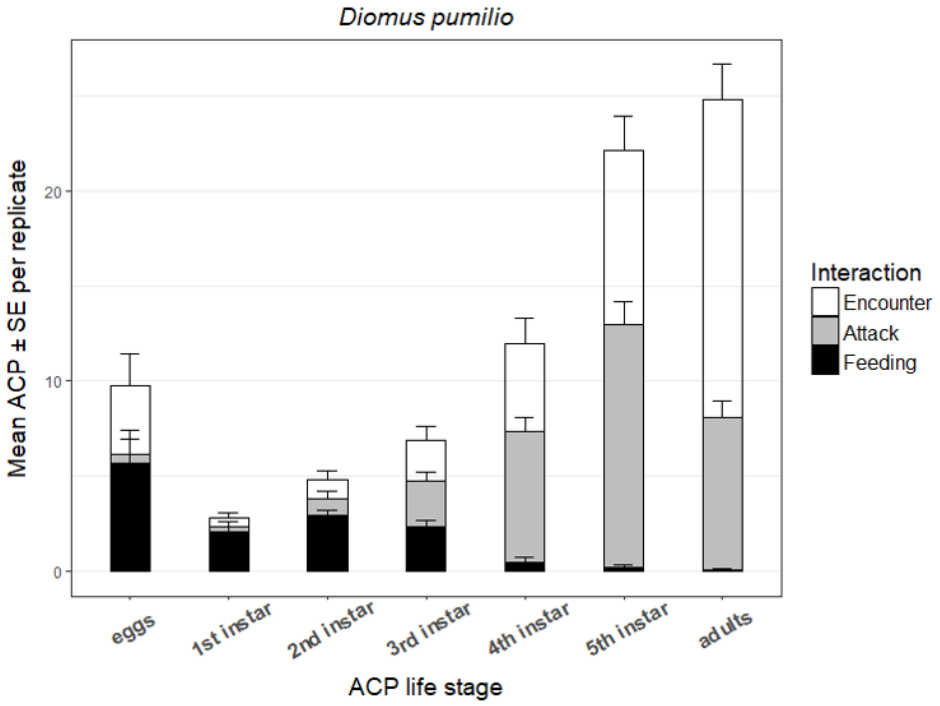

Figure 5.2: *Diomus pumilio* interactions with ACP by instar
Mean ± SE encounters (white bars), attacks (grey bars), and feeding (black bars) on each life stage of ACP by adult *Diomus pumilio*. An encounter is defined as contact between predator and prey for any body part, an attack is defined as a predator probing prey with mouthparts, and feeding is defined as partial or complete consumption of the prey. Bars are superimposed (not stacked) because feeding events are nested in attacks, which are nested in encounters. The top of each white, grey, and black bar represents the mean number of events for that predator/prey stage combination.

Digestion time analysis

All *D. pumilio* tested positive for ACP DNA at 0 hr and 1 hr post-feeding. Over half tested negative at 2 hr, one out of twelve tested positive at 3 hr, two out of twelve tested positive at 4 hr, and zero out of six tested positive at 6 hr after feeding. Amplification of DNA at low levels is subject to stochastic events, which may explain why two tested positive at 4 hr post-feeding while only one tested

positive at 3 hr post-feeding. The DT_{50} for *D. pumilio* was predicted by probit analysis to be 1.9 hr (1 hr 54 min) (Fig 5.3).

Twenty-three out of twenty-five *C. comanche* (92%) tested positive for ACP DNA immediately after feeding. This proportion dropped to 35% after 1 hr, 15% after 2 hr, and 5% after 3 hr. Probit analysis predicted a DT_{50} of 0.33 hr, (~20 min) for *C. comanche*.

