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Trophic Interactions Within a Parasitoid Guild (Hymenoptera: Chalcidoidea) From Pakistan in the Context of Asian Citrus Psyllid Classical Biological Control in California

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Dedication

This Master's Thesis is dedicated to my family. Mom and Dad, thank you for encouraging me to pursue my dreams. I could never have come this far without your love, support, and the occasional home-made dinner. Lindsey, thanks for always being there to help me laugh and remind me that sanity is overrated.

This thesis is also dedicated to my amazing husband. You kept me grounded and made sure my day-to-day life didn't fall apart during grad school. You assured me that this was all worth it when I was in doubt, forced me to relax when I was stressed, and reminded me to eat when schedules got busy. This has been an ordeal, and I literally could not have done this without your constant support. I love you, Andrew. Far more than this thesis (but don't tell it I said so).

ABSTRACT OF THE THESIS

Trophic Interactions Within a Parasitoid Guild (Hymenoptera: Chalcidoidea) From Pakistan in the Context of Asian Citrus Psyllid Classical Biological Control in California

by

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Master of Science, Graduate Program in Entomology
University of California, Riverside, June 2015
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Diaphorina citri Kuwayama (ACP) (Hemiptera: Liviidae) is an important agricultural pest of citrus that was recently introduced into California in 2008. The initial response focused on controlling ACP with pesticides, however these efforts were discontinued in favor of a classical biological control program utilizing *Tamarixia radiata* (Waterston) and *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal). As a part of this biological control program, *D. aligarhensis* underwent non-target safety testing at the University of California Riverside. This testing exposed *D. aligarhensis* to seven non-target psyllid species selected based on the following criteria: (1) taxonomic proximity to ACP; (2) likelihood of being encountered by *D. aligarhensis* near release sites; and (3) psyllid species being utilized for biological control of invasive weeds.

Bactericera cockerelli (Šulc), the pestiferous potato psyllid, was the only non-target species which was successfully parasitized by *D. aligarhensis*, and at low levels (< 14%). Based on safety testing results, *D. aligarhensis* was determined to pose no significant impact to native species, and was released as a part of the ACP biological control program in December 2014.

An additional goal of the ACP biocontrol program has been to identify additional species within the associated parasitoid guild from ACP's home range which could also be utilized for the program in California. Prior records estimated this guild to contain approximately nine species of primary parasitoids. Three such species (*Chartocerus* sp., *Pachyneuron crassiculme* [Waterston], and *Psyllaphycus diaphorinae* [Hayat]) were collected from parasitized ACP mummies in Punjab, Pakistan in April 2013, and believed to be a part of this guild. However, based on choice and no choice exposure trials performed in quarantine at UCR, all three species were confirmed to be obligate hyperparasitoids of *D. aligarhensis* and *T. radiata*, and showed no successful parasitism on ACP nymphs. Because the original record for *P. diaphorinae* characterized this species specifically as a primary parasitoid, further studies were performed to describe the reproductive biology and life history of this parasitoid. Results of these studies suggest that *D. aligarhensis* is a superior host (compared to *T. radiata*), based on a higher number of offspring and higher proportion of females. These findings also support the results of previous studies which found that *D. aligarhensis* is subjected to higher rates of hyperparasitism than *T. radiata* in its home range.

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Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) is a phloem-feeding insect native to Asia and the Middle East, where it is a common citrus pest. ACP was first described by Shigeru Kuwayama in 1908 from specimens collected in Japan by Dr. S. Matsumuka on various *Citrus* (Sapindales: Rutaceae) species (Kuwayama 1908).

In 2008, ACP was first recorded in San Diego and Imperial Counties of southern California (USA), with many other finds recorded soon after in 2009 across Los Angeles County, California. ACP is considered a serious citrus pest because it can vector the phloem-limited fastidious bacterium *Candidatus* Liberibacter asiaticus (*C*Las), one of the causative agents of a highly lethal disease known as citrus greening, or huanglongbing (HLB). Trees which contract HLB suffer foliar dieback, reduced fruit yield, premature fruit drop, production of small, hardened fruits which will not ripen evenly, and will eventually die within about 8 years from the time of initial infection (Bové 2006). *C*Las infected trees are reservoirs of the bacterium, from which uninfected psyllids can acquire bacteria while feeding. Psyllids dispersing from *C*Las-infected trees can potentially carry bacteria to uninfected trees, inoculating them when they commence feeding. *C*Las is undetectable in newly-infected trees for at least one year (Pelz-Stelinkski et al. 2010).

While pesticide treatments can help reduce populations of ACP in commercial groves, the urban landscape of California, where ~36% of homeowners possess at least

one citrus tree on their property (Hoddle and Pandey 2014), provides large reservoirs outside of spray zones from which ACP can migrate into commercial groves. In light of this situation, reduction of the vector population (which would in turn reduce the likelihood that an individual psyllid feeding event would transmit *C*Las between trees) through a classical biological control approach is currently the best management option for reducing ACP densities, thereby helping to protect California's US$3 billion per year citrus industry.

There is currently no feasible treatment for HLB within commercial groves aside from removing and destroying any affected trees to reduce the probability of acquisition and spread by ACP. There has been only one confirmed case of HLB in California to date (March 2012 in Hacienda Heights, Los Angeles County) (Kumagai et al. 2013). The effect of the ACP-HLB complex has been devastating in Florida, where the citrus industry has experienced approximately US$3.6 billion in losses (Keremane et al. 2015). Regarding ACP biocontrol and the national citrus industry, HLB is projected to have lasting impacts on citrus economics, restricting the production of citrus to its current level, or potentially slightly less, in models through FY 2032 (Spreen et al. 2014).

The classical biological control program for ACP in California utilizes two well-recognized natural enemies of ACP, both parasitoids that attack nymphs, collected from Punjab, Pakistan between 2010 and 2013. *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), an ectoparasitoid which preferentially targets fourth and fifth instar ACP nymphs, was first released in Riverside, CA in December 2011 (Hoddle and Pandey

2014). To date, over 1 million individuals have been released in residential and commercial areas throughout southern California. This parasitoid has been recovered widely in urban areas over multiple years suggesting that *T. radiata* may have established self-sustaining populations in California. *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal) (Hymenoptera: Encyrtidae), an endoparasitoid which preferentially utilizes second and third instar ACP nymphs, recently passed non-target safety screening and was released in California beginning December 2014 (Bistline-East et al. 2015). There have been no recoveries at release sites as of April 2015. This non-target safety testing research comprises Chapter 1 of this thesis.

A potential benefit of introducing both of these natural enemies into a novel environment is the likely lack of coevolved hyperparasitoids. Several surveys across ACP's home range have revealed a host of hyperparasitoids attacking one or both primary parasitoid species (see Chapter 3 for a review of species). Presence of hyperparasitoids has been shown to reduce primary parasitoid populations (Gomez-Márco et al. 2015), and in its native range, *D. aligarhensis* may suffer hyperparasitism rates of over 40%. In the context of biological control, there are two competing theories on the significance of hyperparasitoids. The first is that hyperparasitoid species have the potential to compromise the biological control system (Schooler 2013, Gomez-Márco 2015). This theory predicts that hyperparasitoids will either drive local populations of primary parasitoids to extinction, or will reduce primary parasitoids to numbers so low that there is a significant decline in efficacy against the pest being targeted for control.

Conversely, the second theory postulates that hyperparasitoids exert a stabilizing force on a host-parasitoid-hyperparasitoid system (Bodlah and Naeem 2013). This stabilization would come about because hyperparasitoids prevent primary parasitoids from driving their host to local extinction, in a similar manner to a predator-prey model. While total pest eradication may be desirable in augmentation biological control setups, an efficacious stable and self-sustaining pest-natural enemy system is the ultimate goal of classical biological control. In the native range, ACP, *T. radiata*, *D. aligarhensis*, and a complex of hyperparasitoids coexist in the citrus agroecosystem. However, stability does not necessarily guarantee that the pest targeted in the system is being subjected to economically effective levels of population suppression. Therefore, it remains advisable to treat hyperparasitoids with caution when designing biological control systems.

Because hyperparasitoids are an integral part of the ACP-parasitoid system in citrus in their home range, and have the potential to diminish the efficacy of ACP biocontrol, the second and third chapters of this thesis provide an examination of the biology of several hyperparasitoids associated with *T. radiata* and *D. aligarhensis*. Chapter 2 discusses the host association of *Chartocerus* sp. (Hymenoptera: Signiphoridae) and *Pachyneuron crassiculme* (Hymenoptera: Pteromalidae), two species recovered from parasitized ACP mummies collected in Pakistan. Chapter 3 presents an updated host record for *Psyllaphycus diaphorinae* (Hymenoptera: Encyrtidae) as well as a detailed description of its biology as a hyperparasitoid, rather than as a primary parasitoid, as was originally published (Hayat 1972). This distinction is important with regard to biological control of ACP, because it was originally estimated that nine primary

parasitoids were associated with ACP in part of its home range in the Indian subcontinent (Hussain and Nath 1927). The combination of extensive field surveys and experimental evidence from host exposure studies discussed in this thesis indicates that *T. radiata* and *D. aligarhensis* are likely the only primary parasitoid species attacking ACP, and should remain the focus of current and future ACP biological control programs.

References

Bistline-East, A., R. Pandey, M. Keceçi, and M.S. Hoddle. 2015. Host range testing of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) for use in classical biological control of *Diaphorina citri* (Hemiptera: Liviidae) in California. J. Econ. Entomol. doi: 10.1093/jee/tov020

Bodlah, I. and M. Naeem. 2013. Record and trophic associations of three aphid hyperparasitoids from Punjab province of Pakistan. Pakistan J. Zool. 45 (2): 558-562.

Bové, J. M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Pl. Pathol. 88(1): 7-37.

Gomez-Márco, F., A. Urbaneja, J.A. Jaques, P.F. Rugman-Jones, R. Stouthamer, A. Tena. 2015. Untangling the aphid-parasitoid food web in citrus: Can hyperparasitoids disrupt biological control? Biol. Cont. 81: 111-121.

Hoddle, M.S. and R. Pandey. 2014. Host range testing of *Tamarixia radiata* (Hymenoptera: Eulophidae) sourced from the Punjab of Pakistan for classical biological control of *Diaphorina citri* (Hemiptera: Liviidae: Euphyllurinae: Diaphorinini) in California. J. Econ. Entomol. 107 : 125-136.

Keremane, M.L., C. Ramadugu, E. Rodriguez, R. Kubota, S. Shibata, D.G. Hall, M.L. Roose, D. Jenkins, R.F. Lee. 2015. A rapid field detection system for citrus huanglongbing associated '*Candidatus* Liberibacter asiaticus' from the psyllid vector, *Diaphorina citri* Kuwayama and its implications in disease management. Crop Protection. 68: 41-48.

Kumagai, L.B., C.S. LeVesque, C.L. Blomquist, K. Madishetty, Y. Guo, P.W. Woods, S. Rooney-Latham, J. Rascoe, T. Gallindo, D. Schnabel, and M. Polek. 2013. First report of *Candidatus* Liberibacter asiacticus associated with citrus huanglongbing in California. Plant Dis. 97: 283.

Kuwayama S. 1908. Die Psylliden Japans. I. Transactions of the Sapporo Natural History Society 2: 149-189.

Naranjo, S.E., P.C. Ellsworth, and G.B. Frisvold. 2015. Economic value of biological control in integrated pest management of managed plant systems. Annu. Rev. Entomol. 60: 621-645.

Pelz-Stelinski, K.S., R.H. Brlansky, T.A. Ebert, M.E. Rogers. 2010. Transmission parameters for *Candidatus* Liberibacter asiaticus by Asian citrus psyllid (Hemiptera: Psyllidae). J. Econ. Entomol. 103(5): 1531-1541.

Schooler, S.S. P. De Barro, A.R. Ives. 2011. The potential for hyperparasitism to compromise biological control: Why don't hyperparasitoids drive their primary parasitoid hosts extinct? Biol. Cont. 58: 167-173.

Spreen, T.H., J.P. Baldwin, and S.H. Futch. 2014. An economic assessment of the impact of huanglongbing on citrus tree plantings in Florida. Hort. Science 49(8): 1052-1055.

Chapter 1. Host range testing of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) for use in classical biological control of *Diaphorina citri* (Hemiptera: Liviidae) in California

Allison Bistline-East, Raju Pandey, Mehmet Keceçi, and Mark S. Hoddle

Abstract

Host range tests for *Diaphorencyrtus aligarhensis* Shafee, Alam, and Agarwal (Hymenoptera: Encyrtidae), an endoparasitoid of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), sourced from Punjab Pakistan were conducted in quarantine at the University of California, Riverside, California. Seven non-target psyllid species representing four psyllid families were exposed to mated *D. aligarhensis* females in four different treatment types: (1) short sequential no choice treatments, (2) prolonged sequential no choice treatments, (3) prolonged no choice static treatments, and (4) choice treatments. Selection of non-target psyllid species was based on phylogenetic proximity to *D. citri*, likelihood of being encountered by *D. aligarhensis* in the prospective release areas in California, and psyllid species in biological control of invasive weeds. *D. aligarhensis* exhibited high host affinity to *D. citri*, and only parasitized one non-target species, the pestiferous potato psyllid, *Bactericera cockerelli* (Šulc), at low levels (<14%). Based on the results of this study, we conclude that *D. aligarhensis* has a narrow host range and exhibits a high level of host specificity, as it shows a significant attack preference for the target pest, *D. citri*. Results presented here suggest *D. aligarhensis* poses minimal risk to non-target psyllid species in California.

Introduction

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), an invasive pest of citrus, was discovered in southern California, USA, in August 2008 (Gutierrez and Ponti 2013, Grafton-Cardwell 2010). While psyllid feeding can damage citrus flush, the most critical threat presented by *D. citri* is its ability to transmit the bacterium *Candidatus* Liberibacter asiaticus Jagoueix, Bové, and Garnier (*C*Las), a phloem-limited fastidious bacterium (α-Proteobacteria) which is recognized as a causative agent of huanglongbing (HLB), a lethal disease in citrus (Hall et al. 2013). Both nymphal and adult psyllids can become infected with *C*Las when feeding on infected trees (Halbert and Manjunath 2004). Adult *D. citri* vector HLB as they disperse and feed, transmitting the bacterium within salivary excretions into phloem tissue (Bové 2006). Trees infected with HLB suffer foliar dieback, mottled yellow leaves, and reduced fruit yield ranging from 30-100% (Wang and Trivedi 2013). Tree death usually occurs within eight years of HLB contraction, though trees may be asymptomatic for several years, making HLB difficult to diagnose in its early stages (Halbert and Manjunath 2004).

The detrimental effects of *D. citri*-HLB are well known from many commercial citrus growing areas in the world, particularly in Florida, where HLB was first detected in 2005 (Bové 2006), and had caused an estimated $1.7 billion in damage by 2012 (Hodges and Spreen 2012). In April 2012, the first HLB-positive tree was discovered in California (Hacienda Heights, Los Angeles County) (Kumagai et al. 2013), raising serious concerns

for California's citrus industry, which in 2009 was worth ≈ $3 billion, and supplied more than 26,000 jobs (Richards et al. 2014).

Many of California's commercial citrus orchards are in close proximity to urban areas, which can serve as reservoirs from which *D. citri* can migrate into production areas. These residential areas also provide corridors for *D. citri* spread which makes containment and pesticide treatments difficult (Richards et al. 2014). Insecticide applications to suppress *D. citri* populations in residential areas have been costly; Hoddle and Pandey (2014) estimated that ≈ 6% of housing lots in Los Angeles County expected to have citrus were treated chemically at an overall cost of $4.7 million, or about $100 per residence. Due to this cost and difficulty of accessing properties, urban spray programs for suppressing *D. citri* in southern California have been largely abandoned in favor of classical biological control using host-specific parasitoids from *D. citri*'s home range (Hoddle and Pandey 2014).

D. citri has two widely-recognized natural enemies which likely co-evolved with *D. citri* on the Indian subcontinent: *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal) (Hymenoptera: Encyrtidae) (Halbert and Manjunath 2004). In an effort to establish a *D. citri* biological control program in California, a total of six foreign exploration trips to Punjab, Pakistan were conducted between September 2010 and April 2013, to search for both known (*T. radiata*, *D. aligarhensis*) and unknown natural enemies. This was accomplished by collecting parasitized *D. citri* mummies and returning them under

federal and state issued permits to quarantine at the University of California, Riverside (UCR). Adult parasitoids were collected as they emerged from this material. Punjab Pakistan was selected for sourcing natural enemies because it is likely part of the native range of *D. citri* and this area has ≈ 70% climate match to major citrus production areas in California. Good climate match is assumed to be an important factor affecting natural enemy establishment in a new region (Hoddle 2012).

T. radiata has been widely used for the biological control of *D. citri* (Halbert and Manjunath 2004), providing varying levels of control in different areas where is has been introduced, including Réunion Island (excellent control) and Florida (modest control) (Chien et al. 1989, Aubert and Quilici 1984, Qureshi and Stansly 2009). Since its approval for release in December 2011, > 700,000 *T. radiata* have been released at > 600 sites across southern California. Recovery of *T. radiata* at 107 different sites suggests establishment of *T. radiata* is likely (Simmons et al. 2013).

It is anticipated that *D. aligarhensis* could complement *T. radiata* in California in much the same way it is observed in its native range, thereby enhancing *D. citri* biological control. *D. aligarhensis* is an arrhenotokous endoparasitoid which preferentially parasitizes 2nd and 3rd instar *D. citri* (compared to *T. radiata*, an ectoparasitoid, which preferentially parasitizes 4th and 5th instars [Sule et al. 2014]). In addition to parasitization, females can kill *D. citri* nymphs via host feeding (Rohrig et al. 2011). *D. aligarhensis* has been utilized against *D. citri* in various citrus-growing regions including Taiwan, Réunion Island, Saudi Arabia, and Florida (Chien et al. 1989, Al-

Ghamdi and Faragalla 2000, Rohrig et al. 2012), where it has provided low levels of *D. citri* control. Florida populations of *D. aligarhensis*, sourced from Taiwan and China, have failed to establish, despite repeated release efforts (Rohrig et al. 2012).