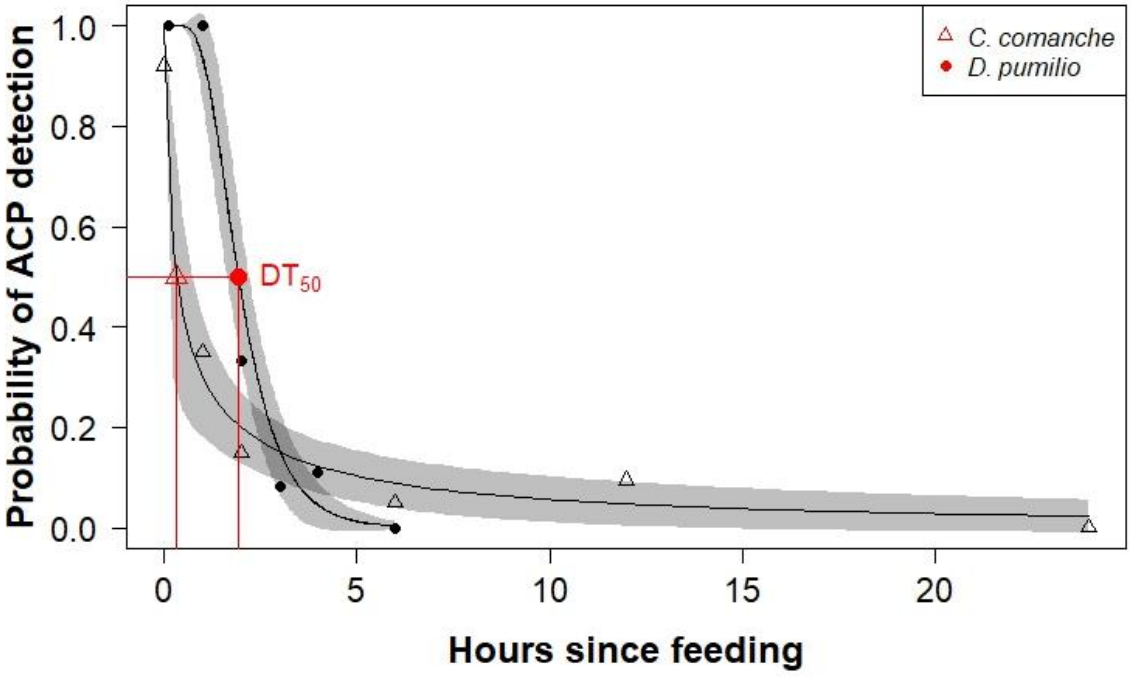

Figure 5.3: ACP predation detectability results for *C. comanche* and *D. pumilio*
Figure shows detection probability curves predicted by probit regression analysis. Gray regions depict 84% confidence intervals (Payton et al., 2003). Detectability half-lives (DT_{50}) are marked for both species. DT_{50} = 0.33 hr for *C. comanche* and 1.9 hr for *D. pumilio.*

Discussion

Prey choice

The results of this prey choice experiment showed that ACP life stage preference is non-random for both predator species. Eggs were a preferred prey

of *C. comanche,* as they were more likely to be eaten following an attack than ACP adults or first, second, or fifth instar nymphs for all three *C. comanche* instars. We observed that second instar *C. comanche* ate 100% of eggs following an attack. It is worth considering that the lacewing larvae used in this experiment were reared on *E. kuehniella* eggs, which could be hypothesized to influence a preference for insect eggs as prey; however, first instar *C. comanche* larvae displayed the same preferences despite being fed nothing between hatching and the prey choice experiment. Adults were the least preferred prey stage for all three larval instars, but third instar *C. comanche* larvae attacked over 60% of adults they encountered, and ate 20% of the adults they attacked. We did not record ACP escape attempts; however, we observed that adult ACP more easily fled predators than did immature ACP. ACP of all life stages did not appear to avoid or escape predators before they were attacked.

Partial consumption of prey was rare for all three *C. comanche* larval stages. Larvae rarely abandoned a prey item before completely draining it of fluid, and often released and re-attacked a drained prey item several times before attacking fresh prey. Cohen (1995) noted that extra-oral digestion in fluid-feeders such as larval Chrysopidae influences the likelihood that predators will completely consume a prey item because extra-oral digestive enzymes are an “indispensible resource” that “must be recovered for further use in the gut if the predator is to exploit their full value.”

Diomus pumilio preferentially preyed upon the four youngest stages of ACP, with feeding/attack ratios declining from 93% for eggs to 49% for third instar nymphs. All three of the oldest ACP stages were less preferred, with feeding/attack ratios declining from 6% for fourth instar nymphs to 1% for adults. Only two *D. pumilio* out of thirty replicates preyed on an ACP adult. It appeared during 30 hr of observation that *D. pumilio* usually could not pierce the integument of fourth instar and older ACP to feed. However, we observed while rearing these beetles that *D. pumilio* larvae, which are fluid feeders, prey on a wider range of ACP life stages than adult beetles, which are chewing predators. We observed that newly eclosed first instar *D. pumilio* larvae were able to feed on ACP eggs and nymphs up to the third instar. Older *D. pumilio* larvae were able to consume eggs and nymphs of any stage. *Diomus pumilio* often accomplished predation on larger nymphs by piercing and then sucking out a nymph's body contents through its leg or antenna. *Diomus pumilio* larvae have been observed feeding on acacia psyllid in the same fashion (Dreistadt and Hagen, 1994).

Formal prey choice observations for the four larval instars of *D. pumilio* would be a valuable addition to this research. Such work would be aided by development or recovery of mass rearing protocols for *D. pumilio*, such as those used to rear and release over 7,000 *D. pumilio* against acacia psyllid during 1972-1975 (Dreistadt and Hagen, 1994). Because no alternate prey species for *D. pumilio* were discovered during this project, each laboratory-reared beetle

required daily hand-feeding of live ACP, an intensive use of both labor and insectary plants. Meyerdirk (1983) reported raising *Diomus flavifrons* (a chimeric synonym based on a junior synonym, *Scymnus flavifrons*) on citrus mealybug eggs; however, the *Diomus* population used in the study may not have been *pumilio*. Voucher specimens were collected from the experimental beetles' area of origin in Loxton, Australia the year after the study was conducted and were deposited in the British Museum. These were examined and tagged as *Diomus* sp. near *pumilio*, but are separated from *D. pumilio* by the head texture, which is described as shining in *D. pumilio* and microreticulate in the specimens submitted by Meyerdirk (Booth, 2016, personal communication) .

Digestion time analysis

We calculated a DT_{50} for *D. pumilio* of 1.9 hr, a period over five times as long as *C. comanche*'s DT_{50} of 0.33 hr. This difference in detectability half-lives indicates that ACP predation detection rates in field-collected predators are biased in favor of *D. pumilio.* In the previous chapter, we reported positive ACP detections in 42.9% of *C. comanche* and 17.5% of *D. pumilio* specimens. Because *C. comanche* field specimens tested positive far more often than *D. pumilio* field specimens, the ranking of these two predators by frequency of ACP detection is supported, not changed, by these DT_{50}s. The predator ranking remains the same as determined by raw detection data, because the predator

with a higher field detection rate also had a shorter detectability half-life in our assay.