Though *D. aligarhensis* is reported to have no other hosts besides *D. citri* in its native range (Aubert and Quilici 1984, Skelley and Hoy 2004), it was required to undergo host range testing in quarantine in California before release permits would be issued by the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS). Determining the potential host range of arthropod biological control agents is important in order to avoid undesired non-target effects which may occur as a result of introducing a novel species into a new area (Babendreier et al. 2006). However, selecting non-target species when faced with the potential of hundreds of subjects and performing host range experiments within a quarantine facility is challenging. It is common practice to test non-target species that are taxonomically closely related to the target species, as they may most likely be targeted by the natural enemy of interest. Other considerations when selecting non-target species for host range testing include ecological or niche similarities, potential geographic overlap, and morphological similarities between target and non-target species. Distantly-related species for inclusion in host range testing may be warranted if the non-target species of concern is a beneficial or endangered species (Kuhlmann et al. 2006).

Methods of testing non-target species to determine the host range of natural enemies are variable, but commonly entail small-scale laboratory experiments designed

to determine attack likelihood (i.e., host range) and preference (i.e., host specificity) of the natural enemy in the presence and absence of target and non-target species (van Lenteren et al. 2006). To determine the host range and host specificity of *D. aligarhensis*, variations on choice and no choice exposure tests using the target pest (i.e., *D. citri*) and seven non-target psyllid species were conducted in quarantine at the University of California Riverside. The results of these exposure trials are presented here.

Materials and Methods

Selection of Non-Target Psyllid Species. California has a rich psyllid fauna, with 164 species represented in 34 genera and four families (Percy et al. 2012). Due to this high diversity, a subset of potential non-target psyllid (NTP) hosts was tested. A key concept in host range testing is that native species that are taxonomically closely related to the target host are likely at the highest risk of experiencing attacks from the potential biological control agent (Kuhlmann et al. 2006). However, in California there are no species in the genus *Diaphorina* or the tribe Diaphorinini, to which *D. citri* belongs, so additional criteria were considered for candidate selection. Candidate species were selected for host range testing using the following criteria: (1) NTP taxonomic relatedness to the target host, *D. citri*, (2) likelihood of *D. aligarhensis* encountering the NTP in wilderness areas in close proximity to citrus orchards or backyard gardens, and

(3) psyllids used as biological control agents of invasive plants. The species selected for host range testing and their selection criteria are listed in Table 1.1.

Source of Insects Used in Experiments. *D. citri* utilized for host specificity experiments were sourced from colonies maintained in the Insectary and Quarantine Facility (IQF) at UC Riverside. *D. citri* were reared on *Citrus volkameriana* V. Ten. & Pasq. and were initiated from southern California-collected *D. citri* adults which were tested molecularly and confirmed to be HLB-free. Non-target psyllid species used in host range testing were collected primarily from local wild populations around California and used to found colonies in IQF (Table 1.1), the exception being *Boreioglycaspis melaleucae*, a weed biocontrol agent targeting *Melaleuca quinquenervia* (Cav.), a weedy tree infesting the Florida Everglades. These NTP were shipped to IQF from USDA-ARS-IPRL (Ft. Lauderdale, FL) under USDA permit number P526P-13-02516 and used to found colonies on *M. quinquenervia*.

D. aligarhensis adults were taken from colonies established in UCR IQF which were originally founded with bi-parental individuals collected from parasitized *D. citri* in Punjab, Pakistan and shipped to UCR IQF under USDA-APHIS permit number P526P-11-00103. All *D. aligarhensis* females used in trials were between 3 and 14 days old, and were assumed to have mated after being exposed to male *D. aligarhensis* for at least 24 h.

Maintenance of *D. citri* Colonies. *C. volkameriana* used for maintaining *D. citri* colonies were obtained as rooted seedlings < 2 yr of age either directly from Willits and Newcomb Inc. citrus nursery (Arvin, CA) or from the CDFA rearing facility at the Mt.

Rubidoux Field Station (Rubidoux, Riverside, CA). All *C. volkameriana* were grown in 10.16 cm-diameter pots with modified UCR type III potting soil mix. Seedlings were maintained in greenhouses (27 ± 2°C; 50% RH; and natural day length) at UCR Agricultural Operations (AgOps), with daily watering and Osmocote Pro granular smart-release fertilizer (The Scotts Company LLC, Marysville, OH) applied approximately every 3 months.

D. citri females oviposit only on very young citrus flush (Hall et al. 2008). In order to promote flush growth, *C. volkameriana* were subjected to regular pruning. Approximately 10 - 12 d after pruning, plants developed suitable flush for *D. citri* oviposition and were moved from AgOps to IQF for use in *D. citri* colonies. In IQF, *C. volkameriana* were pruned to remove foliage unsuitable for *D. citri* oviposition and a nylon stocking sleeve was fitted over the soil to prevent emergence of soil-borne insects. Plants were then placed into a primary colony cage, constructed using two stacked transparent U-shaped acrylic risers 15 x 15 x 15 cm (SW Plastics F2191, Riverside, CA), that formed a rectangular cage 15 x 15 x 30 cm (width x depth x height) with two open sides. One open face was covered with white semi-opaque no-see-um netting (Skeeta, Bradenton, FL) and the other was fitted with a 30 cm-long sleeve sewn from no-see-um netting.

Table 1.1. Psyllid species and selection criteria for host range testing of *Diaphorencyrtus aligarhensis.*

Psyllid Species (Family: Tribe)	Qualifying Criteria for Selection				Host Plant Species Used for Testing	Source of Psyllids/ Collection Site
	Target Species	Close Taxonomic Relatedness	Likely to be Encountered in Nature	Biological Control Agent of a Weed		
Diaphorina citri Kuwayama (Liviidae: Euphyllurinae)	X				*Citrus volkameriana* V. Ten. & Pasq.[a]	UCR IQF colony
Bactericera cockerelli (Sulc) (Triozidae)			X		*Capsicum annum* L.[a]	Trumble Lab (Dept. of Entomology, UCR)
Heteropsylla sp. (Psyllidae: Ciriacreminae)			X		*Acacia farnesiana* (L.) Willd.[a]	UCR Botanic Gardens, Riverside, CA
Arytainilla spartiophylla (Forester) (Psyllidae: Psyllinae)				X	*Cytisus scoparius* (L.) Link[c]	El Dorado Co., CA
Euphyllura olivina (Costa) (Liviidae: Euphyllurinae)		X	X		*Olea europaea* L.[a]	Murrietta, CA
Heteropsylla texana Crawford (Psyllidae: Ciriacreminae)			X		*Prosopis glandulosa* Torr.[a]	UCR Botanic Gardens, Riverside, CA
Diclidophlebia fremontiae (Klyver) (Liviidae: Liviinae)		X			*Fremontodendron californicum* (Torr.) Coville[b]	Big Bear, CA
Boreioglycaspis melaleucae Moore (Aphalaridae)				X	*Melaleuca quinquenervia* (Cav.) S.T. Blake [b]	USDA-ARS- IPRL Ft. Lauderdale, FL

[a]Non target psyllid (NTP) nymphs presented on seedlings in Cone-tainers.
[b]NTP nymphs presented on seedlings in D40 containers.
[c]Seedlings used in experiments were young wild-collected plants transplanted into Cone-tainers in UCR IQF.

Approximately 18 cages per wk (6 each every Monday, Wednesday, and Friday) were prepared for *D. citri* oviposition. Approximately 15 - 20 adult *D. citri* were introduced into each cage and allowed to oviposit for 2 - 4 d, after which they were removed and transferred to a new cage for oviposition. *D. citri* adult mortality was mitigated by supplementing additional individuals sourced from HLB-free California strain lab colonies. Inoculated experimental cages were then placed within a larger secondary outer cage (BugDorm model 6610, MegaView Science, Taiwan), and grouped by inoculation date. This dual-cage system was instituted in compliance with designated protocols under CDFA Permit No. 2870 to ensure *D. citri* adults would not escape from cages.

Colony plants were watered three times per wk (Monday, Wednesday, and Friday). All *D. citri* colonies in IQF rearing rooms were maintained under constant conditions at 29°C, 40% RH, and 14:10 h (L:D) photoperiod. Cages were illuminated artificially (Sylvania Fluorescent Octron 4100K bulbs, Osram Sylvania Inc., Danvers, MA) and they had exposure to natural daylight from a single south-facing window.

Maintenance of NTP Colonies. Native psyllid species (*Heteropsylla* sp., *H. texana*, *D. fremontiae*, and *B. cockerelli*), as well as *B. melaleucae*, were maintained in colonies on their preferred host plants (*Acacia farnesiana*, *Prosopis glandulosa*, *Fremontodentron californicum*, *Solanum melongena*, and *M. quinquenervia*, respectively) in IQF rearing rooms. *Arytainilla spartiophylla* and *Euphyllura olivina*, two non-native species, were not maintained in colonies in IQF because they are not subject

to parasitism in the field (Percy et al. 2012) and nymphs were field-collected on *Cystus scoparius* and *Olea europaea*, respectively, on an as-needed basis.

To initiate non-target psyllid colonies, 4-6 host plants were placed within a large BugDorm (model 2120 MegaView Science, Taiwan) and non-target psyllid adults were introduced into cages. Colonies were maintained by rotating out older host plants for new plants with appropriate growth stages for oviposition on an as-needed basis. *A. farnesiana* and *P. glandulosa* were grown from seed and matured in 19 L pots (16 cm diameter by 18 cm deep) using modified UCR type III potting mix. *S. melongena* ("Long Purple" variety, Botanical Interests, Broomfield, CO), *F. californicum* (Moosa Creek Nursery, Valley Center, CA), and *M. quinquenervia* (Ponto & Sons Wholesale Nursery, Vista, CA) were obtained as seedlings and transplanted into 19 L pots. Host plants were pruned on an as-needed basis to promote flush growth as well as to restrict plant height so plants could fit within cages. All plants were grown in UCR AgOps greenhouses and watered every Monday, Wednesday, and Friday. Miracle-Gro all-purpose plant food (water soluble formula, 24-8-16 NPK; The Scotts Miracle-Gro Company, Marysville, OH) was applied as needed. Non-target host plants were transferred to IQF and placed within the appropriate colony cage when needed. All NTP colonies were maintained in compliance with CDFA Permit Nos. 2976 and 2958 in IQF at 25°C with 40% RH and 14:10 h (L:D).

Plant Preparation for Testing. All NTP were tested on their preferred host plant (Table 1.1) except *B. cockerelli*, which was kept in colony on *S. melongena* but tested on *Capsicum anuum* ("California Wonder", Ferry Morse Seed Company, Felton, KY). All

host plants used in experiments, with the exception of *C. scoparius*, *F. californicum*, and *M. quinquenervia*, were young seedlings, ≈ 5 - 7 cm tall, grown from seed in white plastic Ray Leach Cone-tainers (SC7 Stubby, 114 mL, 3.8 cm diameter, Stuewe and Sons Inc., Portland, OR) in outdoor greenhouses at CDFA Mt. Rubidoux Field Station and delivered as needed to UCR AgOps. *C. scoparius* seedlings were harvested at the site of *A. spartiophylla* collection in El Dorado County, CA, and transported to IQF under CDFA Permit No. 2977, where seedlings were pruned, transplanted into Cone-tainers, and allowed to root before being used.

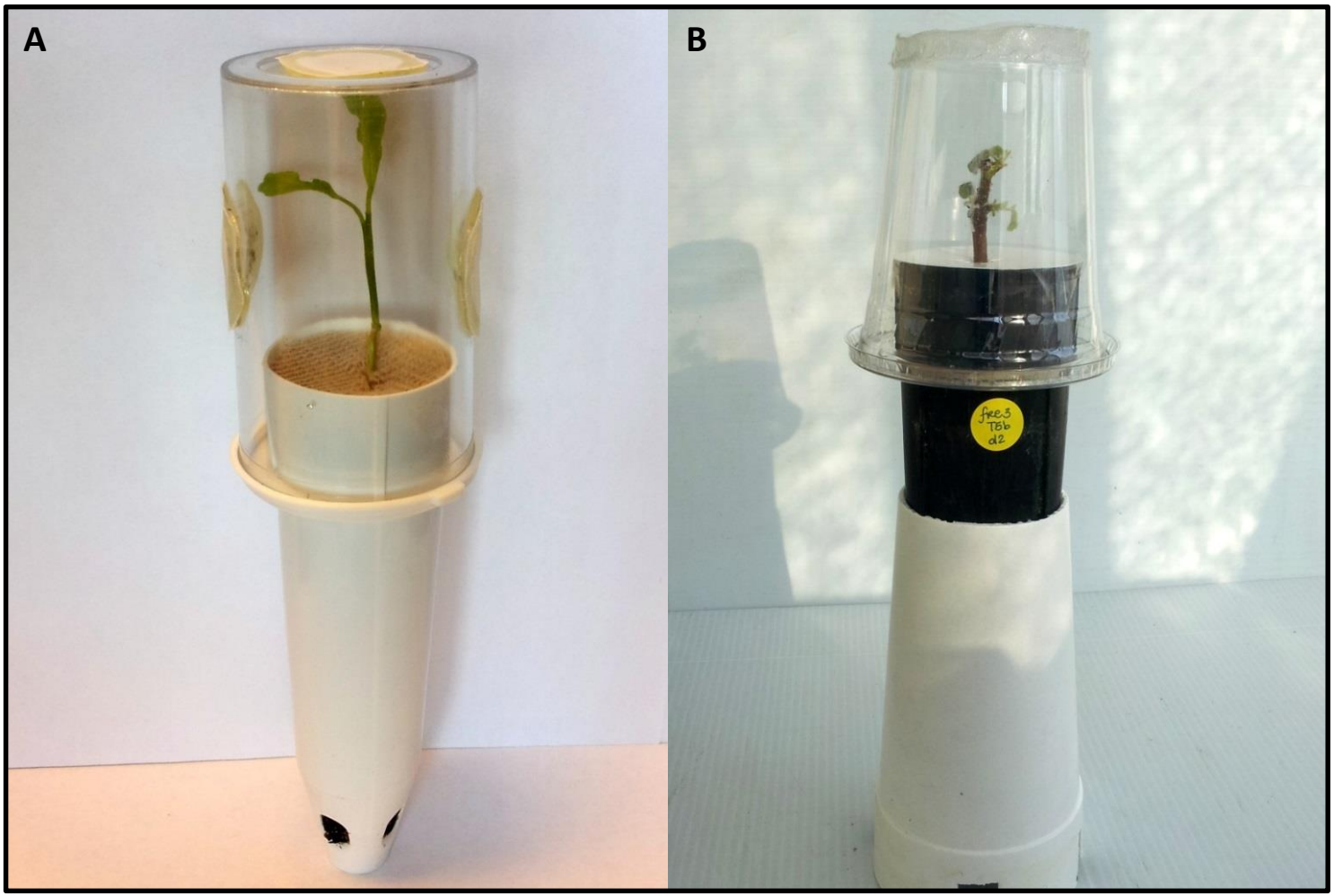
Seedlings were transported from AgOps greenhouses to an IQF Insectary-level laboratory for preparation at least 2 h before testing commenced. Exposure vials were assembled by taking seedlings in Cone-tainers and stripping them of excess foliage. A 3.7 cm diameter netted upholstery foam disc was fitted around the base of the seedling stalk to completely cover the soil in order to prevent soil pest contamination of testing vials, as well as to prevent both psyllid nymphs and *D. aligarhensis* from becoming lost in soil. A ventilated 148 mL transparent plastic vial (Thornton Plastic Co., Salt Lake City, UT) was then affixed to the Cone-tainer to provide containment for insects: a 3.7cm diameter hole was cut into the vial lid, which allowed it to be fitted around the top of the Cone-tainer, and was held in place using brass thumbtacks. Vials were ventilated with three 2 cm diameter holes (two on either side and one on the base) covered with fine organza mesh, inverted, and placed over the top of the seedling and fastened onto its corresponding lid (Fig. 1.1A). Five second to third instar *D. citri* nymphs were transferred with a fine-hair paintbrush from colony plants onto prepared *C. volkameriana* seedlings, and five second

to third instar nymphs of the NTP being tested were transferred in a similar manner from their respective colony plants onto corresponding host seedlings.

Because *F. californicum* and *M. quinquenervia* were obtained in D40 conical growing containers (6.5 cm diameter, 25 cm deep) and were too large to transplant into Cone-tainers, a modified experimental design was developed for testing these two NTP species (Fig. 1.1B). A system similar to the Cone-tainer setup was adapted using 470 mL clear plastic cups and corresponding 10.5 cm diameter lids instead of ventilated vials. A 6 cm diameter hole was cut in the center of each lid, which was then fitted around the top of the D40 cone and held in place with a rubber band. The base of each cup was removed and covered with white no-see-um netting to provide ventilation. A 7 cm paper disc was fitted around the base of the seedling and affixed to the top rim of the D40 in order to cover the soil in the manner of the foam discs in Cone-tainers. Five each of *D. fremontiae* and *B. melaleucae* nymphs were transferred onto seedlings in these modified D40 growing containers for testing in the same manner as other NTP species. Two small drops of wild clover honey were applied to the inner walls of all testing arenas to provide a carbohydrate source to *D. aligarhensis*.