Although 0.33 hr and 1.9 hr are among the shortest detectability half-lives reported in the literature for gut content analyses, the difference in magnitude between two predator species tested with the same prey molecule is not unprecedented (Greenstone et al., 2014; Greenstone et al., 2010). Two previous studies which compared detectability half-lives of *Chrysoperla* sp. with coccinellid species found a shorter DT_{50} for *Chrysoperla* using DNA-based methods (Chen et al., 2000; Fournier et al., 2008). (Interestingly, Fournier et al. (2006) found the opposite time-detectability relationship for *Chrysoperla carnea* and *Harmonia axyridis* using an ELISA assay.) At 88 bp, the sequence we used to detect predation is shorter than almost all the others that have been used in DNA-based gut analyses (Greenstone et al., 2014). Common sense indicates that a shorter sequence would remain detectable longer; however, meta-analysis did not confirm this relationship (Greenstone et al., 2014).

Morris and Campos (2006), using ELISA-based digestion time analysis, reported that prey protein titer was significantly higher for a longer period of time in *Chrysoperla carnea* larvae that were fed a “chaser diet” after consuming the target prey than larvae which were starved after they consumed the target prey. Taking this into consideration, the 0.33 hr DT_{50} we calculated in laboratory conditions may be shorter than the DT_{50} of free-living field specimens. It is unlikely that a *C. comanche* larva would fail to feed in the 24 hr prior to eating

ACP, or that it would cease feeding after eating no more than a single second instar nymph.

It is not unusual for a portion of *Chrysoperla* sp. to test negative at t=0 hr in digestion time analysis experiments (Chen et al., 2000; De León et al., 2006), as occurred in this study. *Chrysoperla* larvae's fluid-feeding predation habit may contribute to this pattern, since a high level of pre-oral digestion may decrease detection rates (Sunderland et al., 2005, p. 370). Future studies utilizing molecular gut content analyses of *Chrysoperla* spp. or other fluid-feeders would likely benefit from incorporating a chaser diet, thereby bringing satiation, and likely the prey detectability interval, closer to levels found in the field. However, this would be difficult to standardize between multiple species when an alternate prey species is not available for all species being tested, or when predators being tested cannot all be fed the same alternate prey species.

In this study, ranking the two predator species by frequency of predation was uncomplicated. However, in cases where multiple species are involved, it is necessary to weight the results obtained from field specimens with the values obtained from digestion time analysis. There is not yet a widely settled upon mathematical procedure for adjusting field predation rates according to DT_{50} values (Gagnon et al., 2011). Two methods have been applied in the literature. First, it has been suggested that the ratios of DT_{50}s can be used as multipliers for field rates to obtain adjusted values (Chen et al., 2000; Gagnon et al., 2011; Greenstone et al., 2014). This calculation is simple but might be misleading in

our case. For example, multiplying *C. comanche*'s 42.9% detection rate by 1.9 hr/0.33 hr would yield an adjusted rate greater than 100%. Using the values obtained in this study, *C. comanche* would have an adjusted predation rate fourteen times higher than *D. pumilio*'s (42.9% positive x 1.9/0.33 = 247% for *C. comanche* vs. 17.5% for *D. pumilio*). However, *C. comanche*'s DT_{50} may have been artificially low in this study due to preoral digestion and the lack of chaser prey. Chelex DNA extractions of Chrysopidae did not reliably amplify PCR product, so the Qiagen DNeasy® Blood & Tissue kit was used for all molecular work performed with *C. comanche* specimens reported in this dissertation.

The rate of ACP predation detection for field-collected *Diomus pumilio* reported in Chapter 4 might have been artificially low due to the use of Chelex extractions for those specimens. The Chelex protocol was chosen for use in molecular gut content analysis of field-collected coccinellid specimens because it appeared to be quite sensitive for detecting ACP eaten by *Hippodamia convergens* in preliminary experiments. However, Chelex extractions of ACP-fed *D. pumilio* in preliminary trials for this study amplified unreliably, with only 7 out of 23 specimens (30.4%) testing positive at t=0 hrs. This detection rate was insufficient to construct a DT_{50} curve: probit analysis generated a negative DT_{50} value based on the detection data. We then switched to use of the Qiagen DNeasy® Blood & Tissue kit for *D. pumilio* DNA extraction. This method was far more reliable, with 100% of 32 DNeasy kit-extracted specimens testing positive at t=0 hrs. To utilize the results of the laboratory DT_{50} trials to adjust rates of ACP

prey detection obtained from field-collected *D. pumilio*, we calculated a correction factor for DNA extraction method. Using the 30.4% detection rate for the 23 ACP-fed, Chelex-extracted specimens analyzed in preliminary experiments, we calculated a correction factor for Chelex extraction of 1 + (100%-30.4%) = 1.696. Adjusting the 17.5% overall field predation rate of Chelex-extracted *D. pumilio* with this correction factor gives 0.175 x 1.696 = 0.296, or 29.6%. This correction factor adjusts the pre-dawn detection rate of 7.4% to 12.6%, and adjusts the late afternoon rate of 32.2% to 54.6%, bringing *D. pumilio*'s diurnal predation rate as high as the 54% positive detection rate of 2nd-3rd instar *C. comanche*, the highest found in any group of field-collected predators.

With these corrected values, the method of Chen et al. (2000) for adjusting field predation detection rates predicts that *C. comanche's* DT_{50} -adjusted predation rate is 8.3 times as high as *D. pumilio*'s (42.9% positive x 1.9/0.33 = 247% for *C. comanche* vs. 29.6% for *D. pumilio*). These adjusted rates should be considered with caution, as we cannot assume that field-collected *D. pumilio*, if extracted with DNeasy kits, would have tested positive precisely 1.696 times more often than the Chelex-extracted specimens did.

A second method, used by Greenstone et al. (2010) and Szendrei et al. (2010), is a two-step calculation which produces adjusted predation rates that, although they do not have ecological significance, can be used to create a ranking for predator groups which accounts for both field predation rates and DT_{50}. In this procedure, each species/life stage's field detection rate is fed back

into its probit model to calculate a theoretical time since feeding. This value is then fed into the probit equation for a selected index species to solve for percent of specimens positive. Although not representative of field predation rates, the resulting values can be arranged to form a reasonable ranking of predation frequency.

Secondary predation and scavenging may cause spurious positives in field specimens (Foltan et al., 2005; Harper et al., 2005; Hosseini et al., 2008; Juen and Traugott, 2005; Sheppard et al., 2005), and long detectability intervals for primary predators worsen the problem (Sheppard et al., 2005). Several methods can be used to address this uncertainty. A proposed approach for identifying likely cases of secondary predation is to carry out multiplex PCR which tests for the DNA of other predators as well as for DNA of the target prey (Harper et al., 2005; Sheppard et al., 2005). Protein analyses of predator gut contents can also address this question by targeting and distinguishing between specific protein markers of both live and dead prey (Mansfield and Hagler, 2016). Laboratory feeding assays to determine predation habits can help distinguish between primary predators, secondary predators, and scavengers, because species-specific molecules originating from dead prey can be as easily detectable as those from living prey (Juen and Traugott, 2005). In this case, our prey choice experiments established that both *C. comanche* and *D. pumilio* readily feed on living ACP.

Conclusion

These experiments have determined detectability half-lives and described ACP life stage preferences for *C. comanche* and *D. pumilio*, two newly identified predators of ACP established in southern California. The short detectability half-lives, high rates of field predation detection, and ability of multiple stages of each predator to feed on multiple stages of prey are promising with respect to both predator species. We have established that exposure to resident predatory arthropods reduces ACP survival in the field in southern California (Chapter 2), that *C. comanche* and *D. pumilio* are abundant within the same location (Chapter 3), that field-collected specimens frequently test positive for ACP predation (Chapter 4), and, in this chapter, that the predator readily consumes ACP in laboratory observations. The most pressing question to address before incorporating these species into a biological control program or integrated pest management plan is whether an increased population density of either or both of these predators can reduce the population density of ACP in the field. I recommend testing the ability of both *C. comanche* and *D. pumilio* to reduce ACP on a field scale, with the primary step of reviving or establishing mass rearing protocols for each species. Subsequent decisions about whether to use these species in biological control programs should take into account epidemiological recommendations that predict the monetary value of reducing ACP populations by specified target levels (Monzo and Stansly, 2017) to enable comparisons

between the cost of a predator program and the benefits of the predicted reduction in ACP population.

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Chapter 6:

Conclusions

Following initial observations that ACP was subject to substantial natural mortality in southern California, we undertook to quantify the reduction in ACP population levels caused by predatory arthropods and to identify species causing this mortality.

The work presented in Chapter 1 established by means of predator exclusion experiments that predatory arthropods significantly diminished populations of ACP in an unsprayed southern California orchard. ACP survival to adulthood in colonies protected by predator exclusion cages was significantly lower than survival to adulthood in uncaged colonies for five of the seven months (March-September) during which enough ACP were present to conduct the study. During the other two months, June and July, there was no significant difference in the ACP survival rates between caged and uncaged colonies. ACP survival to adulthood in completely unprotected colonies was 10.4% overall, with a minimum of < 1% survival in March and a maximum of 24% survival in July. ACP survival in completely protected colonies ranged from 32% in May to 48% in September, or 39.7% overall, 3.8 times as high as in unprotected cohorts. Within individual months, survival in closed predator exclusion cages ranged from 1.5 times as high as in unprotected cohorts in June, to 51.7 times as high in March. These findings strongly indicate the importance of predators as natural enemies of ACP in southern California and suggest their potential usefulness in improved biological control of this pest.

To begin identifying the predators responsible for this reduction, I conducted a year-long foliar survey by powered vacuum collection in two orchards, with collections taken before dawn and during late afternoon for each sampling date. Analysis of our samples revealed ten predatory insects and spiders represented by at least 90 specimens each. The insects included *Chrysoperla comanche* Banks (Chrysopidae), *Stethorus punctum* LeConte, *Diomus pumilio* Weise, *Cryptolaemus montrouzieri* Mulsant, and *Rhyzobius lophanthae* Blaisdell (Coccinellidae). Spiders included *Sassacus vitis* Cockerell (Salticidae), *Anyphaena pacifica* Banks (Anyphaenidae), *Hibana incursa* Chamberlin (Anyphaenidae), *Trachelas pacificus* Chamberlin & Ivie (Trachelidae), and *Theridion* spp., a mixture of *T. submissum* and *T. dilutum* (Theridiidae). 90% of spiders collected were juveniles. This survey provided the first record of *D. pumilio* on a non-acacia host plant.