Experimental Design. To perform exposure trials, one mated female *D. aligarhensis* was introduced into the Cone-tainer or enclosed D40 cone containing psyllid nymphs on their respective host plants. Experiments were set up in a block design comprised of eight treatment types per NTP species, so that all treatments were tested

Figure 1.1. Experimental setup of seedlings used in host range testing. (A) Cone-tainer setup used for testing *D. citri*, *B. cockerelli*, *Heteropsylla* sp., *A. spartiophylla*, *E. olivina*, and *H. texana*. (B) Modified D40 setup used for testing *D. fremontiae* and *B. melaleucae*.



simultaneously, and each block of treatments lasted 48 h. Each NTP trial was replicated 8 - 10 times at the average rate of 2 replicates per wk over a 4 wk period. All experiments took place in a quarantine laboratory at 27°C, 40% RH, and a 14:10 h (L:D) photoperiod.

A combination of sequential no choice, static no choice, and choice tests were used to assess the propensity of *D. aligarhensis* to attack different psyllid species, as well as the impact on *D. citri* and NTP nymph survivorship (Table 1.2). Control treatments were run for both *D. citri* and NTP in the absence of *D. aligarhensis* to provide baseline measurements of psyllid nymph mortality and survivorship under identical conditions as parasitoid exposure treatments. Following completion of testing, surviving *D. aligarhensis* were immediately preserved in 95% ethanol.

Sequential No Choice Tests (T1, T2, T5, T6). Sequential no choice treatments exposed *D. aligarhensis* to either *D. citri* or NTP for the first testing period, which varied according to either short (i.e., 4 h) or prolonged (24 h) exposures, and subsequently *D. aligarhensis* was transferred onto the opposite host type (i.e., *D. citri* to NTP or NTP to *D. citri*) for the second testing interval of the same exposure period. This sequential setup evaluated whether the order of host exposure and the total length of exposure time were significant factors influencing attack rates.

Table 1.2. Summary of treatment types and experimental design of host range trials.

Treatment	Exposure Type	Psyllid Species Exposed to *D. aligarhensis*			
		Day 1		Day 2	
		1st 4 h	2nd 4 h	1st 4 h	2nd 4 h
T1	Short Sequential[a]	*D. citri*	NTP[b]	*D. citri*	NTP
T2	Short Sequential[a]	NTP	*D. citri*	NTP	*D. citri*
		24 h		24 h	
T3	Choice	*D. citri* + NTP		*D. citri* + NTP	
T4	Prolonged Static	*D. citri*		*D. citri*	
T5	Prolonged Sequential	*D. citri*		NTP	
T6	Prolonged Sequential	NTP		*D. citri*	
T7	Prolonged Static	NTP		NTP	
T8	Control	*D. citri* + NTP		*D. citri* + NTP	

[a]*D. aligarhensis* in short-term treatments held overnight (16 h) with access to honey droplets at 13.2-14.4°C.
[b]Non target psyllid (NTP).

Short sequential no choice treatments had female *D. aligarhensis* in vials with either 5 *D. citri* (T1) or 5 NTP (T2) nymphs on their respective host plants and allowed to oviposit for 4 h. Females were subsequently removed and directly transferred to a vial with the opposite psyllid type (i.e., onto NTP for T1 and onto *D. citri* for T2) and allowed to oviposit for an additional 4 h. After this second 4 h exposure period, females were removed in 2 mL O-ring microcentrifuge tubes with honey droplets and stored overnight for 16 h at ~14°C. The same exposure sequence was followed on day 2 using the same *D. aligarhensis* female as day 1 (Table 1.2). In prolonged sequential exposures**,** female *D. aligarhensis* were introduced into vials containing either 5 *D. citri* (T5) or 5 NTP (T6) nymphs on their respective host plants on day 1 and allowed to oviposit for 24 h. On day 2, females were transferred directly to new vials with the reciprocal host species and given an additional 24 h to oviposit before removal.

Prolonged Static No Choice Tests (T4, T7). Static tests exposed *D. aligarhensis* to the same host species over the entire testing cycle. Females were introduced into vials with either 5 *D. citri* (T4) or 5 NTP (T7) nymphs for the initial 24 h exposure; they were then directly transferred to a second vial containing the same host species for a second 24 h exposure period. This allowed *D. aligarhensis* time to host feed during the first testing period, and was designed to accommodate an increased likelihood of oviposition on psyllid hosts presented on the second day of testing.

Choice Tests (T3). Choice tests presented both target and non-target hosts simultaneously in a shared testing arena to assess host preferences when foraging females

could choose between host species. Unenclosed *D. citri*-infested *C. volkameriana* seedlings and NTP-infested seedlings were used in choice tests and allowed free access to both species of psyllid by female *D. aligarhensis*. Seedlings were placed within a choice testing arena (a mesh and acrylic cage, 15 x 15 x 20 cm for NTP species in Cone-tainers, and 15 x 15 x 30 cm for NTP species in D40s), into which one *D. aligarhensis* female was released. After 24 h, nymphs were removed from the testing arena and replaced with new hosts of the same species to which the same *D. aligarhensis* female was exposed to for an additional 24 h period. At the end of each exposure period, all choice treatment plants were fitted with ventilated vials to contain emerging psyllids and parasitoids.

Control (T8). One vial each of *D. citri* and NTP were prepared in an identical manner as exposure treatments, but nymphs were not exposed to parasitoids. These control treatments measured psyllid survivorship in the absence of *D. aligarhensis* under the same handling procedures and experimental conditions as exposure treatments. Controls were run for both day 1 and day 2 of each replicate.

Data Recording and Statistical Analyses. All test vials were observed at 6-8 d, 12-14 d, and 21 d after initial replicate setup to record psyllid and parasitoid emergence rates. Any remaining nymphs (i.e., no emergence of parasitoids or eclosion of adult psyllids) counted during this third observation were considered dead. Recorded outcomes for psyllid nymphs fell into four categories: live adult (i.e., psyllid nymph successfully eclosed), mummy (i.e., successful parasitism by *D. aligarhensis* resulted in a mummy), dead (i.e., dead psyllid nymph recovered), and missing (i.e., psyllid nymph not

recovered). Missing nymphs were not included in data figures because their fate could not be conclusively determined. Parasitized psyllid nymphs (mummies) were monitored for emergence of adult parasitoids, which were counted and sexed.

Operating under the null hypothesis that the rate of psyllid mortality was independent of the presence or absence of parasitoids, Fisher's Exact Test for count data was run to compare each treatment against the control mortality for both *D. citri* and NTP. Experiment-wide error rate of multiple comparisons was controlled using the sequential Bonferroni correction method, where experimental P-values were ranked lowest to highest and compared in a step-down method against a significance level of 0.05/*n*, 0.05/*n*-1, 0.05/*n*-2, etc. (where *n* = the number of comparisons). One species (*B. cockerelli*) out of the seven NTP tested was parasitized by *D. aligarhensis* (see Results) so a second Fisher's exact test with sequential Bonferroni correction was run using a pairwise comparison of treatments between *D. citri* and *B. cockerelli* to determine if *D. aligarhensis* exhibited a preference between these two psyllid species. Analyses of psyllid nymph mortality were conducted using basic packages included in the R statistical programming environment (R version 3.0.2, R Core Team 2013, The R Foundation for Statistical Computing, Vienna, Austria).

Results

***D. citri* Mortality for Control and Parasitoid Exposure Treatments.** *D. citri* in control cages experienced an average mortality rate of approximately 16% across all trials, with ≈ 58% successfully eclosing as adult psyllids. The ≈ 26% discrepancy is attributed to psyllid nymphs which were unaccounted for (i.e., missing) at time of data collection. There was no parasitism recorded in any control treatment, indicating unintentional exposure of nymphs to *D. aligarhensis* did not occur at any time.

Parasitism of *D. citri* by *D. aligarhensis* occurred in every treatment type and in every replicate. Total *D. citri* nymph mortality (death due to unknown causes + death by parasitism) was significantly elevated across all treatments, regardless of exposure type or length, in most replicates (see Table 1.3 for full summary of results and statistical analyses). For those sets of *D. citri* exposures which were not significantly elevated (i.e., the *H. texana*, *D. fremontiae*, and *B. melaleucae* exposure experiments), this resulted from high mortality in control treatments, from unknown causes. Rates of *D. citri* parasitism by *D. aligarhensis* across all exposure types in all replicates averaged 22%, and ranged from 4% to 50% within individual treatments (Table 1.3).

Short sequential no choice trials with *D. citri* exposed first (T1) had an overall mortality rate (death by unknown causes + parasitism) across all replicates of 46% and ranged from 20-52%. NTP-first short sequential no choice exposures (T2) had an overall *D. citri* mortality rate of 38% with a range of 16-49% across all replicates. A mortality

range of 30 - 45% was observed in choice exposures (T3), with an overall mortality rate across all replicates of 38%. Prolonged static exposures (T4) consistently yielded higher mortality rates than other treatment types in nearly all experiment sets, with an overall rate of 46% which ranged from 28 - 58%. The overall mortality rate for prolonged sequential exposures when *D. citri* was exposed first (T5) was 41%, with a range of 30 - 60%. Prolonged sequential exposures where NTP were exposed first (T6) showed an overall mortality of 37% and a range of 12 - 54% (Table 1.3).

Non-Target Psyllid Mortality. Of the seven tested NTP species, *D. aligarhensis* parasitized only *B. cockerelli*, the pestiferous potato psyllid. A total of 67 nymphs out of the 480 exposed (14%) were successfully parasitized. Levels of psyllid nymph mortality (death + parasitism) in all treatments were significantly higher than mortality in control treatments (24 - 44% vs. 4%, respectively) (Table 1.3). However, rates of *D. citri* mortality were always higher when exposed to *D. aligarhensis*, regardless of treatment, than that experienced by *B. cockerelli*. When *B. cockerelli* was examined for host preference through a secondary Fisher's Exact Test directly comparing mortality between *D. citri* and *B. cockerelli* treatments, rates of NTP mortality were significantly elevated in T1, T2, T4, and T7 treatments (Table 1.4). It is likely that elevated mortality rates in the prolonged static no choice exposure trials (i.e., T4 and T7) were due, in part, to prolonged confined exposure which prevented patch abandonment by *D. aligarhensis*. In prolonged sequential tests where *D. citri* was presented first, there was a significantly higher rate of *D. citri* (60%) vs. *B. cockerelli* (36%) mortality, though there was no significant difference when *B. cockerelli* was presented before *D. citri*. An analysis between *D. citri*

Table 1.3. Mortality and parasitism rates for second and third instar *D. citri* and non-target psyllid (NTP) nymphs when exposed to *Diaphorencyrtus aligarhensis* females under different exposure treatments.

Treatment	Total nymphs exposed	Parasitism (%)	Total mortality (%)	P-value	Total nymphs exposed	Parasitism (%)	Total mortality (%)	P-value
	D. citri				*B. cockerelli*			
Short sequential *D. citri* first	100	39	52	<0.01[a]	90	13	32	<0.01[a]
Short sequential NTP first	100	40	46	<0.01[a]	95	19	24	<0.01[a]
Choice test	100	27	33	<0.01[a]	95	17	31	<0.01[a]
Prolonged sequential *D. citri* first	50	46	60	<0.01[a]	50	8	36	<0.01[a]
Prolonged sequential NTP first	50	38	54	<0.01[a]	50	10	44	<0.01[a]
Prolonged exposure (static)	95	51	58	<0.01[a]	100	12	35	<0.01[a]
Control (No parasitoid)	105	0	6	-	95	0	4	-
	D. citri				*Heteropsylla* sp.			
Short sequential *D. citri* first	80	28	48	<0.01[a]	80	0	8	0.31
Short sequential NTP first	80	19	44	<0.01[a]	80	0	5	0.71
Choice test	75	21	35	<0.01[a]	75	0	16	<0.01[a]
Prolonged sequential *D. citri* first	40	40	60	<0.01[a]	40	0	5	0.64
Prolonged sequential NTP first	40	25	53	<0.01[a]	35	0	11	0.10
Prolonged exposure (static)	80	31	48	<0.01[a]	80	0	9	0.19
Control (No parasitoid)	85	0	11	-	90	0	3	-

	D. citri				*A. spartiophylla*			
Short sequential *D. citri* first	80	18	34	0.02	80	0	39	0.44
Short sequential NTP first	75	16	33	0.03	75	0	32	1.00
Choice test	80	29	40	<0.01[a]	80	0	13	<0.01[a,b]
Prolonged sequential *D. citri* first	40	20	33	0.07	30	0	17	0.11
Prolonged sequential NTP first	40	3	13	0.61	40	0	23	0.31
Prolonged exposure (static)	70	31	44	<0.01[a]	70	0	30	0.87
Control (No parasitoid)	90	0	18	-	105	0	32	-
	D. citri				*E. olivina*			
Short sequential *D. citri* first	80	21	35	<0.01[a]	75	0	20	0.70
Short sequential NTP first	65	29	49	<0.01[a]	70	0	20	1.00
Choice test	80	18	39	<0.01[a]	80	0	13	0.23
Prolonged sequential *D. citri* first	40	18	33	0.03[a]	35	0	6	0.06
Prolonged sequential NTP first	40	18	23	0.32	40	0	33	0.13
Prolonged exposure (static)	75	41	55	<0.01[a]	70	0	19	0.85
Control (No parasitoid)	100	0	15	-	100	0	20	-
	D. citri				*H. texana*			
Short sequential *D. citri* first	85	20	38	0.08	85	0	24	0.86
Short sequential NTP first	90	20	32	0.33	90	0	13	0.05
Choice test	90	23	46	<0.01[a]	90	0	8	<0.01[a,b]
Prolonged sequential *D. citri* first	45	11	31	0.54	45	0	11	0.08

Prolonged sequential NTP first	45	18	38	0.24	45	0	18	0.40
Prolonged exposure (static)	85	14	28	0.62	85	0	26	1.00
Control (No parasitoid)	100	0	25	-	100	0	25	-
		D. citri				*D. fremontiae*		
Short sequential *D. citri* first	80	8	33	0.18	80	0	11	0.63
Short sequential NTP first	80	23	48	<0.01[a]	80	0	14	0.35
Choice test	75	15	32	0.23	75	0	8	1.00
Prolonged sequential *D. citri* first	40	18	35	0.20	35	0	20	0.12
Prolonged sequential NTP first	25	16	40	0.13	40	0	20	0.09
Prolonged exposure (static)	80	31	50	<0.01[a]	70	0	7	0.78
Control (No parasitoid)	100	0	23	-	100	0	9	-
		D. citri				*B. melaleucae*		
Short sequential *D. citri* first	75	11	20	0.85	75	0	3	1.00
Short sequential NTP first	80	11	16	0.84	80	0	1	0.63
Choice test	70	9	30	0.09	70	0	13	0.01[a]
Prolonged sequential *D. citri* first	40	10	30	0.17	40	0	8	1.00
Prolonged sequential NTP first	35	23	37	0.03[a]	40	0	0	0.26
Prolonged exposure (static)	80	16	33	0.04[a]	70	0	4	0.68
Control (No parasitoid)	100	0	18	-	105	0	3	-

P values generated using Fisher's Exact Test for count data. Some percentages may not add up to 100% due to rounding.

[a]Fisher's Exact Test with sequential Bonferroni correction comparing mortality vs. control was significant.

[b]Results were significant due to treatments having significantly lower mortality than control.

and *B. cockerelli* mortality rates (27% and 17%, respectively) in choice cages showed no statistical difference (Table 1.4).

E. olivina, an invasive pest infesting olive trees, was chosen as a taxonomically closely related species to *D. citri* for testing (representing a different tribe within Liviidae [Table 1.1]). Mortality of *E. olivina* was ≤ 20% in all treatment types except prolonged exposure where NTP was presented first, which was 33%. No mortality for any treatment type when exposed to *D. aligarhensis* was significantly elevated from the control rate (20%). *D. citri* control mortality was 15% for exposure trials with *E. olivina*, and all treatments exposed to *D. aligarhensis*, with the exception of prolonged sequential no choice exposures where NTP was presented first, were significantly elevated (Table 1.3).

The native California psyllid, *D. fremontiae*, was the second species chosen for its phylogenetic proximity to *D. citri* (representing a third tribe within Liviidae [Table 1.1]). Control mortality of *D. fremontiae* was 9%, and there was no significant difference between control and any NTP treatment exposed to *D. aligarhensis* (mortality rates ranged from 7 - 20%). While *D. citri* mortality rates were significantly higher than control (23%) only in short sequential no choice tests (NTP first) and prolonged static no choice exposures to *D. aligarhensis*, mortality levels across all *D. citri* exposures was significantly higher being > 30% when compared to *D. fremontiae* (Table 1.3).

H. texana nymph mortality in control treatments was 25%, and though there were significant differences between levels of NTP control vs. *D. aligarhensis* exposure mortality in short sequential no choice tests with NTP exposed first and choice exposures,

this significance was due to mortality rates in exposure cages being markedly lower than in control cages (13%, 8%, and 11%, respectively). *D. citri* mortality ranged from 28 (prolonged static no choice exposures) to 46% (choice trials), though only the choice treatment yielded significantly higher mortality as compared to control cages (25%) (Table 1.3).

Heteropsylla sp. control mortality (3%) was not significantly different from the NTP mortality rates observed in any *D. aligarhensis* exposure treatments, which ranged from 5 (short sequential no choice exposures presenting NTP first) to 11% (prolonged sequential no choice exposures presenting NTP first). Conversely, *D. citri* mortality was significantly elevated in every treatment exposed to *D. aligarhensis*, ranging from 35% (choice) to 60% (prolonged sequential presenting *D. citri* first), as compared with an 11% mortality rate in the control treatment (Table 1.3).