Mean predator count per sample was positively correlated with mean ant count per sample, but mean adult ACP count per sample was not. Predatory insects and adult ACP were captured in higher numbers during afternoon collections, while more Argentine ants were collected before dawn. The spiders classified as nocturnal were more commonly collected during pre-dawn sampling than during the afternoon, while spiders classified as diurnal were equally likely to be caught during either collection period. This validated our choice to collect a nocturnal sample as well as a diurnal sample. The justification for doing so had been to ensure that the abundance of nocturnally active species was properly

monitored, and to collect night-feeding specimens with fresh gut contents for later analysis.

To determine which of the ten most abundant predatory insects and spiders frequently ate ACP, I designed, with colleagues, a DNA-based assay to test for ACP remains in total DNA extractions of field-collected predator specimens. This test amplifies an 88 base pair sequence of ACP's ribosomal ITS-II gene with qPCR. The test returned >10% positive results for *C. comanche, D. pumilio,* and *R. lophanthae* and all five spiders, while only one out of 100 *C. montrouzieri* and one out of 273 *S. punctum* (including larvae and adults for both species) tested positive. Within species that tested positive for ACP predation, second/third instar *C. comanche* tested positive three times as often as first instar *C. comanche* (54% vs. 18%), while afternoon-collected *D. pumilio* tested positive more than three times as often as pre-dawn specimens (24% vs 7%). No other differences in detection rates were observed between life stages, time of day, or study sites. These findings indicate that *C. comanche*, *D. pumilio*, and perhaps *R. lophanthae* have potential as augmentative or conservation biological control agents of ACP. Further study of the spider community in southern California citrus may reveal its potential to reduce ACP levels through conservation biological control management practices.

We selected *C. comanche* and *D. pumilio* for further study because they were the two insect predators of ACP at our study sites with the highest rates of positive predation tests on ACP. We conducted prey life stage choice

experiments to determine which ACP life stages they prey upon, and used the results of this test to select an appropriate life stage (second instar nymphs) which could be fed to both species and reliably consumed for digestion time analysis. Digestion time analysis allowed us to compare prey detection results in field-collected specimens because digestion rates vary between species, with prey becoming undetectable more quickly in some species than others.

Prey choice tests showed that both species readily feed on living ACP. *Chrysoperla comanche* larvae preferentially feed on ACP eggs, with lesser preferences for most nymphal stages. Adults were the least preferred prey life stage. Second and third instar *C. comanche* consumed significantly more ACP individuals of every life stage than did first instars. *Diomus pumilio* adults preferentially feed on ACP eggs through third instar nymphs. Rearing observations indicated that *D. pumilio* larvae feed on a wider range of ACP life stages than do adults, and that they can be reared to maturity on a diet of immature ACP. Digestion time analysis determined a detectability half-life (DT_{50}) for the predation assay DNA sequence of 1.9 hr for *D. pumilio* and 0.33 hr for *C. comanche.* Use of these values to adjust field predation rates suggests that *C. comanche* larvae prey on ACP twelve times more often than do adult *D. pumilio.* When *D. pumilio* field predation rates are corrected for DNA extraction method as described in Chapter 5, DT_{50}-adjusted predation frequency suggests that *C. comanche* preys on ACP 8.3 times as often as does *D. pumilio.* However, *D. pumilio* should be given due consideration as a natural enemy for ACP because

it preys on ACP during its entire lifespan, whereas *C. comanche* is only predatory during larval development.

We recommend field testing with both *C. comanche* and *D. pumilio* to determine whether increasing their populations reduces ACP levels measurably and to a degree that supports management goals. Management plans should account for economic benefits from reductions in ACP levels as well as per-unit cost of mass-reared natural enemies. Predatory insects used in augmentation biological control could be applied either for early-season control of ACP at the beginning of leaf flushes, or to supplement the natural enemy community during months when field populations of the most effective predators have declined and ACP survival is high, as was the case during June and July in our study (Chapter 3). Because spiders prey on ACP (Chapter 4), spider conservation may support ACP management as well. As with predatory insects, effective incorporation of spider conservation into management plans requires prior field testing to verify and measure the contribution of spider management to ACP reduction. When compatible, techniques to increase conservation of spiders in citrus orchards would likely involve adding mulch to increase hunting spider habitat, increasing plant structural complexity through secondary plantings, supporting recolonization by maintaining natural vegetation near orchards, and/or minimizing cultivation and insecticide application when possible (Maloney et al., 2003).

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Appendix

Supplementary table 1

Non-coccinellid species proposed as predators of ACP in literature records.