A. spartiophylla, a self-introduced species in northern California, is a fortuitous biological control agent of the invasive noxious weed, Scotch broom, *C. scoparius*. Mortality rates in *A. spartiophylla* control cages (32%) did not differ significantly when compared to treatments exposed to *D. aligarhensis* (which ranged from 23 - 39%), except in choice cages, where NTP nymph mortality was significantly lower than the controls (Table 1.3). *A. spartiophylla* nymphs likely suffered elevated mortality due to the nature of their field collection, excessive handling, and transportation to IQF before testing. *D. citri* mortality was significantly higher in all treatments exposed to *D. aligarhensis* than

in control cages (33 - 44% vs. 18%), except in prolonged sequential no choice trials (13% and 33%) (Table 1.3).

B. melaleucae mortality across all exposure treatments was low (0 - 13%), and only the mortality rates observed from choice trials (13%) were significantly higher than the observed < 3% control mortality (Table 1.3). It is unknown what may have contributed to this elevated mortality rate in choice cages, but no parasitism was observed. Associated *D. citri* treatments experienced a lower rate of parasitism and combined mortality, with only the prolonged sequential no choice exposures being significantly higher than controls (37 and 33% vs. 18%; all other exposures were < 30%) (Table 1.3).

Table 1.4. Comparison of *D. aligarhensis* parasitism and nymph mortality between *D. citri* and *B. cockerelli* across different treatment types.

Treatment	Host species	Total nymphs exposed	Parasitism (%)	Total mortality (%)	P-value
Sequential (*D. citri* first)	*D. citri*	100	39	52	<0.01[a]
	B. cockerelli	90	13	32	
Sequential (NTP first)	*D. citri*	100	40	46	<0.01[a]
	B. cockerelli	95	19	24	
Choice test	*D. citri*	100	27	33	0.76
	B. cockerelli	95	17	31	
Prolonged exposure (*D. citri* first)	*D. citri*	50	46	60	0.03[a]
	B. cockerelli	50	8	36	
Prolonged exposure (NTP first)	*D. citri*	50	38	54	0.42
	B. cockerelli	50	10	44	
Prolonged exposure (static)	*D. citri*	95	51	58	<0.01[a]
	B. cockerelli	100	12	35	

P values generated using Fisher's Exact Test for count data.
[a]Fisher's exact test with sequential Bonferroni correction was significant

Discussion

D. aligarhensis is reportedly highly host-specific, with records indicating *D. citri* as its only known host (Aubert and Quilici 1984, Skelley and Hoy 2004). Host range trials for *D. aligarhensis* conducted here were comprised of seven NTP species spanning four families and seven tribes, including four species native to California, one invasive pest species, and two non-native species used for biological control of invasive weeds. Results from no choice and choice trials presented here largely support this observation, as only one non-target species, the highly pestiferous potato psyllid, *B. cockerelli*, was successfully parasitized.

Under a combination of short sequential, prolonged sequential, prolonged static no choice, and choice exposure trials, *D. aligarhensis* was shown to successfully parasitize only *D. citri* (target host) and *B. cockerelli* (non-target). Compared to the attack rates on *D. citri* (≈ 40% parasitism), *B. cockerelli* experienced an overall average parasitism rate of 14% by *D. aligarhensis*. Because these two psyllid species are not closely related phylogenetically, it is likely that observed parasitism was the result of artificial testing conditions or a similarity in size and/or superficial morphology of *D. citri* nymphs. A similar result with *B. cockerelli* was observed in *T. radiata* host range testing (Hoddle and Pandey 2014), but parasitism of *B. cockerelli* by *T. radiata* in the field has not been observed even in areas of very close sympatry (i.e., infested host plants growing together in gardens) (MSH, unpublished). These low levels of non-target

parasitism can often be the result of small-scale testing schemes, which artificially inflate the proportion of non-target species estimated to be at risk of attack in the field (van Lenteren et al. 2006). There is also a possibility that host volatiles, which many parasitoids utilize for recognition of suitable hosts (Zuk and Kolluru 1998), are similar between the two species, leading *B. cockerelli* to be mistakenly identified by *D. aligarhensis* as a suitable host in small cage trials. However, additional research into chemical cues employed by various psyllid species and associated parasitoids is needed to determine if this is the case (Arras et al. 2012). Unlike *B. cockerelli*, *E. olivina* and *D. fremontiae*, the two NTP most closely related taxonomically to *D. citri,* experienced no parasitism. This result may support the suggestion that *D. aligarhensis* non-target host selection relies more on size or morphology similarity of nymphs than species relatedness to the target, *D. citri*.

Results presented here suggest that potential for *D. aligarhensis* to inflict significant non-target impacts in nature will likely be negligible, and attacks on *B. cockerelli*, should they occur in the field, are unlikely to affect populations of this pest. Additionally, *D. aligarhensis* will be faced with competition from a rich guild of native parasitoids that attack native California psyllids (Percy et al. 2012). This type of biotic resistance may be important for minimizing population-level non-target impacts by parasitoids released for *D. citri* biological control (Hoddle and Pandey 2014). Data from this research indicate that *D. aligarhensis* is very unlikely to have deleterious effects on *A. spartiophylla* or *B. melaleucae*, two important psyllid species attacking either Scotch

broom or melaleuca, invasive weeds found in northern California and the Florida Everglades, respectively.

The ability of *D. aligarhensis* to establish populations in California is of high interest. Though this parasitoid has been continuously released in Florida since 1998, there remains no conclusive evidence that it has established (Rohrig et al. 2011). Unlike the strains released in Florida, which were all-female and confirmed to be infected with *Wolbachia* (which can influence survivorship, fecundity, and offspring sex ratio) (Skelley and Hoy 2004, Rohrig et al. 2012), the population of *D. aligarhensis* used in these studies is bi-parental. It is also worth noting that, while the majority of *D. aligarhensis* releases in Florida took place in commercial citrus groves, which may have been subjected to pesticide treatments, the majority of California releases of *D. aligarhensis* are expected to occur primarily in residential areas, where pesticide use is very low and *D. citri* infested citrus is common.

Possible competition with *T. radiata* is another concern in establishing *D. aligarhensis* as a biological control agent of *D. citri*. *T. radiata* has a higher reproductive rate and shorter generation time (nearly two generations per one generation of *D. aligarhensis*) which may allow *T. radiata* to competitively exclude *D. aligarhensis* in areas where they could be competing for *D. citri* nymphs. Additionally, *T. radiata* females have been recorded to kill nearly twice as many *D. citri* nymphs in their lifetime as *D. aligarhensis* through a combination of parasitism and host feeding (Skelley and Hoy 2004). In cases of direct competition, *T. radiata* has been demonstrated to

successfully parasitize *D. citri* nymphs within five days following initial oviposition by *D. aligarhensis* (Rohrig et al. 2012). The advantage posed by the situation in southern California is although *T. radiata* has small established populations as a result of the biological control program targeting *D. citri* (Hoddle and Pandey 2014), there remain large areas where *D. citri* is present that have no established *T. radiata* populations. Selection of these areas (e.g., San Diego and Imperial Counties [see distribution map in Morgan et al. 2014]) for *D. aligarhensis* releases may increase establishment rates because of reduced interspecific competition for *D. citri* nymphs. Additionally, varied climatic conditions throughout major citrus production areas in California may provide climate niches more favorable to either *D. aligarhensis* or *T. radiata*. Some notable past biological control successes of citrus pests in California have required more than one natural enemy because of differential performances by biological control agents in citrus production areas with different climates (Quezada and DeBach 1973). However, there is debate over the number of natural enemy species that need to be established to achieve successful insect pest suppression (Denoth et al. 2002).

It is possible that California could provide a permissive environment for proliferation by *D. aligarhensis* because the new range into which this parasitoid may be introduced could lack hyperparasitoids. In regions where *D. aligarhensis* is native, it is known to be attacked by at least 10 species of hyperparasitoids which resulted in ≈ 40% hyperparasitism in Taiwan, compared to < 1% of *T. radiata* being hyperparasitized (Chien et al. 1989). In Pakistan, where the California populations of *D. aligarhensis* were sourced, it is also attacked by several species of hyperparasitoid (Hoddle et al. 2013,

Bistline-East and Hoddle 2014). Consequently, introduction into novel areas free of co-evolved hyperparasitoids may facilitate *D. aligarhensis* establishment and allow the development of higher populations in California than those observed in its native range (Simmons et al. 2013).

A goal of the classical biological control program in California is to reconstitute the guild of primary *D. citri* parasitoids from Punjab, Pakistan, an area with a very good climate match to citrus production areas in California (Hoddle 2012). Should *D. aligarhensis* and *T. radiata* both establish in southern California, greater control of *D. citri* populations may be expected, due to complementarity, than would be possible with either species of parasitoid individually. Use of biological control to suppress *D. citri* populations, especially in areas where pest populations are high but insecticide treatments are unlikely for area-wide suppression of pest management, may reduce the spread of HLB from urban zones (HLB was first detected in a backyard tree in California [Kumagai et al., 2013]) to commercial production areas as vector prevalence is minimized (Pelz-Stelinski et al., 2010). This reduction of vector densities in residential areas to reduce the rate of HLB spread into commercial citrus production zones is a key goal of California's *D. citri* biological control program. On 1 November 2013, an 86-page Environmental Assessment Report detailing the research reported here was submitted to USDA-APHIS. On 26 October 2014, following review of this report, USDA-APHIS issued a finding of no significant impact (FONSI) for *D. aligarhensis*, and on 24 November 2014 USDA-APHIS issued permit P526P-14-04034 authorizing the

release of *D. aligarhensis* from quarantine allowing releases in California to begin in December 2014.

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References

Al-Ghamdi, K.M.S. and A. Faragalla. 2000. Parasitism of *Diaphoria* [sic] *citri* (Kuwayama) (Homoptera: Psyllidae) by *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) on Mexican lime trees, western Saudi Arabia. Bio-Science Research Bulletin 16(2):99-105.

Arras, J., W. Hunter, and B. Bextine. 2012. Comparative analysis of antennae sensory arrays in Asian citrus psyllid, *Diaphorina citri*, and potato psyllid, *Bactericera cockerelli* (Hemiptera). Southwest. Entomol. 37(1): 1-12.

Aubert, B. and S. Quilici. 1984. Biological control of the African and Asian citrus psyllids (Homoptera: Psylloidea), through eulophid and encyrtid parasites (Hymenoptera: Chalcidoidea) in Réunion Island. *In* S. M. Garnsey, L. W. Timmer, and J. A. Dodds (eds.) Proceedings of the 9th Conference of International Organization of Citrus Virologists. International Organization of Citrus Virologists, Riverside, CA, pp. 100-108.

Babendreier, D., F. Bigler, and U. Kuhlmann. 2006. Current status and constraints in the assessment of non-target effects, pp. 1-14. *In* F. Bigler, D. Babendreier, and U. Kuhlmann (eds.), Environmental impact of invertebrates for biological control of arthropods: methods and risk assessment. CABI Publishing, Wallingford, United Kingdom.

Bistline-East, A. and M.S. Hoddle. 2014. *Chartocerus* sp. (Hymenoptera: Signiphoridae) and *Pachyneuron crassiculme* (Hymenoptera: Pteromalidae) are obligate hyperparasitoids of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and possibly *Tamarixia radiata* (Hymenoptera: Eulophidae). Fla. Entomol. 97(2): 562-566.

Bové, J.M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J. Pl. Pathol. 88(1): 7-37.

Chien, C.C., S.C. Chiu, and S.C. Ku. 1989. Biological control of *Diaphorina citri* in Taiwan. Fruits 44: 401-407.

Denoth, M., L. Frid, and J.H. Myers. 2002. Multiple agents in biological control: improving the odds? Biol. Cont. 24: 20-30.

Grafton-Cardwell, E. 2010. How serious is the threat of Asian citrus psyllid and Huanglongbing? Citrograph 1: 8-10.

Gutierrez, A.P. and L. Ponti. 2013. Prospective analysis of the geographic distribution and relative abundance of Asian citrus psyllid (Hemiptera: Liviidae) and citrus greening disease in North America and the Mediterranean Basin. Fla. Entomol. 96(4): 1375-1389.

Halbert, S.E. and K.L. Manjunath. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: a literature review and assessment of risk in Florida. Fla. Entomol. 87(3): 330-349.

Hall, D.G., M.G. Hentz, and R.C. Adair. 2008. Population ecology and phenology of *Diaphorina citri* (Hemiptera: Psyllidae) in two Florida citrus groves. Environ. Entomol. 37: 914-924.

Hall, D.G., H.L. Richardson, E. Ammar, and S.E. Halbert. 2013. Asian citrus psyllid, *Diaphorina citri*, vector of citrus huanglongbing disease. Entomol. Exp. Appl. 146: 207-223.

Hoddle, M.S. 2012. Foreign exploration for natural enemies of Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Pysllidae), in the Punjab of Pakistan for use in a classical biological control program in California USA. Pak. Entomol. 34: 1-5.

Hoddle, C.D., M.S. Hoddle, and S.V. Triapitsyn. 2013. *Marietta leopardina* (Hymenoptera: Aphelinidae) and *Aprostocetus* (*Aprostocetus*) sp. (Hymenoptera: Eulophidae) are obligate hyperparasitoids of *Tamarixia radiata* (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae). Fla. Entomol. 96(2): 643-646.

Hoddle, M.S. and R. Pandey. 2014. Host Range Testing of *Tamarixia radiata* (Hymenoptera: Eulophidae) sourced from the Punjab of Pakistan for classical biological control of *Diaphorina citri* (Hemiptera: Liviidae: Euphyllurinae: Diaphorinini) in California. J. Econ. Entomol. 107 : 125-136.

Hodges, A.W. and T.H. Spreen. 2012. Economic impacts of citrus greening (HLB) in Florida, 2006/07-2010/11. EDIS document FE903, Food and Resource Economics Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL. (http://edis.ifas.ufl.edu) (accessed 5 August 2014).

Kuhlmann, U., U. Schaffner, and P.G. Mason. 2006. Selection of non-target species for host specificity tests, pp. 15-37. *In* F. Bigler, D. Babendreier, and U. Kuhlmann (eds.), Biological control of arthropods using invertebrates: methods for environmental risk assessment. CABI Publishing, Wallingford, United Kingdom.

Kumagai, L.B., C.S. LeVesque, C.L. Blomquist, K. Madishetty, Y. Guo, P.W. Woods, S. Rooney-Latham, J. Rascoe, T. Gallindo, D. Schnabel, and M. Polek. 2013. First report of *Candidatus* Liberibacter asiacticus associated with citrus huanglongbing in California. Plant Dis. 97: 283.

Morgan, D., G. Radebough, and A. Muniz. 2014. ACP biological control moves toward grid release strategy. Citrograph, 5(2): 63-67.

Pelz-Stelinski, K.S., R.H. Brlansky, T.A. Ebert, and M.E. Rogers. 2010. Transmission Parameters for *Candidatus* Liberibacter asiaticus by Asian Citrus Psyllid (Hemiptera: Psyllidae). J. Econ. Entomol. 103(5): 1531-1541.

Percy, D. M., A. Rung, and M.S. Hoddle. 2012. An annotated checklist of the psyllids of California (Hemiptera: Psylloidea). Zootaxa 3193: 1-27.

Quezada, J.R. and P. DeBach. 1973. Bioecological and population studies of the cottony-cushion scale, *Icerya purchasi* Mask., and its natural enemies, *Rodolia cardinalis* Mul. and *Cryptochaetum iceryae* Will., in southern California. Hilgardia 41: 631-688.

Qureshi, J.A. and P.A. Stansly. 2009. Exclusion techniques reveal significant biotic mortality suffered by Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae) populations in Florida citrus. Biol. Control 50(2): 129-136.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Richards, T.J., D.W. Shanafelt, and E.P. Fenichel. 2014. Foreclosures and invasive insect spread: the case of Asian citrus psyllid. Amer. J. Agr. Econ. 96: 615-630.

Rohrig, E.A., D.G. Hall, J.A. Qureshi, and P.A. Stansly. 2012. Field release in Florida of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae), an endoparasitoid of *Diaphorina citri* (Homoptera: Psyllidae), from mainland China. Fla. Entomol. 95(2): 479-481.

Rohrig, E., P.D. Shirk, D.G. Hall, and P.A. Stansly. 2011. Larval Development of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae), an endoparasitoid of *Diaphorina citri* (Hemiptera: Psyllidae). Ann. Entomol. Soc. Am. 104: 50-58.

Simmons, G., D. Morgan, M.S. Hoddle, R. Pandey, A. Soper, R. Stouthamer, C.D. Hoddle, A. Bistline, R. Zhao, B. Munoz, M. Pitcairn, B. Taylor, and V. Mellano. 2013. Update on the Asian citrus psyllid cooperative biological control program. Citrograph 4(5): 40-48.

Skelley, L.H. and M.A. Hoy. 2004. A synchronous rearing method for the Asian citrus psyllid and its parasitoids in quarantine. Biol. Cont. 29: 14-23.

Sule, H., R. Muhamad, D. Omar, and A.K.W. Hee. 2014. Parasitism rate, host stage preference and functional response of *Tamarixia radiata* on *Diaphorina citri*. Int. J. Agric. Biol. 16(4): 783-788.

van Lenteren, J.C., M.J.W. Cock, T.S. Hoffmeister, and D.P.A. Sands. 2006. Host specificity in arthropod biological control, methods for testing and interpretation of data, pp. 38-63. *In* F. Bigler, D. Babendreier, and U. Kuhlmann (eds.), Environmental impact of invertebrates for biological control of arthropods: methods and risk assessment. CABI Publishing, Wallingford, United Kingdom.

Wang, N. and P. Trivedi. 2013. Citrus huanglongbing: a newly relevant disease presents unprecedented challenges. Phytopathology 103: 652-663.

Zuk, M. and G.R. Kolluru. 1998. Exploitation of sexual signals by predators and parasitoids. Q. Rev. Biol. 73: 415-438.

Chapter 2. *Chartocerus* sp. (Hymenoptera: Signiphoridae) and *Pachyneuron crassiculme* (Hymenoptera: Pteromalidae) are obligate hyperparasitoids of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and possibly *Tamarixia radiata* (Hymenoptera: Eulophidae)

Allison Bistline-East and Mark S. Hoddle

Abstract

Two species of suspected hyperparasitoids, *Chartocerus* sp. and *Pachyneuron crassiculme*, emerged from parasitized *Diaphorina citri* nymphs collected in Punjab Pakistan over 15 - 22 April 2013. Exposure tests conducted in quarantine on *D. citri* nymphs parasitized by *Tamarixia radiata* and *Diaphorencyrtus aligarhensis*, as well as unparasitized *D. citri* nymphs, confirmed that *Chartocerus* sp. and *P. crassiculme* are hyperparasitoids. Both *Chartocerus* sp. and *P. crassiculme* successfully reproduced on *D. aligarhensis*, with one instance of *P. crassiculme* reproducing on *T. radiata*. There was no emergence from unparasitized *D. citri*.

Introduction

Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), was discovered in California USA in 2008. *D. citri* vectors '*Candidatus* Liberibacter asiaticus', a putative causative agent of huanglongbing (HLB), a lethal disease of citrus (Hoffman et al. 2013, Wang & Trivedi 2013). HLB was detected in California in March 2012 (Leavitt 2012). To mitigate the threat posed by *D. citri*-HLB to California's citrus industry, a biological control program using *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) sourced from Pakistan was initiated (Hoddle 2012). *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal) (Hymenoptera: Encyrtidae), a second parasitoid of *D. citri* also collected from Pakistan, is currently in quarantine at the University of California, Riverside (UCR).

Figure 2.1. *Chartocerus* sp. male (A) and female (B).

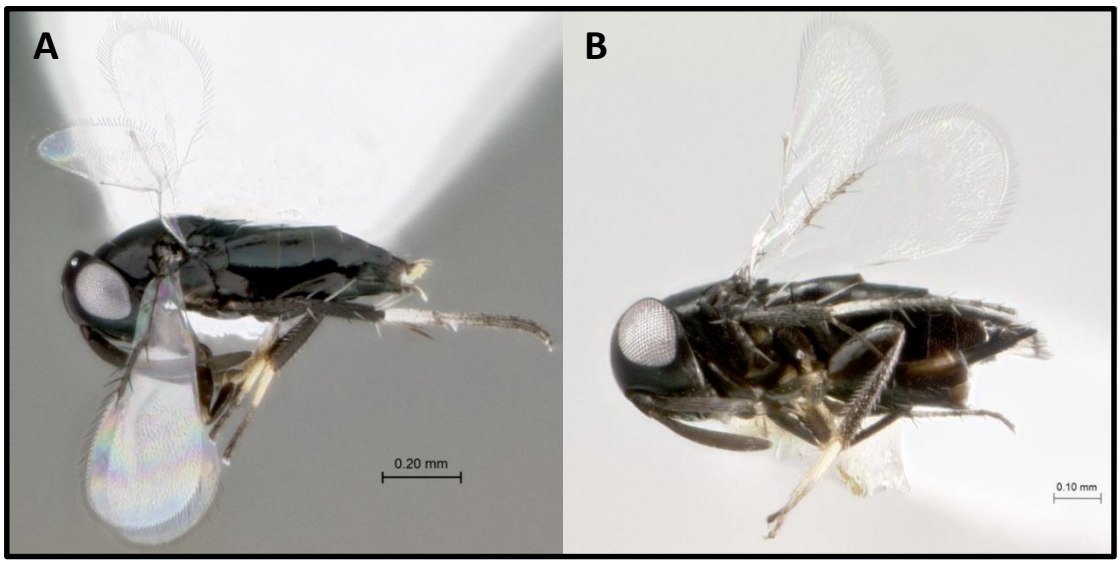
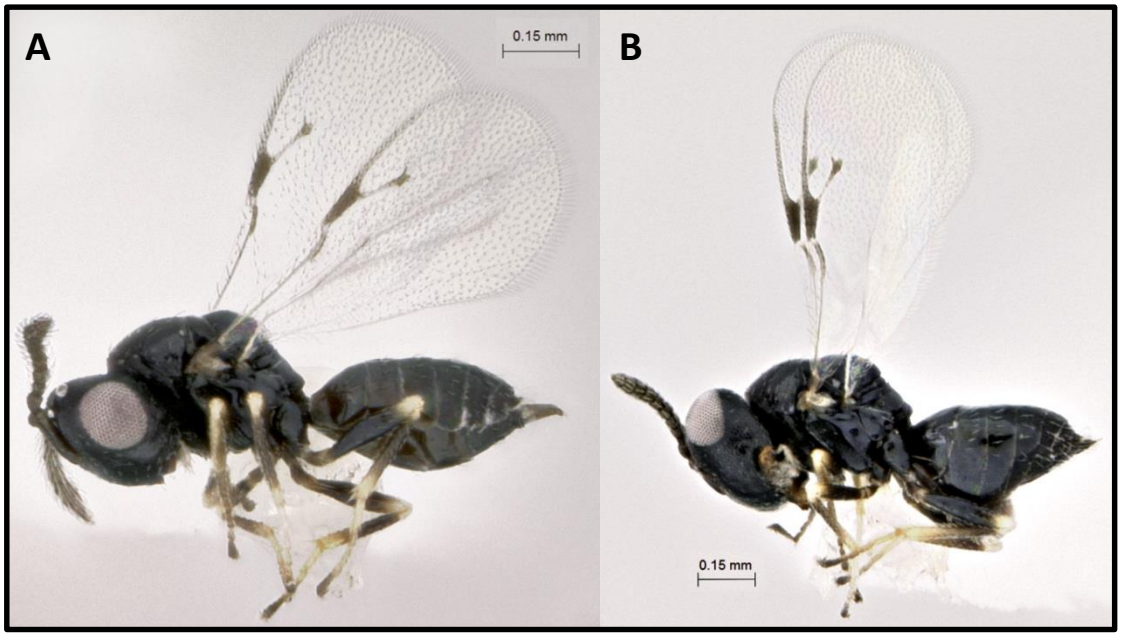


Figure 2.2. *Pachyneuron crassiculme* male (A) and female (B).



Materials and Methods

Parasitized *D. citri* host material returned from Punjab Pakistan to quarantine at UCR (15 - 22 April 2013) yielded previously collected *T. radiata* and *D. aligarhensis*, along with several species of known (*Marietta leopardina* Motschulsky [Hymenoptera: Aphelinidae], *Aprostocetus* (*Aprostocetus*) sp. [Hymenoptera: Eulophidae] [Hoddle et al., 2013]) or suspected (*Chartocerus* sp. [Hymenoptera: Signiphoridae], *Pachyneuron crassiculme* Waterston [Hymenoptera: Pteromalidae] and *Psyllaphycus diaphorinae* [Hymenoptera: Encyrtidae]) hyperparasitoids.

Experimental Design. To confirm that *Chartocerus* sp. (Fig. 2.1A. male, B. female) and *P. crassiculme* (Fig. 2.2A. male, B. female) are not primary parasitoids of *D. citri*, exposure trials using 10 sets of 4 - 7 *Chartocerus* sp. and 10 pairs of 1 male and 1 female *P. crassiculme* that emerged from material collected in Pakistan were rotated through each of 4 treatment types between 26 April and 24 May, 2013 in quarantine at UCR. It was not possible to reliably sex live *Chartocerus* sp., so this species was exposed in groups (assumed to contain at least 1 female each) unless a pair was otherwise observed mating. Exposure treatments consisted of: (A) nymphs parasitized by *T. radiata* (n = 8 replicates of 5 - 10 parasitized nymphs for *Chartocerus* sp. and 9 replicates of 5 parasitized nymphs for *P. crassiculme*), 5-9 days post-exposure to *T. radiata*; (B) nymphs parasitized by *D. aligarhensis* (n = 8 replicates of 5 - 10 parasitized nymphs for *Chartocerus* sp. and 10 replicates of 5 for *P. crassiculme*), 10 - 14 days post-exposure to

D. aligarhensis; (C) unparasitized third to fourth instar *D. citri* nymphs (*n* = 9 replicates of 5 - 10 unparasitized nymphs for *Chartocerus* sp. and 10 replicates of 5 nymphs for *P. crassiculme*); and (D) each of the 3 previously listed host types (A, B, and C) presented simultaneously in a choice cage (*n* = 9 replicates of 5-10 of each host type for *Chartocerus* sp. and 9 replicates of 5 of each host type for *P. crassiculme*).

Each replicate was comprised of host material for each treatment type exposed to a group of potential hyperparasitoids for 24 hours each. Hosts were exposed sequentially in a different order for each replicate to prevent bias due to presentation order. Emergence rates of *T. radiata* (*n* = 5 parasitized nymphs on each of 10 cuttings) and *D. aligarhensis* (*n* = 5 parasitized nymphs on each of 10 cuttings) determined baseline mortality for primary parasitoids in the absence of hyperparasitoids. Unparasitized *D. citri* nymphs (*n* = 5 fourth instar nymphs on each of 10 plants) provided data on nymph mortality in the absence of hyperparasitoids. Mummies of *T. radiata* and *D. aligarhensis* used in exposure experiments were sourced from colonies maintained in quarantine at UCR.

D. citri nymphs parasitized by either *T. radiata* or *D. aligarhensis* for no-choice treatments were presented on small *Citrus volkameriana* cuttings. *C. volkameriana* seedlings grown in 114 mL Cone-tainers^{TM} (SC7 Stubby, 3.8 cm diameter, Stewe and Sons Inc., Oregon) and infested with *D. citri* nymphs were used to expose unparasitized *D. citri* nymphs to *Chartocerus* sp. and *P. crassiculme*. Clear plastic vials (Thornton Plastic Co. 148 mL capacity, Salt Lake City, Utah) with three 12 mm diameter ventilation

holes covered with ultra-fine organza were inverted and placed over the top of the plant and fitted into the corresponding vial lid, which had a hole cut in the center to allow it to be fitted around the cone (Irvin et al. 2009).

Choice treatments were set up in 30 cm x 15.3 cm x 15.3 cm (h x w x d) clear plastic boxes (S&W Plastics, Riverside, California) with a 30 cm sleeve sewn from no-see-um netting (Skeeta Mosquito & Other Insect Protection Products, Bradenton, Florida). Unparasitized *D. citri* nymphs in Cone-tainers and *T. radiata*- and *D. aligarhensis*-parasitized nymphs on *C. volkameriana* cuttings in water were placed in the cage without ventilated vials on top to allow free access to all three host types simultaneously. After 24 h, each host type was enclosed with an inverted ventilated vial to contain all insects that emerged from each host type. All experiments were conducted in quarantine at UCR's Insectary and Quarantine Facility, at 27°C, 40% RH, and 14:10 h L:D. Replicates were observed daily after initial exposure, and total numbers of each emerged species were recorded per treatment.

Results

No choice treatments resulted in *Chartocerus* sp. reproducing successfully only on *D. aligarhensis* (Table 2.1). Mean emergence time for *Chartocerus* sp. offspring from *D. aligarhensis* was 18.36 d ± 2.34 (SE). *P. crassiculme* produced progeny on *D. aligarhensis* and *T. radiata* in no choice treatments, though parasitism was much higher on *D. aligarhensis* (Table 2.2). Mean emergence times for males and females were 12.83 d ± 2.48 (SE) and 11.33 d ± 2.05 (SE), respectively. *P. crassiculme* had a single male emerge from *T. radiata* after 11 d. Emergence rates for control treatments of *T. radiata, D. aligarhensis*, and *D. citri* were 84%, 88%, and 88%, respectively. *Chartocerus* sp. and *P. crassiculme* failed to reproduce on unparasitized *D. citri* nymphs.

Immature *D. aligarhensis* exposed to *Chartocerus* sp. in no choice tests experienced 47% parasitism, 17% died from undetermined causes, 3% were unaccounted for, and 33% emerged as adult *D. aligarhensis*. In 20% of trials (i.e., 2 of 10 replicates) *Chartocerus* sp. exhibited superparasitism, with 11 adults emerging from 9 *D. aligarhensis* mummies in one replicate, and 6 adults emerging from 3 mummies in the second. In no choice tests, immature *T. radiata* exposed to *Chartocerus* sp. exhibited 0% parasitism, 27% of mummies died from unknown causes, 6% disappeared, and 67% emerged as adult *T. radiata*.

In no choice tests where *P. crassiculme* was exposed to immature *D. aligarhensis*, 28% of hosts were parasitized by *P. crassiculme*, 19% died from unknown causes, and 53% emerged as adult *D. aligarhensis*. On *T. radiata*, *P. crassiculme* successfully parasitized only 2% of host material (i.e., one host), 40% died from unknown causes, 7% were unaccounted for, and 51% emerged as adult *T. radiata*. Unknown mortality may be attributable to superparasitism, host feeding, or a combination of both by *P. crassiculme*.

There was no successful parasitism of any host in choice tests for either *Chartocerus* sp. or *P. crassiculme*. However, elevated mortality rates were observed for *T. radiata* (26% when exposed to *Chartocerus* sp.; 28% for *P. crassiculme*) and *D. aligarhensis* (29%; 13%). In comparison, control mortality for *T. radiata* and *D. aligarhensis* were < 13% in the absence of these hyperparasitoids. When viewed collectively, data from exposure trials demonstrates that *Chartocerus* sp. and *P. crassiculme* are obligate hyperparasitoids within the *D. citri-Tamarixia-Diaphorencyrtus* system, and they are likely specialists on *D. aligarhensis*. Immediately following the conclusion of trials, all *Chartocerus sp.* and *P. crassiculme* material was killed in quarantine and preserved in 95% ethanol.

Table 2.1. Emergence and mortality rates for *Chartocerus* sp. exposed to unparasitized third and fourth instar *D. citri* nymphs, and nymphs parasitized by *T. radiata*, and *D. aligarhensis* in no-choice and choice treatments.

	No Choice					Choice			
Host	Total No. Exposed	% Host Emergence	% Parasitism	% Dead[5]	% Missing[6]	Total No. Exposed	% Host Emergence	% Dead[5]	% Missing[6]
D. citri	65	72.31%[1]	0.00%	9.23%	18.46%	65	81.54%[1]	13.85%	4.61%
T. radiata	67	67.16%[2]	0.00%	26.87%	5.97%	62	66.13%[2]	25.81%	8.06%
D. aligarhensis	60	33.33%[3]	46.67%[4]	16.67%	3.33%	65	53.85%[3]	29.23%	16.92%

[1]Percentage of *D. citri* adults that emerged from unparasitized nymphs
[2]Percentage of *T. radiata* adults that emerged from parasitized nymphs
[3]Percentage of *D. aligarhensis* adults that emerged from parasitized nymphs
[4] Percentage of hosts killed by parasitism. Actual number of host killed = 28; actual number of *Chartocerus* sp. adults emerged = 33, demonstrating 2 observed instances of superparasitism.
[5] Percentage of hosts found dead
[6] Percentage of hosts unaccounted for at time of data collection

Table 2.2. Emergence and mortality rates for *Pachyneuron crassiculme* exposed to unparasitized third and fourth instar *D. citri* nymphs, and nymphs parasitized by *T. radiata* and *D. aligarhensis* in no-choice and choice treatments.

	No Choice					Choice			
Host	Total No. Exposed	% Host Emergence	% Parasitism[4]	% Dead[5]	% Missing[6]	Total No. Exposed	% Host Emergence	% Dead[5]	% Missing[6]
D. citri	50	68.00%[1]	0.00%	24.00%	8.00%	45	73.33%[1]	13.33%	13.33%
T. radiata	45	51.11%[2]	2.22%	40.00%	6.67%	46	71.74%[2]	28.26%	0.00%
D. aligarhensis	53	52.83%[3]	28.30%	18.87%	0.00%	46	86.96%[3]	13.04%	0.00%

[1]Percentage of *D. citri* adults that emerged from unparasitized nymphs
[2]Percentage of *T. radiata* adults that emerged from parasitized nymphs
[3]Percentage of *D. aligarhensis* adults that emerged from parasitized nymphs
[4]Percentage of *Pachyneuron crassiculme* adults that successfully emerged from parasitized hosts
[5]Percentage of hosts found dead
[6]Percentage of hosts unaccounted for at time of data collection

Discussion

Assuming *Chartocerus* sp. and *P. crassiculme* are specialists on *D. aligarhensis* as these exposure trial data suggest, the frequency of *Chartocerus* sp. and *P. crassiculme* emergence in quarantine from material collected from Punjab Pakistan in April 2013 was significant in comparison to *D. aligarhensis* emergence rates. *Chartocerus* sp. (237 individuals reared), *P. crassiculme* (181), and *D. aligarhensis* (743) represented 20%, 16%, and 64% of material reared, respectively, within this complex. A total of 292 *T. radiata* were reared from April 2013 collections. Exposure trials suggest that the lower numbers of *T. radiata* obtained from Pakistan in April 2013 were not likely due to hyperparasitism.

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Table 2.3. Emergence rates of unparasitized third and fourth instar *D. citri* nymphs and nymphs parasitized by *T. radiata* and *D. aligarhensis* in control treatments not exposed to hyperparasitoids.

Host	Total No. Exposed	No. Adults Emerged	No. Dead Hosts[4]	No. Missing Hosts[5]
D. citri	60	44[1]	1	0
T. radiata	50	42[2]	6	2
D. aligarhensis	52	46[3]	3	1

[1]Total number of *D. citri* adults that matured from unparasitized nymphs
[2]Total number of *T. radiata* adults that emerged from parasitized nymphs
[3]Total number of *D. aligarhensis* adults that emerged from parasitized nymphs
[4]Total number of hosts found dead
[5]Total number of hosts unaccounted for at time of data collection

Table 2.4. Specimen accession numbers for all species used in exposure trials and deposited in the Entomology Museum at the University of California Riverside.