Order or Subclass	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Acari	Anystidae	*Anystis agilis*	USA (California)	(Kistner et al., 2016)	no	yes
Acari	Phytoseiidae	*Amblyseius swirskii*	laboratory only	(Juan-Blasco et al., 2012)	yes	no
Acari	Phytoseiidae	*Neoseiulus barkeri*	Widespread/commercially available	(Fang et al., 2013)	yes	no
Acari	Phytoseiidae	*Neoseiulus cucumeris*	Widespread/commercially available	(Fang et al., 2013)	yes	yes
Araneae	Anaphaenidae	*Hibana velox*	USA (Florida)	(Michaud, 2002)	yes	yes
Araneae	Anaphaenidae	*Hibana velox*	USA (Florida)	(Michaud, 2004)	no	yes
Araneae	Anyphaenidae	*Anyphaena pacifica*	USA (California)	(Kistner et al., 2016)	no	yes
Araneae	Gnaphosidae	*Zelotes* sp.	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Araneae	Miturgidae	*Cheiracanthium inclusum*	USA (Florida)	(Michaud, 2002)	no	yes
Araneae	Miturgidae	*Cheiracanthium insulanum*	Pakistan (Faisalaband)	(Vetter et al., 2013)	no	no
Araneae	Miturgidae	*Cheirachanthium* sp.	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Araneae	Oxyopidae	*Oxyopes* sp.	USA (Florida)	(Michaud, 2002)	no	yes
Araneae	Salticidae	*Hentzia palmorum*	USA (Florida)	(Michaud, 2002)	yes	yes
Araneae	Salticidae	*Marpissa tigrina*	India	(Sadana, 1991)	yes	yes
Blattodea	Ectobiidae	*Blattella asahinai*	USA (Florida)	(Qureshi and Stansly, 2009)	no	no
Coleoptera	Carabidae	*Egaploa crenulata*	Saudi Arabia	(Al-Ghamdi, 2000)	no	no
Coleoptera	**Coccinellidae**	**See Table 1B**				
Coleoptera	Histeridae	*Saprinus chalcites*	Saudi Arabia	(Al-Ghamdi, 2000)	no	no
Diptera	Syrphidae	*Allobaccha sapphirina*	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Diptera	Syrphidae	*Allograpta exotica*	USA (California)	(Kistner et al., 2016)	no	yes
Diptera	Syrphidae	*Allograpta obliqua*	USA (California)	(Kistner et al., 2016)	no	yes
Diptera	Syrphidae	*Allograpta obliqua*	USA (Florida)	(Michaud, 2002)	yes	yes
Diptera	Syrphidae	*Allograpta obliqua*	USA (Florida)	(Michaud, 2004)	no	no
Diptera	Syrphidae	*Allograpta* sp.	USA (Florida)	(Hall et al., 2008)	no	no
Diptera	Syrphidae	*Allograpta* sp.	Nepal	(Aubert, 1987)	no	no
Diptera	Syrphidae	*Allograpta* spp.	Reunion Island	(Aubert, 1987)	no	no
Diptera	Syrphidae	*Ocyptamus fuscipennis*	USA (Florida)	(Michaud, 2004)	no	no
Diptera	Syrphidae	*Ocyptamus* sp.	Cuba	(González et al., 2000)	yes	no
Diptera	Syrphidae	*Ocyptamus* sp.	Cuba	(González et al., 2002)	no	yes
Diptera	Syrphidae	*Ocyptamus* sp.	Cuba	(Rodrıguez-Toledo et al., 2008)	no	yes

Order or Subclass	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Diptera	Syrphidae	*Pseudodorus clavatus*	USA (Florida)	(Michaud, 2002)	yes	no
Hemiptera	Reduviidae	*Zelus longipes*	USA (Florida)	(Hall et al., 2008)	no	no
Hemiptera	Reduviidae	*Zelus longipes*	Mexico (Michoacán)	(Miranda-Salcedo and López-Arroyo, 2010)	no	no
Hymenoptera	Formicidae	*Dorymyrmex bureni*	USA (Florida)	(Michaud, 2002)	no	yes
Hymenoptera	Formicidae	*Dorymyrmex bureni*	USA (Florida)	(Michaud, 2004)	no	yes
Hymenoptera	Formicidae	*Pseudomyrmex gracilis*	USA (Florida)	(Michaud, 2002)	no	yes
Hymenoptera	Formicidae	*Pseudomyrmex gracilis*	USA (Florida)	(Michaud, 2004)	no	yes
Hymenoptera	Formicidae	*Pseudomyrmex* spp.	USA (Florida)	(Monzo et al., 2014)	no	yes
Hymenoptera	Vespidae	*Brachygastra mellifica*	Mexico	(Reyes-Rosas et al., 2013; Reyes-Rosas et al., 2011)	no	yes
Neuroptera	Chrysopidae	*Ceraeochrysa claveri*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Neuroptera	Chrysopidae	*Ceraeochrysa cubana*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Neuroptera	Chrysopidae	*Ceraeochrysa everes*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Neuroptera	Chrysopidae	*Ceraeochrysa* near *cincta*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Neuroptera	Chrysopidae	*Ceraeochrysa* sp.	USA (Florida)	(Michaud, 2001)	no	yes
Neuroptera	Chrysopidae	*Ceraeochrysa* sp.	USA (Florida)	(Michaud, 2004)	no	yes
Neuroptera	Chrysopidae	*Ceraeochrysa* spp.	USA (Florida)	(Michaud, 2002)	yes	yes
Neuroptera	Chrysopidae	*Ceraeochrysa* spp.	Mexico (Nayarit)	(Rodríguez-Palomera et al., 2012)	no	yes
Neuroptera	Chrysopidae	*Ceraeochrysa valida*	Mexico (Veracruz)	(Jose-Pablo et al., 2017)	yes	no
Neuroptera	Chrysopidae	*Ceraeochrysa valida*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Neuroptera	Chrysopidae	*Ceraochrysa* sp.	USA (Florida)	(Qureshi and Stansly, 2008)	no	no
Neuroptera	Chrysopidae	*Chrysopa* sp.	Cuba	(González et al., 2002)	no	yes
Neuroptera	Chrysopidae	*Chrysopa* sp.	India	(Husain and Nath, 1927)	no	yes
Neuroptera	Chrysopidae	*Chrysopa* spp.	Cuba	(Rodrıguez-Toledo et al., 2008)	no	yes
Neuroptera	Chrysopidae	*Chrysopa* spp.	USA (Florida)	(Hall et al., 2008)	no	no
Neuroptera	Chrysopidae	*Chrysopa vulgaris*	Saudi Arabia	(Al-Ghamdi, 2000)	no	no
Neuroptera	Chrysopidae	*Chrysoperla carnea*	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Neuroptera	Chrysopidae	*Chrysoperla comanche*	USA (California)	(Kistner et al., 2016)	no	yes
Neuroptera	Chrysopidae	*Chrysoperla rufilabris*	USA (Florida)	(Michaud, 2002)	yes	yes
Neuroptera	Chrysopidae	*Chrysoperla rufilabris*	Mexico (Michoacán)	(Miranda-Salcedo and López-Arroyo, 2010)	no	no
Neuroptera	Chrysopidae	*Chrysoperla rufilabris*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no