Species	Accession No.
D. citri[1]	UCRC_ENT00334428
T. radiata[2]	UCRC_ENT00334402-334418
D. aligarhensis[2]	UCRC_ENT00334426-334427
Chartocerus sp.[2]	UCRC_ENT00417173-00417182
P. crassiculme[2]	UCRC_ENT00417183-00417187

[1]Multiple individuals of Pakistani *D. citri* preserved in a single vial of 95% ethanol
[2]Point-mounted individuals

References

Hoddle, M.S. 2012. Has the Asian citrus psyllid parasitoid, *Tamarixia radiata*, established in California? http://cisr.ucr.edu/blog/asian-citrus-psyllid-2/radiata-established-in-california/ (Last accessed 23 Aug 2013).

Hoddle, M.S., and C.D. Hoddle. 2013. Classical biological control of Asian citrus psyllid with *Tamarixia radiata* in urban southern California. Citrograph 4(2): 52-58.

Hoddle, C.D., M.S. Hoddle, and S.V. Triapitsyn. 2013. *Marietta leopardina* (Hymenoptera: Aphelinidae) and *Aprostocetus* (*Aprostocetus*) sp. (Hymenoptera: Eulophidae) are obligate hyperparasitoids of *Tamarixia radiata* (Eulophidae) and *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae). Florida Entomol. 96: 643-646.

Hoffman, M.T., M.S. Doud, L. Williams, M. Zhang, F. Ding, E. Dtover, D. Hall, S. Zhang, L. Jones, M. Gooch, L. Fleites, W. Dixon, D. Gabriel, and Y. Duan. 2013. Heat treatment eliminates '*Candidatus* Liberibacter asiaticus' from infected citrus trees under controlled conditions. Phytopathology 103 (1), 15-22.

Irvin, N.A., M.S. Hoddle, and J. Suarez-Espinoza. 2009. The functional response of *Gonatocerus ashmeadi* and the 'new association' parasitoid *G. tuberculifemur* attacking eggs of *Homalodisca vitripennis.* Environ. Entomol. 38: 1634-1641.

Leavitt, R. 2012. Huanglongbing confirmed in California. Citrograph 3(3): 8-9.

Wang, N., and P. Trivdei. 2013. Citrus Huanglongbing: A newly relevant disease presents unprecedented challenges. Phytopathology 103(7): 652-665.

Chapter 3. Biology of *Psyllaphycus diaphorinae* Hayat (Hymenoptera: Encyrtidae), a hyperparasitoid of *Diaphorencyrtus aligarhensis* and *Tamarixia radiata*

Allison Bistline-East and Mark S. Hoddle

Abstract

The biology and ecology of *Psyllaphycus diaphorinae* Hayat, a potential natural enemy of Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is poorly understood. In April 2013, six *P. diaphorinae* females from Punjab, Pakistan emerged from parasitized ACP nymphs in quarantine at the University of California, Riverside and were used to found colonies. Contrary to previous claims, *P. diaphorinae* was found to be an obligate hyperparasitoid and not a primary parasitoid attacking ACP nymphs. *P. diaphorinae* was able to successfully reproduce on both *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Shafee, Alam, and Agarwal) (Hymenoptera: Encyrtidae) pupae. No reproduction on unparasitized ACP nymphs was observed. *D. aligarhensis* appears to be a preferred host in comparison to *T. radiata*, as *P. diaphorinae* produced a higher number of offspring and a higher proportion of females on *D. aligarhensis*. This preference supports results from previous studies documenting elevated rates of hyperparasitism by multiple species on *D. aligarhensis* throughout its native range.

Introduction

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), was first discovered in California (USA) in Imperial and San Diego counties in 2008, and in Los Angeles county in 2009 (Grafton-Cardwell 2010). Since these initial finds, pest populations expanded rapidly, and *D. citri* spread through urban areas in southern California. In 2014, ACP was detected in the San Joaquin Valley (the major citrus production region in California), and isolated finds as far north as San Jose and San Francisco have been documented (Civerolo 2015). The greatest threat posed by ACP is its ability to vector the bacterium *Candidatus* Liberbacter asiaticus (*C*Las), a fastidious phloem-limited bacterium that is one of the causative agents of huanglongbing (HLB), or "citrus greening disease" (Hall et al. 2013).

HLB is a lethal citrus disease which causes foliar dieback, irregular fruit ripening, premature fruit drop, and overall yield reductions. Trees may be asymptomatic for up to 2 years after initial infection, making HLB difficult to detect in its early stages. Infected trees usually die within 8 years of acquiring *C*Las (Halbert and Manjunath 2004). In March 2012, the first *C*Las-positive citrus tree was found in a residential property in Hacienda Heights, Los Angeles County (Kumagai et al. 2013). To date, this is the only confirmed case of HLB in California. However, the ACP-HLB complex in California is considered a significant threat to the state's ~ US$3 billion per year citrus industry. The impact to California's citrus industry is expected to be similar to that experienced in

Florida, where ACP was discovered in 1998 and HLB was detected in 2005. Since this detection of HLB in Florida, ACP-HLB has caused over US$ 3.6 billion in losses to the Florida citrus industry, as well as the loss of over 6,000 jobs and an increase in citrus production costs of 40% (Keremane et al. 2015, Salifu et al. 2012).

In an attempt to reduce the ACP-HLB threat to California, a classical biological control program using host specific natural enemies from ACP's home range was initiated with the intent of suppressing psyllid populations in urban areas where ACP populations are greatest. By reducing the number of psyllids capable of acquiring and transmitting *C*Las, there is a reduced likelihood that healthy trees will be inoculated with bacteria. Six foreign exploration trips to Punjab, Pakistan were conducted between September 2010 and April 2013 to collect ACP natural enemies, in particular parasitoids that attack ACP nymphs. Punjab is purportedly within *D. citri*'s native range, and this region has a close climatic match to major citrus production regions of California (Hoddle 2012). Hussain and Nath (1927) indicated that a rich guild of ACP parasitoids could exist in Punjab, with up to nine species of primary parasitoids possibly attacking ACP nymphs. Several species of parasitoid were reared from ACP collected from Pakistan in quarantine at the University of California Riverside (UCR). From this material, two primary parasitoids were identified, *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* Shafee, Alam, and Agarwal (Hymenoptera: Encyrtidae), both of which have been used previously for ACP biocontrol (Halbert and Manjunath 2004). These two parasitoids are being utilized in

California in a classical biological control program targeting ACP (Hoddle and Pandey 2014, Bistline-East et al. 2015).

One of the goals of the ACP biocontrol program in California was to identify the remaining seven primary parasitoid species in Punjab alluded to by Hussain and Nath (1927) with the intent of potentially replicating this native parasitoid guild in California. Other parasitoid species reared from parasitized ACP nymphs in quarantine at UCR included *Aprostocetus* (*Aprostocetus*) sp. (Hymenoptera: Eulophidae), *Marietta leopardina* Motschulsky (Hymenoptera: Aphelinidae), *Chartocerus* sp. (Hymenoptera: Signiphoridae), *Pachyneuron crassiculme* Waterston (Hymenoptera: Pteromalidae), and *Psyllaphycus diaphorinae* Hayat (Hymenoptera: Encyrtidae). Through a series of systematic exposure trials, *Aprostocetus* (*Aprostocetus*) sp., *M. leopardina*, *Chartocerus* sp., and *P. crassiculme* were demonstrated to be obligate hyperparasitoids of either or both *T. radiata* and *D. aligarhensis* (Hoddle et al. 2013, Bistline-East and Hoddle 2014, Hoddle et al. 2014). The guild of nine unidentified parasitoid species documented by Hussain and Nath (1927) in Punjab does not appear to exist. The high estimate of parasitoid species attacking ACP nymphs likely resulted from the mis-association of parasitoids with ACP that emerged from other very small plant-feeding insects (e.g., parasitoids which emerged from leaf hopper or lace bug eggs) that co-infested citrus foliage with ACP nymphs (Hoddle et al. 2014)

P. diaphorinae was originally described by Hayat (1972), based on an examination of 4♂ 6♀ specimens collected in the Punjab region of India (a region

immediately adjacent to Punjab, Pakistan) in 1968. These described specimens were reared from parasitized *Diaphorina aegyptica* (= *D. cardiae* Crawford) nymphs (Hayat 1972). As Hayat originally described *P. diaphorinae* as an uncommon primary parasitoid on *D. citri* there was interest in assessing the potential of this natural enemy for the classical biological control of ACP in California.

P. diaphorinae (*n* = 3♂ 7♀) recovered from an October 2011 collecting trip to Punjab Pakistan were preserved, slide-mounted, and compared with the original holotype and paratype specimens described by Hayat (1972). Minor morphological differences between specimens from India and Pakistan were observed, but the Pakistan-collected specimens were confirmed morphologically as *P. diaphorinae* (Triapitsyn *et al.* 2013). Material returned from an April 2013 collecting trip to Punjab Pakistan yielded the highest number of live *P. diaphoroinae* individuals (*n* = 6♀). Because there is almost no information on the biology and ecology of *P. diaphorinae*, the assumption that this wasp is a primary ACP parasitoid (and possibly of other psyllid species [Hayat 1972, Mehrnejad and Emami 2005, Triapitsyn et al. 2013]) needed to be confirmed. Experiments investigating host utilization (i.e., unparasitized ACP nymphs and ACP mummies parasitized by *T. radiata* and *D. aligarhensis*) by *P. diaphorinae* are detailed here in an effort to better understand the reproductive biology of this parasitoid.

Materials and Methods

Material Sources for Experiments. Insects used as hosts in exposure trials (ACP, *T. radiata*, *D. aligarhensis*) were sourced from colonies maintained at the University of California Riverside Insectary and Quarantine Facility (UCR IQF). ACP colonies were established from field-collected material in Riverside, California and moved to UCR IQF under California Department of Food and Agriculture (CDFA) permit no. 2870. All ACP colonies were confirmed to be HLB-free through PCR analysis of 30 individual psyllids and reared on *Citrus volkameriana* V. Ten. & Pasq. (Rutaceae). Small citrus cuttings containing parasitized ACP nymphs ("mummies") collected from multiple sites in Punjab, Pakistan were hand carried under USDA-APHIS permit number P526P-11-00103 to UCR IQF (Hoddle 2012). ACP mummies maintained in small collection vials (no. 55- 3.5 dram, Thornton Plastic Co., Salt Lake City, UT) were held in an emergence cage (90 x 45 x 45 cm, length x width x height) and examined daily in quarantine. All parasitoids which emerged were collected in 200 μL O-ring microcentrifuge vials (model 89004-308, VWR, Radnor, PA) provisioned with wild clover honey, identified to species when possible, sexed, and tallied. *T. radiata* and *D. aligarhensis* recovered from these Pakistan collections were used to establish colonies on ACP nymphs in UCR IQF.

Six *P. diaphorinae* females (no males) were reared from ACP mummies collected in Pakistan between 23 April and 3 May 2013 and were used to establish colonies in

UCR IQF. Original Pakistan-collected and colony-reared *P. diaphorinae* were maintained in 200 µL microcentrifuge tubes at ~14°C on a diet of wild clover honey for use in experiments.

Preparation of Experimental Plants. ACP nymphs exposed to *P. diaphorinae* were presented on *C. volkameriana* seedlings grown in 114 mL plastic growing cones (Ray Leach Cone-tainers, SC7 Stubby, Stuewe and Sons Inc., Portland, OR) or on 1-2 year old *C. volkameriana* grown in 10.16 cm diameter pots contained within sleeve cages. Sleeve cages were constructed from clear U-shaped acrylic risers (S&W Plastics F2191, Riverside, CA) stacked vertically to form a rectangular cage 15 x 15 x 30 cm (width x depth x height) with two open sides. The open back was covered with white no-see-um netting (Skeeta, Bradenton, FL), and the front was fitted with a 30 cm sleeve sewn from no-see-um netting.

C. volkameriana seedlings in Cone-tainers were grown from seed and potted plants were obtained either as young bare-root trees from Willits and Newcomb, Inc. citrus nursery (Arvin, CA) or grown from seed at UCR Agricultural Operations (AgOps). Plants were grown at AgOps in greenhouses (27 ± 2°C; 50% RH; natural day length) using a modified UCR Type III potting mix with daily watering and Osmocote Pro granular fertilizer (The Scotts Company LLC, Marysville, OH) applied every 3 months. Potted *C. volkameriana* were pruned regularly to promote flush growth necessary to stimulate ACP oviposition (Hall et al. 2008), as well as to maintain them at sizes suitable for cages.

To conduct *P. diaphorinae*-ACP exposure trials, plants were transported from AgOps to a UCR IQF insectary-level laboratory for preparation. Cone-tainers with *C. volkameriana* seedlings were fitted with upholstery foam discs covering the soil to prevent emergence of soil borne insects and inverted 148 mL ventilated vials (Thornton Plastic Co., Salt Lake City, UT) were placed over seedlings to contain experimental insects on seedlings (Bistline-East et al. 2015). Ten psyllid nymphs, ranging from second to late fourth instars, were manually transferred with a fine-haired paintbrush from *D. citri* colony plants onto experimental plants and allowed to settle for at least 1 h before they were exposed to *P. diaphorinae*. Cages containing potted *C. volkameriana* infested with ACP nymphs were taken directly from the ACP colony and nymphs were not manipulated.

ACP mummies parasitized by *T. radiata* or *D. aligarhensis* were presented to *P. diaphorinae* on *C. volkameriana* cuttings. Each cutting had 15 mummies. *T. radiata* and *D. aligarhensis* larvae developing within parasitized ACP nymphs were 5 and 13 d old, respectively. Mummies were taken from colonies of these primary ACP parasitoids maintained in UCR IQF. Cuttings with mummies of each parasitoid species were placed in 148 mL ventilated vials for exposure to *P. diaphorinae*. All experimental vials were labeled and maintained within BugDorms (model 2120; MegaView Science, Taiwan) to prevent accidental escape of experimental insects.

Determination of Host Use by *P. diaphorinae*. No choice exposure trials were used to determine which species (i.e., ACP or ACP mummies containing developing *T.*

radiata, and *D. aligarhensis* larvae) could serve as hosts for *P. diaphorinae*. The original literature described *P. diaphorinae* as a primary parasitoid of *D. citri* (and possibly other psyllid species [Hayat 1972, Mehrnejad and Emami 2005, Triapitsyn et al. 2013]). To test this hypothesis, female *P. diaphorinae* reared from parasitized psyllid nymphs collected in Pakistan were presented with ACP nymphs on *C. volkameriana* plants. These exposure experiments used small ACP infested *C. volkameriana* seedlings in Cone-tainers in some replicates and larger ACP infested *C. volkameriana* in sleeve cages for other replicates.

Four Cone-tainers with *C. volkameriana* seedlings each infested with 10 ACP nymphs ranging from second to fourth instar were enclosed with an inverted ventilated vial and sequentially exposed to female *P. diaphorinae* for 24 h. Two *P. diaphorinae* females were selected from the cohort obtained from Pakistan, and were released together into the inverted vial of the Replicate 1 Cone-tainer for 24 h, then removed and immediately released in the Replicate 2 Cone-tainer for a subsequent 24 h. This process was repeated to complete four replicated exposures.

Four sleeve cage replicates containing individual *C. volkameriana* plants infested with approximately 150 - 200 second to fourth instar ACP nymphs were exposed to *P. diaphorinae* females that were released into cages for 2 - 4 d. These females were then transferred into a subsequent cage containing a *C. volkameriana* infested with mixed stages of *D. citri* nymphs for an additional 2 - 4 d. The number of *P. diaphorinae* females used in cage exposure experiments ranged from two to five, and depended on ACP density and the available number of *P. diaphorinae* not in use in other trials. Female *P.*

diaphorinae were stored in 200 µL microcentrifuge tubes with honey at 14°C until used in exposure experiments. ACP nymphs in both Cone-tainers and cages were monitored daily for *P. diaphorinae* emergence.

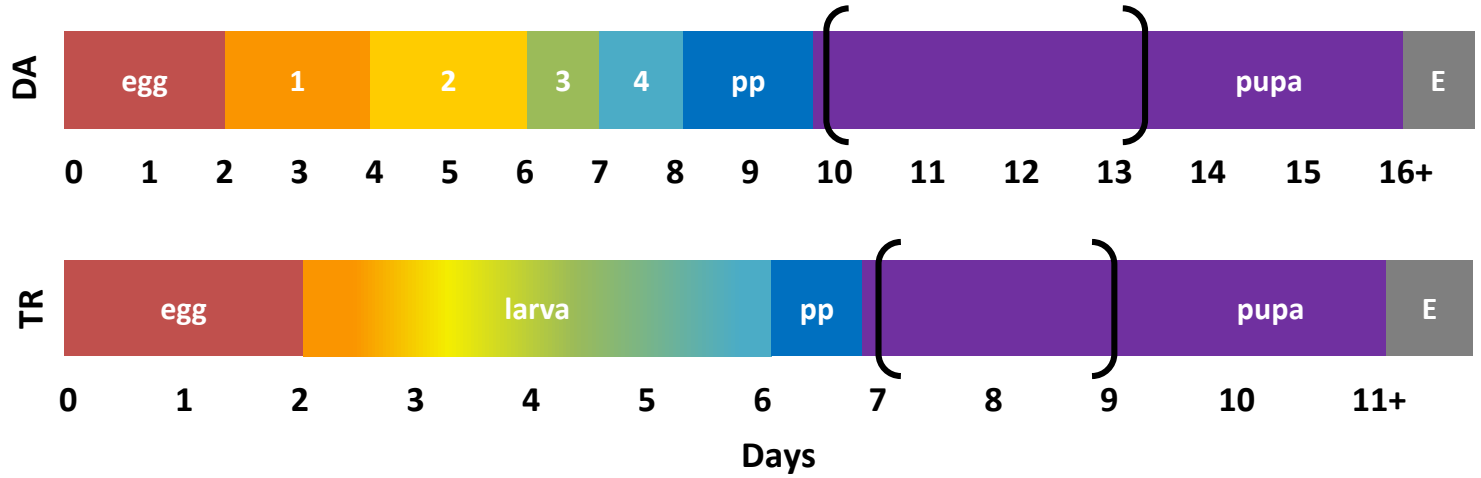
Following exposure to ACP nymphs, *P. diaphorinae* were sequentially exposed to *T. radiata* and *D. aligarhensis* mummies, and one additional replicate of unparasitized ACP nymphs. Two female *P. diaphorinae* were put into an exposure vial containing 15 *D. aligarhensis* mummies (14 d old) on citrus twigs excised from the *D. aligarhensis* colony. Parasitoids were allowed to forage for 24 h. The same two females were subsequently transferred to a second vial where they were given access to 15 *T. radiata* mummies (6 d old) on citrus twigs removed from the *T. radiata* colony and allowed to oviposit for an additional 24 h. For the third 24 h exposure period, females were removed from the *T. radiata* vial and put onto a *C. volkameriana* seedling infested with 10 third and fourth instar ACP nymphs in a Cone-tainer and enclosed with an inverted ventilated vial. Following the final exposure, parasitoids were removed and stored in honeyed vials at 14^{o}C. *D. aligarhensis* and *T. radiata* mummies were monitored daily for *P. diaphorinae* emergence. Parasitoids that emerged from different host exposure trials were counted, identified to species, and sexed.

Establishment of *P. diaphorinae* Colonies. Based on data from exposure trials (see Results), colonies of *P. diaphorinae* were maintained in acrylic sleeve cages (see above) containing potted *C. volkameriana* plants infested with ACP nymphs parasitized by *D. aligarhensis* or *T. radiata* at 27°C ± 2°, 40% RH, and a 14:10 h (L:D) photoperiod.

Plants in colony cages with *D. aligarhensis* or *T. radiata* hosts were prepared according to the following procedure for each host species. Preliminary studies assessing host use by *P. diaphorinae* revealed that this hyperparastoid may only parasitize the pupal stage of *T. radiata* and *D. aligarhensis* (Fig. 3.1) (ABE, unpublished). Consequently, pupae of these primary ACP parasitoids were exposed to *P. diaphorinae* in these studies.

T. radiata colony cages were created by exposing fourth instar *D. citri* nymphs (11 - 13 d old at 29ºC), a life stage preferred for parasitism by this parasitoid (Skelley and Hoy 2004), on potted *C. volkameriana* to approximately 10♂ 15♀ *T. radiata* for 3 - 4 d. Parasitized nymphs were held for 7 - 9 d following initial exposure to allow *T. radiata* larvae to develop to pupal stage within ACP hosts (Fig. 3.1). Approximately 5♂ 10♀ (or similar numbers in ~ 1:2 ratio, depending on host density) *P. diaphorinae* were released into cages and allowed to forage for *T. radiata* mummies for 3 - 4 d before being removed.

Figure 3.1. Developmental timelines of *Diaphorencyrtus aligarhensis* ("DA") and *Tamarixia radiata* ("TR"). Numbered stages refer to larval instars; "pp" = prepupa; "E" = eclosion. Brackets indicate ideal window for *Psyllaphycus diaphorinae* oviposition.



D. aligarhensis cages were prepared in a similar manner to *T. radiata*. Second to third instar *D. citri* nymphs (8 - 10 d old at 29ºC), the preferred life stage for parasitism by *D. aligarhensis* (Rohrig et al. 2011), were exposed to 10♂ 5♀ *D. aligarhensis* for 2 - 4 d. *D. aligarhensis* mummies were held for 10 d after initial exposure and matured to pupal stage (Fig. 3.1). Mated male and female *P. diaphorinae* in 1:2 or 1:3 ratio (depending on host density) were released into the cage and allowed to forage on *D. aligarhensis* mummies for 3 - 4 d before being removed.

Cages containing *D. aligarhensis* and *T. radiata* mummies exposed to *P. diaphorinae* were monitored daily for parasitoid emergence. The date of emergence, number, and sex of parasitoids that emerged were recorded per cage. *P. diaphorinae* offspring were maintained in microcentrifuge tubes with honey and stored at ~14°C and used for maintaining *P. diaphorinae* colonies.

Statistical Analysis to Determine Host Preference. Numbers of *P. diaphorinae* that emerged from *T. radiata* and *D. aligarhensis* host cages were compared across 20 generations. The number of *P. diaphorinae* offspring and development rates on different host species were compared to determine if host utilization by *P. diaphorinae* differed between primary parasitoid species. Because both emergence numbers and developmental rates from colony cages were not normally distributed and had significantly differing degrees of variance, comparisons were made using a non-parametric Wilcoxon rank-sum test with a 0.05 level of significance in R (R version 3.0.2, R Core Team 2013, The R Foundation for Statistical Computing, Vienna, Austria).

Measurement of *P. diaphorinae* Longevity. A subset of individuals from colonies were tracked to determine the average male and female *P. diaphorinae* longevity under colony conditions. Because greater numbers of *P. diaphorinae* were reared on *D. aligarhensis* than *T. radiata* (see Results), only individuals emerging from the *D. aligarhensis* host colony were used. Longevity of males and females were measured under solitary and paired conditions. Recently emerged *P. diaphorinae* (< 24 h old) were collected from *D. aligarhensis* cages and maintained in 200 µL microcentrifuge tubes with drops of wild clover honey. Solitary treatments were comprised of one male or female *P. diaphorinae* per vial. Vials with paired treatments each contained one male and one female *P. diaphorinae*. All experimental vials were maintained under colony conditions (27°C ± 2°, 40% RH, and a 14:10 h [L:D] photoperiod) and honey was refreshed as needed, approximately once per week.

Longevity vials were monitored daily until all *P. diaphorinae* died. Dead *P. diaphorinae* were preserved in 95% ethanol and stored at -4^{o}C. Date of emergence (i.e., the date *P. diaphorinae* was collected from a colony cage) and date of death were recorded for each individual and used to calculated age in days. Differences in longevity between solitary males, solitary females, paired males, and paired females were analyzed using a one-way ANOVA in R using a 0.05 significance level. A Tukey's HSD test with 0.05 significance level (analyzed in R) was used to determine which treatments differed significantly from one another.

Estimation of *P. diaphorinae* Fecundity. The average fecundity of *P. diaphorinae* females was evaluated by presenting vials of *D. aligarhensis* mummies (as this was the best host for this hyperparasitoids [see Results]) over the course of seven days, then monitoring mummies for *P. diaphorinae* and *D. aligarhensis* emergence. Mummy mortality, which could reflect hyperparasitoid host feeding, was also recorded. *C. volkameriana* cuttings with four to seven 9 - 15 d old *D. aligarhensis* mummies (i.e., pupae) were taken from *D. aligarhensis* colony cages and placed inside a sealed 148 mL ventilated plastic vial). One mated 1 d old female *P. diaphorinae* was placed in the vial and allowed to forage on *D. aligarhensis* mummies for 24 h. Females were removed and transferred to a subsequent vial with four to seven more 9 - 15 d old mummies for an additional 24 h. Each female was exposed to *D. aliagarhensis* mummies in one vial per day for 7 consecutive days. Five replicates, each utilizing a single mated female P. *diaphorinae* were performed exposing a total of 183 *D. aligarhensis* mummies.

Ten control vials with *D. aligarhensis* mummies not exposed to *P. diaphorinae* were run simultaneously with fecundity trials. Cuttings containing 5 *D. aligarhensis* mummies each were placed in ventilated vials and maintained under identical conditions. Control vials determined baseline *D. aligarhensis* emergence and mummy mortality rates under experimental conditions and handling procedures in the absence of exposure to *P. diaphorinae*.

All fecundity exposure vials were maintained under quarantine laboratory conditions (27°C ± 2°, 40% RH, and a 14:10 h (L:D) photoperiod) and vials were

maintained inside BugDorms. Experimental vials were monitored daily, and numbers of emerging *D. aligarhensis* and *P. diaphorinae* were recorded. The number of dead mummies was recorded. Mummies were classified as "dead" if there was no parasitoid eclosion within 3 wk of initial exposure. The mortality rate, represented by the number of mummies producing *P. diaphorinae* (i.e., the number of mummies hyperparasitized) + the number of dead mummies, was calculated across the five replicates for each trial date. Mortality rates were compared to rates of naturally-occurring mortality in control vials using a Fisher's Exact Test for count data in R. These analyses were conducted to determine if *D. aligarhensis* mortality was elevated when exposed to *P. diaphorinae*. Mean parasitism rates were compared between all trail dates in a pairwise manner using Games and Howell's Test in R, to evaluate whether *P. diaphorinae* parasitism levels varied through time.

Results

***P. diaphorinae* Development and Host Preference.** *P. diaphorinae* did not successfully parasitize ACP nymphs regardless of instar. However, *P. diaphorinae* parasitized immature *D. aligarhensis* and *T. radiata* developing inside ACP nymphs (Table 3.1). Initial exposure of unmated *P. diaphorinae* reared in UCR IQF from ACP nymphs collected in Pakistan to *D. aligarhensis* resulted in 10♂ *P. diaphorinae* and 5 dead mummies. *T. radiata* exposure yielded 5♂ *P. diaphorinae* and 3♂ 7♀ *T. radiata*,

with no observed mummy mortality. *P. diaphorinae* offspring emerged over a 3 d period between 10 and 13 d after exposure to *D. aligarhensis* mummies, and 12 d after exposure to *T. radiata* mummies. Males (n = 15) produced in these exposure trials were mated with the original females from Pakistan, and these newly-mated females were used to found two colonies of *P. diaphorinae*, one maintained on *T. radiata* and a second on *D. aligarhensis*.

Daily emergence for *P. diaphorinae* on both parasitoid hosts was recorded through 20 generations. *T. radiata* colonies produced 458 male and 265 female *P. diaphorinae* (n = 52 cage replicates; 37% female sex ratio), and *D. aligarhensis* colonies yielded 1,077 males and 1,596 females (n = 80 cages; 60% female sex ratio) between May 2013 and February 2015 (Table 3.2). Mean time to emergence for *P. diaphorinae* males and females reared on *T. radiata* were 16 ± 0.15 d (SE) and 18 ± 0.24 d (SE), respectively. Male and female *P. diaphorinae* emerged from *D. aligarhensis* colony cages after an average of 16 ± 0.09 d (SE) and 17 ± 0.075 d (SE), respectively.

P. diaphorinae produced more offspring and developed faster on *D. aligarhensis* than on *T. radiata*. *D. aligarhensis* colony cages produced significantly higher numbers of *P. diaphorinae* per cage than *T. radiata* ($P < 0.0001$). The number of males produced per cage was significantly elevated on *D. aligarhensis* ($P < 0.001$), as well as the difference in the number of females produced from *D. aligarhensis* compared to *T. radiata* ($P < 0.0001$) (Fig. 3.2).

Table 3.1. Exposure trial results of *Psyllaphycus diaphorinae* to Asian citrus psyllid, *Tamarixia radiata*, and *Diaphorencyrtus aligarhensis*.

Host	Total No. Exposed	Total Emergence Numbers			No. Dead Hosts
		P. diaphorinae	Adult ACP	Primary Parasitoid	
ACP nymphs[1]	50	0	46	0	3
T. radiata	15	5	0	10	0
D. aligarhensis	15	10	0	0	5

[1]One ACP nymph could not be accounted for, so was not included in counts as either adult or dead.

Table 3.2. *Psyllaphycus diaphorinae* emergence on *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* in colony over 20 generations.

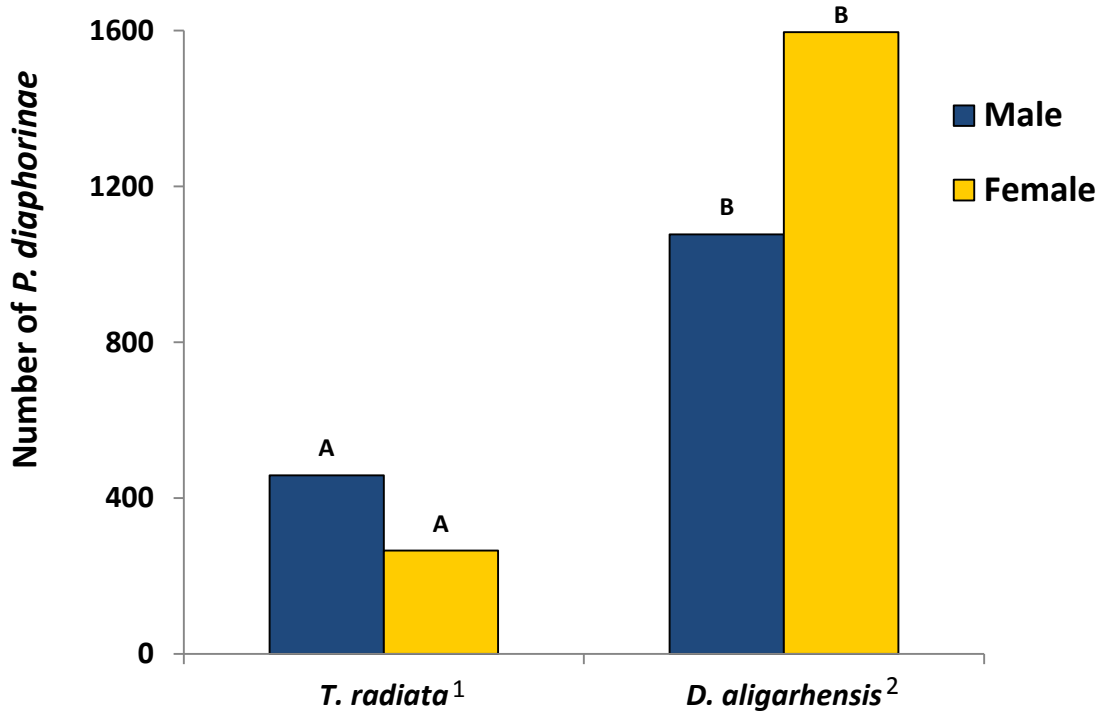
Host	Mean Host Age (days)	Emergence Numbers			Mean Development Time (days)[1]	
		Male	Female	Total	Male	Female
T. radiata	5 (± 0.18 SE)	458	265	723	16 ± 0.15	18 ± 0.24
D. aligarhensis	11 (± 0.19 SE)	1,077	1,596	2,673	16 ± 0.09	17 ± 0.075

[1]Mean developmental time of *P. diaphorinae* ± Standard Error

Overall developmental rate was significantly faster on *D. aligarhensis* compared to *T. radiata* (P = 0.003). Individually, males (P = 0.04) and females (P < 0.0001) developed significantly faster on *D. aligarhensis* than on *T. radiata*. Taken together, these data suggest that *D. aligarhensis* is a better host for *P. diaphorinae* within the ACP-*Tamarixia-Diaphorencyrtus* complex in its home range.

***P. diaphorinae* Longevity.** Both male and female *P. diaphorinae* lived longer, on average, in paired treatment vials compared with those in solitary vials. Males and females had a mean longevity of 17 ± 2.5 d (SE) and 20 ± 2.7 d (SE), respectively, in solitary vials. Paired males had a mean longevity of 20 ± 2.1 d (SE) and paired females had a mean of 30 ± 2.5 d (SE) (Fig. 3.3). A one-way ANOVA returned a significant difference (F = 3.834, df = 3, P = 0.014) between the four treatment types (solitary male, solitary female, paired male, and paired female). However, the only significant difference in longevity when pairs of treatments were compared was solitary males and paired females (P = 0.02) (Fig. 3.3)

Figure 3.2. Total number of *Psyllaphycus diaphorinae* emerged from *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* in colony. Different letters above bars indicate significant differences yielded from Wilcoxon rank-sum test ($P < 0.0001$).



[1]Emergence on *T. radiata*: $n = 458$ (male), $n = 265$ (female)
[2]Emergence on *D. aligarhensis*: $n = 1{,}077$ (male), $n = 1{,}596$ (female)

Estimation of *P. diaphorinae* Fecundity. Hyperparasitism by *P. diaphorinae* occurred in every replicate on every day of exposure except days 4 and 5. Because all replicates were run simultaneously and mummies were sourced from the same colony cages on each day, it was determined that the likely reason for this lack of parasitism is that the mummies used on days 4 and 5 were slightly too young to be successfully parasitized. Because of this inability for *P. diaphorinae* to utilize these nymphs, days 4 and 5 were excluded from statistical analyses. There was no host survivorship in any replicate when *D. aligarhensis* mummies were exposed to *P. diaphorinae* females. Most mortality was due to hyperparasitism. However, some mummies (primarily those from days 4 and 5) died from other causes, which may indicate that *P. diaphorinae* females exhibit host feeding behavior, or mortality resulted from unsuccessful attempts at parasitism. In control vials, only 3 mummies failed to emerge and were classified as dead. One mummy went missing, and 46 produced live *D. aligarhensis*.

The mean daily fecundity of *P. diaphorinae* females was 4 ± 0.25 progeny (SE), out of an average of 5 mummies (range, 4 - 7 mummies) exposed per day (Fig. 3.4). Across the five analyzed days, mean total fecundity measured was 20 ± 1.39 progeny (SE), out of a total average of 26 (± 1 SE) mummies exposed. Compared to control treatments, mortality rates in each replicate were significantly elevated ($P < 0.0001$). However, there was no significant difference in the mean amount of parasitism between any of the seven days of the trial.