Order or Subclass	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Neuroptera	Chrysopidae	*Chrysoperla* sp.	Cuba	(González et al., 2000)	no	no
Neuroptera	Chrysopidae	*Chrysoperla* sp.	USA (Florida)	(Qureshi and Stansly, 2008)	no	no
Neuroptera	Chrysopidae	*Chrysoperla* spp.	USA (Florida)	(Michaud, 2004)	no	yes
Neuroptera	Chrysopidae	*Chrysoperla* spp.	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Neuroptera	Chrysopidae	*Mallada boninensis*	Taiwan	(Lin et al., 1973)	unknown	unknown
Neuroptera	Hemerobiidae	*Micromus posticus*	USA (Florida)	(Michaud, 2002)	no	yes
Odonata	Gomphidae	*Erpetogomphus sabaleticus*	Colombia	(Kondo et al., 2015)	no	yes
Thysanoptera	Thripidae	*Frankliniella occidentalis*	USA (California)	(Kistner et al., 2016)	no	no
Thysanoptera	Thripidae	*Scolothrips sexmaculatus*	USA (California)	(Kistner et al., 2016)	no	no

Supplementary table 2

Coccinellid species proposed as predators of ACP in literature records.

Order	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Coleoptera	Coccinellidae	*Adalia bipunctata*	Pakistan (Sarghoda)	(Khan et al., 2014)	no	no
Coleoptera	Coccinellidae	*Adalia bipunctata*	Widespread/commercially available	(Khan et al., 2016)	yes	no
Coleoptera	Coccinellidae	*Anegleis (Verania) cardoni*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Anegleis cardoni*	Pakistan (Sarghoda)	(Khan et al., 2014)	no	no
Coleoptera	Coccinellidae	*Arawana sp.*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Coleoptera	Coccinellidae	*Azya orbigera*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Coleoptera	Coccinellidae	*Brachiacantha dentipes*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Brumus suturalis*	India	(Husain and Nath, 1927)	no	yes
Coleoptera	Coccinellidae	*Brumus suturalis*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Brumus suturalis*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Cheilomenes sexmaculata*	India	(Husain and Nath, 1927)	no	yes
Coleoptera	Coccinellidae	*Cheilomenes sexmaculata*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Cheilomenes sexmaculata*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Cheilomenes sexmaculata*	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Coleoptera	Coccinellidae	*Cheilomenes sexmaculata*	Pakistan (Sarghoda)	(Khan et al., 2014)	no	no
Coleoptera	Coccinellidae	*Cheilomenes sexmaculata*	Taiwan	(Lin et al., 1973)	unknown	unknown
Coleoptera	Coccinellidae	*Chilocorus cacti*	Cuba	(González et al., 2000)	no	no
Coleoptera	Coccinellidae	*Chilocorus cacti*	Cuba	(González et al., 2002)	no	yes
Coleoptera	Coccinellidae	*Chilocorus cacti*	Cuba	(Rodrıguez-Toledo et al., 2008)	no	yes
Coleoptera	Coccinellidae	*Chilocorus cacti*	Mexico (Nayarit)	(Rodríguez-Palomera et al., 2012)	no	yes
Coleoptera	Coccinellidae	*Chilocorus cacti*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Coleoptera	Coccinellidae	*Chilocorus cacti*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Chilocorus cirumdatus*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Chilocorus nigrita*	India	(Husain and Nath, 1927)	no	yes
Coleoptera	Coccinellidae	*Chilocorus nigritus*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Chilocorus nigritus*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Chilocorus stigma*	Mexico (Veracruz)	(González-Cárdenas et al., 2012)	yes	no
Coleoptera	Coccinellidae	*Chilocorus stigma*	USA (Florida)	(Chong et al., 2010)	no	no
Coleoptera	Coccinellidae	*Cladis nitidula*	Puerto Rico	(Pluke et al., 2005)	yes	no

Order	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Coleoptera	Coccinellidae	*Coccinella repanda*	India	(Husain and Nath, 1927)	no	yes
Coleoptera	Coccinellidae	*Coccinella rependa*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Coccinella septempunctata*	India	(Husain and Nath, 1927)	no	yes
Coleoptera	Coccinellidae	*Coccinella septempunctata*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Coccinella septempunctata*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Coccinella septempunctata*	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Coleoptera	Coccinellidae	*Coccinella septempunctata*	Pakistan (Sarghoda)	(Khan et al., 2014)	no	no
Coleoptera	Coccinellidae	*Coccinella undecimpunctata*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Coelophora inaequalis*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Coelophora inaequalis*	Puerto Rico	(Pluke et al., 2008)	no	no
Coleoptera	Coccinellidae	*Coelophora inequalis*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Coelophora inequalis*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Coelophora saugeti*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Coleomegilla innonata*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Coleomegilla maculata*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Coleomegilla maculata fuscilabris*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Cryptolaemus montrouzieri*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	Mexico (Veracruz)	(González-Cárdenas et al., 2012)	yes	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Chong et al., 2010)	no	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Hall et al., 2008)	no	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Michaud and Olsen, 2004)	yes	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Qureshi and Stansly, 2007)	no	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Qureshi and Stansly, 2008)	no	no
Coleoptera	Coccinellidae	*Curinus coeruleus*	USA (Florida)	(Qureshi and Stansly, 2009)	no	no
Coleoptera	Coccinellidae	*Cycloneda devestita*	Venezuela (Lara)	(Solano et al., 2014)	no	yes
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Cuba	(González et al., 2000)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Cuba	(González et al., 2002)	no	yes