Figure 3.3. Longevity of *Psyllaphycus diaphorinae* males and females reared from *Diaphorencyrtus aligarhensis* held in honeyed vials either individually or as male-female pairs. Different letters above bars indicate significant differences in longevity (P = 0.02) as analyzed by Tukey HSD pairwise comparison.

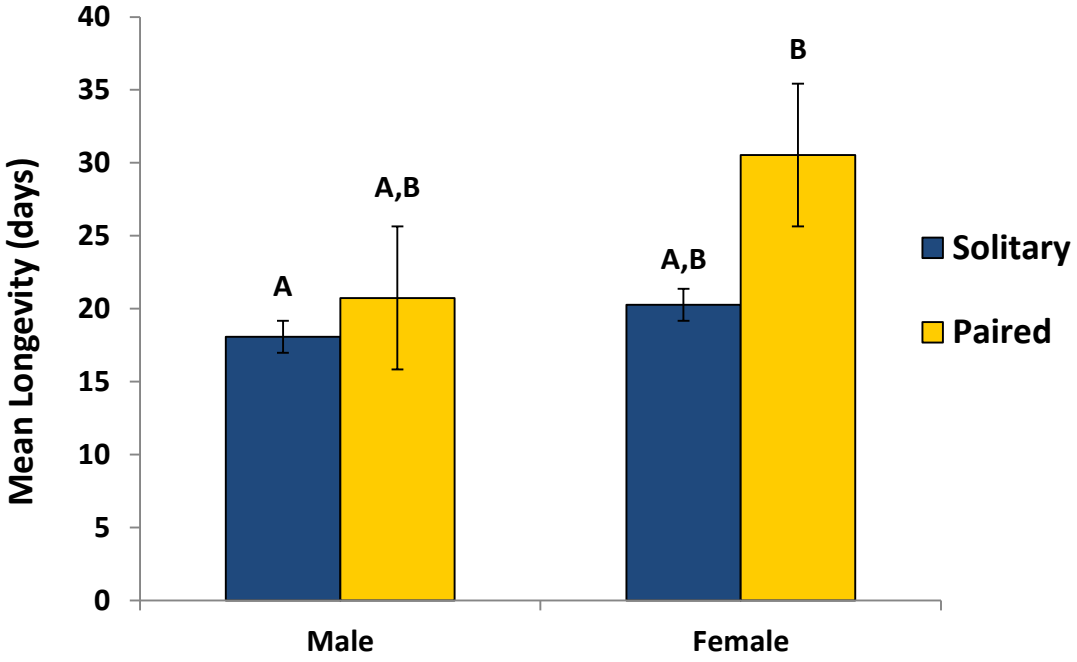
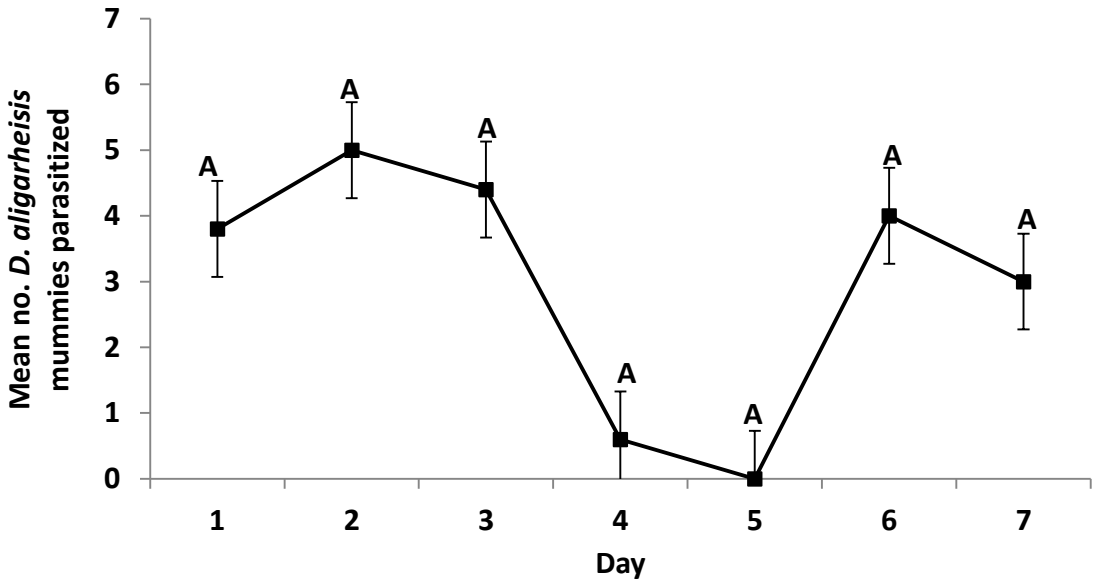


Figure 3.4. Daily mean (± SE) *Psyllaphycus diaphorinae* female fecundity on *D. aligarhensis* mummies over a 7 day period.



Discussion

Hussain and Nath (1927) stated that a probable guild of up to nine primary parasitoids of ACP may exist in Punjab, Pakistan. This number is almost certainly an overestimate (Hoddle et al. 2014), possibly due in part, to the recording of unidentified species of hyperparasitoids emerging from ACP mummies. One of these unnamed species reared by Hussain and Nath (1927) was likely *P. diaphorinae*. To date, four species likely comprising a portion of these original nine have been experimentally tested and conclusively discounted as primary parasitoids (Bistline-East and Hoddle 2014, Hoddle et al. 2013, Hoddle et al. 2014). After a series of no-choice exposure trials on ACP, *T. radiata*, and *D. aligarhensis*, *P. diaphorinae* can now also be eliminated as a potential primary parasitoid of ACP (Hayat 1972, Triapitsyn et al. 2013). *P. diaphorinae* failed to reproduce on unparasitized ACP nymphs but successfully reproduced on both *T. radiata* and *D. aligarhensis* indicating that it is almost certainly an obligate hyperparasitoid in the ACP-*Tamarixia-Diaphorencyrtus* system in citrus.

Considering both reproductive output and developmental times, it was concluded that *D. aligarhensis* is a superior host for *P. diaphorinae* when compared to *T. radiata.* High levels of hyperparasitism observed in Asia (Chien et al. 1989, Qing 1990) also suggest that *D. aligarhensis* is preferred by many hyperparasitoids in comparison to *T. radiata*. It is not certain whether this higher suitability is due to ease of accessibility (being an endoparasitoid, whereas *T. radiata* is an ectoparasitoid), or whether targeting

D. aligarhensis mummies (which have a 6 day pupal stage at 26°C [Rohrig et al. 2011], versus *T. radiata* which have a 4 day pupal stage at 25°C [Mann and Stelinski 2010]) simply allows for more time to be located and utilized by hyperparasitoids. However, this demonstrated preference (as well as similar preferences of other hyperparasitoids) could potentially explain why *D. aligarhensis* is not as successful as *T. radiata* in parasitizing ACP in areas where high levels of hyperparasitism naturally occur (Chien 1989, Qing 1990, Hoddle et al. 2014).

Hyperparasitoid biology may have significant implications in the context of biological control. Schooler *et al.* (2011) demonstrated in greenhouses that hyperparasitoids can drive their associated primary aphid parasitoid extinct. In the resulting absence of primary parasitoids, population densities of the aphids remained significantly higher than populations in greenhouses with primary parasitoids which lacked hyperparasitoids. In field crops, hyperparasitoids may not necessarily cause local extinction of primary parasitoids. They may, however, still disrupt biological control both by significantly reducing primary parasitoid populations (Schooler et al. 2011) or by disrupting sex ratios of primary parasitoids causing them to become male biased because larger mummies containing female parasitoids are preferred hosts (Gomez-Márco et al. 2015).

Classical biological control of ACP in California has the benefit of introducing primary parasitoids into a novel area devoid of coevolved hyperparasitoids. It is possible that the absence of this hyperparasitoid guild could favor *D. aligarhensis* which is heavily

attacked in many parts of its native range by hyperparasitoids (Chien 1989, Qing 1990). Surveys of ACP natural enemies from China, Taiwan, and Pakistan indicated that *D. aligarhensis* can experience hyperparasitism rates of over 40% (Table 3.3). While hyperparasitoid species in the native range of *D. aligarhensis* are absent in California, congeneric species of many native-range hyperparasitoids are present in California as members of hemipteran-parasitoid systems. These genera of resident California hyperparasitoids (Table 3.3) may have the potential to shift hosts and attack primary parasitoids (especially *D. aligarhensis*) released for the classical biological control of ACP (Sullivan and Völkl 1999). Ongoing research with *P. diaphorinae* is currently investigating this species' ability and affinity for utilizing *Tamarixia triaozae* pupae within *Bactericera cockerelli* nymphs, a novel host complex which it has no evolutionary history with, to demonstrate this possibility. Surveys for hyperparasitoids attacking *D. aligarhensis* and *T. radiata* in California are warranted as releases of these two species continue.

Table 3.3. Hyperparasitoids reared from Asian citrus psyllid mummies parasitized *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* in their native range.

Hyperparasitoid species	Country	Primary parasitoid target		Genus with hyperparasitoids present in California? [e]
		D. aligarhensis	*T. radiata*	
Pachyneuron concolor[a,b] (= *Pachyneuron muscarum*)	Taiwan	x	x	yes
Pachyneuron crassiculme[d]	Pakistan	x		
Chartocerus walkeri[a,b]	Taiwan, China	x	x	yes
Chartocerus sp.[d]	Pakistan	x	x	
Encarsia sp. n. *shafeei*[a,b]	Taiwan	x	x	
Encarsia sp. A[b]	China	x	x	yes
Encarsia sp. B[b]	China	x	x	
Coccophagus ceroplastae[a,b]	Taiwan	x		yes
Coccophagus sp. [a,b]	Taiwan	x		
Marietta leopardina[a,c]	Taiwan, Pakistan	x	x	yes
Syrphophagus taiwanus[a,b]	Taiwan, China	x	x	yes
Cheiloneurus sp.[a]	Taiwan	x		yes
Aprostocetus (*Aprostocetus*) sp.[c]	Pakistan	x	x	yes
Ageniaspis sp. [a,b]	Taiwan, China	x		no
Tetrastrichus sp. [a,b]	Taiwan, China	x	x	no
Psyllaephagus sp. [b]	Taiwan, China	x	x	no
Psyllaphycus diaphorinae	Pakistan	x	x	no

[a]Chien et al. 1989
[b]Qing 1990
[c]Hoddle et al. 2013
[d]Bistline-East and Hoddle 2014
[e]Noyes 2015

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References

Bistline-East, A. and M.S. Hoddle. 2014. *Chartocerus* sp. (Hymenoptera: Signiphoridae) and *Pachyneuron crassiculme* (Hymenoptera: Pteromalidae) are obligate hyperparasitoids of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and possibly *Tamarixia radiata* (Hymenoptera: Eulophidae). Fla. Entomol. 97(2): 562-566.

Bistline-East, A., R. Pandey, M. Keceçi, and M.S. Hoddle. 2015. Host range testing of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) for use in classical biological control of *Diaphorina citri* (Hemiptera: Liviidae) in California. J. Econ. Entomol. doi: 10.1093/jee/tov020

Chien, C.C., S.C. Chiu, and S.C. Ku. 1989. Biological control of *Diaphorina citri* in Taiwan. Fruits 44: 401-407.

Civerolo, E. 2015. ACP and HLB detection in California. Citrograph 6: 8-9.

Gomez-Márco, F., A. Urbaneja, J.A. Jaques, P.F. Rugman-Jones, R. Stouthamer, and A. Tena. 2015. Untangling the aphid-parasitoid food web in citrus: Can hyperparasitoids disrupt biological control? Biol. Cont. 81: 111-121.

Grafton-Cardwell, E. 2010. How serious is the threat of Asian citrus psyllid and Huanglongbing? Citrograph 1: 8-10.

Halbert, S.E. and K.L. Manjunath. 2004. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. Fla. Entomol. 87(3): 330-349.

Hall, D.G., M.G. Hentz, and R.C. Adair. 2008. Population ecology and phenology of *Diaphorina citri* (Hemiptera: Psyllidae) in two Florida citrus groves. Environ. Entomol. 37: 914-924.

Hall, D.G., H.L. Richardson, E. Ammar, and S.E. Halbert. 2013. Asian citrus psyllid, *Diaphorina citri*, vector of citrus huanglongbing disease. Entomol. Exp. Appl. 146: 207-223.

Hayat, M. 1972. Descriptions of two new genera and species of Encyrtidae (Hymenoptera, Chalcidoidea), with notes on some described species. Acta ent. bohemoslov. 69: 207-214.

Hoddle, M.S. 2012. Foreign exploration for natural enemies of Asian citrus psyllid, *Diaphorina citri* (Hemiptera: Pysllidae), in the Punjab of Pakistan for use in a classical biological control program in California USA. Pak. Entomol. 34: 1-5.

Hoddle, C.D., M.S. Hoddle, and S.V. Triapitsyn. 2013. *Marietta leopardina* (Hymenoptera: Aphelinidae) and *Aprostocetus* (*Aprostocetus*) sp. (Hymenoptera: Eulophidae) are obligate hyperparasitoids of *Tamarixia radiata* (Hymenoptera: Eulophidae) and *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae). Fla. Entomol. 96(2): 643-646.

Hoddle, M.S. and R. Pandey. 2014. Host Range Testing of *Tamarixia radiata* (Hymenoptera: Eulophidae) sourced from the Punjab of Pakistan for classical biological control of *Diaphorina citri* (Hemiptera: Liviidae: Euphyllurinae: Diaphorinini) in California. J. Econ. Entomol. 107 : 125-136.

Hoddle, M.S., C.D. Hoddle, S.V. Triapitsyn, S.Z. Khan, and M.J. Arif. 2014. How many primary parasitoid species attack nymphs of *Diaphorina citri* (Hemiptera: Liviidae) in Punjab, Pakistan? Fla. Entomol. 97(4): 1825-1828.

Hussain, M.A. and D. Nath. 1927. The citrus psylla (*Diaphorina citri* Kuw.) (Psyllidae: Homoptera). Mem. Dept. Agric. India, Entomol. Ser. 10: 5-27.

Keremane, M.L., C. Ramadugu, E. Rodriguez, R. Kubota, S. Shibata, D.G. Hall, M.L. Roose, D. Jenkins, and R.F. Lee. 2015. A rapid field detection system for citrus huanglongbing associated '*Candidatus* Liberibacter asiaticus' from the psyllid vector, *Diaphorina citri* Kuwayama and its implications in disease management. Crop Protection. 68: 41-48.

Kumagai, L.B., C.S. LeVesque, C.L. Blomquist, K. Madishetty, Y. Guo, P.W. Woods, S. Rooney-Latham, J. Rascoe, T. Gallindo, D. Schnabel, and M. Polek. 2013. First report of *Candidatus* Liberibacter asiacticus associated with citrus huanglongbing in California. Plant Dis. 97: 283.

Liu, D. and J.T. Trumble. 2007. Comparative fitness of invasive and native populations of the potato psyllid (*Bactericera cockerelli*). Entom. Exp. et Appl. 123: 35-42.

Mann, R.S. and L.L. Stelinski. 2010. An Asian citrus psyllid parasitoid *Tamarixia radiata* (Waterston) (Insecta: Hymenoptera: Eulophidae). University of Florida Institute of Food and Agricultural Sciences (UF/IFAS), EENY-475. http://edistt.ifas.ufl.edu/pdffiles/IN/IN85800.pdf. Last accessed: 27 March 2015.

Mehrnejad, M.R. and S.Y. Emami. 2005. Parasitoids associated with the common pistachio psylla, *Agonoscena pistaciae*, in Iran. Biol. Cont. 32: 385-390.

Noyes, J.S. 2015. Universal Chalcidoidea Database. World Wide Web electronic publication. http://www.nhm.ac.uk/chalcidoids. Last accessed: 27 April 2015.

Qing, T.Y. 1990. On the parasite complex of *Diaphorina citri* Kuwayama (Homoptera: Psyllidae) in Asian-Pacific and other areas. Proceedings of the 4th International Asia Pacific Conference on Citrus Rehabilitation. 240-245.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rohrig, E., P.D. Shirk, D.G. Hall, and P.A. Stansly. 2011. Larval development of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae), an endoparasitoid of *Diaphorina citri* (Hemiptera: Psyllidae). Ann. Entomol. Soc. Am. 104: 50-58.

Rojas, P., E. Rodriguez-Leyva, J.R. Lomeli-Flores, and T.X. Liu. 2015. Biology and life history of *Tamarixia triozae*, a parasitoid of the potato psyllid, *Bactericera cockerelli*. Biol. Cont. 60: 27-35.

Salifu, A.W., K.A. Grogan, T.H. Spreen, and F.M. Roka. 2012. Economic analysis of strategies to combat HLB in Florida citrus. Southern Agrigultural Economics Association Annual Meeting. Birmingham, AL. 4-7 Feb. 2012.

Schooler, S.S., P. De Barro, and A.R. Ives. 2011. The potential for hyperparasitism to compromise biological control: Why don't hyperparasitoids drive their primary parasitoid hosts extinct? Biol. Cont. 58: 167-173.

Skelley, L.H. and M.A. Hoy. 2004. A synchronous rearing method for the Asian citrus psyllid and its parasitoids in quarantine. Biol. Cont. 29: 14-23.

Sullivan, D.J. and W. Völkl. 1999. Hyperparasitism: Multitrophic ecology and behavior. Annu. Rev. Entomol. 44: 293-315.

Triapitsyn, S.V., C.D. Hoddle, M. Hayat, and M.S. Hoddle. 2013. Taxonomic notes on *Psyllaphycus diaphorinae* (Hymenoptera: Encyrtidae) and its host associations in Pakistan. Fla. Entomol. 96(1): 212-218.