Order	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Cuba	(Rodrıguez-Toledo et al., 2008)	no	yes
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Mexico (Michoacán)	(Miranda-Salcedo and López-Arroyo, 2010)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Mexico (Nayarit)	(Rodríguez-Palomera et al., 2012)	no	yes
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Mexico (Veracruz)	(González-Cárdenas et al., 2012)	yes	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Chong et al., 2010)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Hall et al., 2008)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Michaud, 2001)	no	yes
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Michaud and Olsen, 2004)	yes	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Qureshi and Stansly, 2007)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Qureshi and Stansly, 2008)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea*	USA (Florida)	(Qureshi and Stansly, 2009)	no	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea limbifer*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Cycloneda sanguinea limbifer*	Puerto Rico	(Pluke et al., 2008)	no	no
Coleoptera	Coccinellidae	*Cycloneda* spp.	USA (California)	(Kistner et al., 2017)	no	yes
Coleoptera	Coccinellidae	*Delphastus* sp.	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Coleoptera	Coccinellidae	*Exochomus childreni*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Exochomus childreni*	USA (Florida)	(Michaud and Olsen, 2004)	yes	no
Coleoptera	Coccinellidae	*Exochomus cubensis*	Cuba	(González et al., 2000)	yes	no
Coleoptera	Coccinellidae	*Exochomus cubensis*	Cuba	(González et al., 2002)	no	yes
Coleoptera	Coccinellidae	*Exochomus inequalis*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Exochomus margipennis*	Mexico (Colimas)	(Palomares-Pérez et al., 2015)	yes	yes
Coleoptera	Coccinellidae	*Exochomus nigripennis*	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (California)	(Kistner et al., 2017)	no	yes
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Chong et al., 2010)	no	yes
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Hall et al., 2008)	no	no

Order	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Michaud, 2001)	no	yes
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Michaud and Olsen, 2004)	yes	no
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Qureshi and Stansly, 2007)	no	no
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Qureshi and Stansly, 2008)	no	no
Coleoptera	Coccinellidae	*Harmonia axyridis*	USA (Florida)	(Qureshi and Stansly, 2009)	no	no
Coleoptera	Coccinellidae	*Hippodamia convergens*	Mexico (Michoacán)	(Miranda-Salcedo and López-Arroyo, 2010)	no	no
Coleoptera	Coccinellidae	*Hippodamia convergens*	Mexico (Veracruz)	(González-Cárdenas et al., 2012)	yes	no
Coleoptera	Coccinellidae	*Hippodamia convergens*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Nephus sp.*	Mexico (Nayarit)	(Rodríguez-Palomera et al., 2012)	no	yes
Coleoptera	Coccinellidae	*Olla v-nigrum*	Mexico (Michoacán)	(Miranda-Salcedo and López-Arroyo, 2010)	no	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	Mexico (Nayarit)	(Rodríguez-Palomera et al., 2012)	no	yes
Coleoptera	Coccinellidae	*Olla v-nigrum*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Hall et al., 2008)	no	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Michaud, 2001)	no	yes
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Michaud, 2002)	yes	yes
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Michaud and Olsen, 2004)	yes	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Qureshi and Stansly, 2007)	no	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Qureshi and Stansly, 2008)	no	no
Coleoptera	Coccinellidae	*Olla v-nigrum*	USA (Florida)	(Qureshi and Stansly, 2009)	no	no
Coleoptera	Coccinellidae	*Pentilia sp.*	Mexico (Nayarit)	(Rodríguez-Palomera et al., 2012)	no	yes
Coleoptera	Coccinellidae	*Rodolia cardinalis*	USA (Florida)	(Michaud, 2004)	no	no
Coleoptera	Coccinellidae	*Scymnus levaillanti*	Iran	(Rakhshani and Saeedifar, 2013)	no	no
Coleoptera	Coccinellidae	*Scymnus distinctus*	Cuba	(González et al., 2000)	no	no
Coleoptera	Coccinellidae	*Scymnus distinctus*	Cuba	(González et al., 2002)	no	yes
Coleoptera	Coccinellidae	*Scymnus nubilus*	India (Kanpur)	(Sharga, 1948)	no	yes
Coleoptera	Coccinellidae	*Scymnus spp.*	Brazil	(Gravena et al., 1996)	no	no

Order	Family	Species	Region	Reference	Observed consuming ACP	
					Laboratory	Field
Coleoptera	Coccinellidae	*Scymnus sp.*	Puerto Rico	(Pluke et al., 2005)	yes	no
Coleoptera	Coccinellidae	*Serangium parcesetosum*	India	(Shivankar et al., 2000)	no	no
Coleoptera	Coccinellidae	*Zagloba sp.*	Mexico (Yucatán)	(Contreras and Argumedo, 2012)	no	no

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