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Biology of *Psyllaphycus diaphorinae* (Hymenoptera: Encyrtidae), a Hyperparasitoid of *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) and *Tamarixia radiata* (Hymenoptera: Eulophidae)

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Abstract

The biology and ecology of *Psyllaphycus diaphorinae* Hayat, a potential natural enemy of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), is poorly understood. In April 2013, six *P. diaphorinae* females collected from Punjab, Pakistan emerged from parasitized Asian citrus psyllid nymphs in quarantine at the University of California, Riverside and were used to initiate colonies. Contrary to previous claims, *P. diaphorinae* was found to be an obligate hyperparasitoid and not a primary parasitoid attacking Asian citrus psyllid 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, both primary parasitoids of Asian citrus psyllid. No reproduction on unparasitized Asian citrus psyllid 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*.

Key words: classical biological control, *Diaphorina citri*, California

The Asian citrus psyllid, *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, Asian citrus psyllid populations have increased and Asian citrus psyllid spread rapidly through urban areas in southern California. In 2014, Asian citrus psyllid 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 Asian citrus psyllid is its ability to vector the bacterium *Candidatus* Liberbacter asiaticus (CLas), 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 yr after initial infection, making HLB difficult to detect in its early stages. Infected trees usually die within 8 yr of acquiring CLas (Halbert and Manjunath 2004). To date, there have been just two confirmed cases of HLB in California. The first was at a residential property in Hacienda Heights, Los Angeles County (Kumagai et al. 2013), and the second find in July 2015 was in a residential garden in San Gabriel (Los Angeles County), ∼24 km from the first HLB find site (Mohan 2015). Asian citrus psyllid-vectored CLas is considered a significant threat to the state's ∼ US$3 billion per year citrus industry. The impact to California's citrus industry is expected to be similar to that experienced in Florida, where Asian citrus psyllid was discovered in 1998 and HLB was detected in 2005. Since the detection of HLB in Florida, Asian citrus psyllid-HLB has caused over US$ 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% (Salifu et al. 2012, Keremane et al. 2015).

In an attempt to reduce the Asian citrus psyllid–HLB threat to California, a classical biological control program using host-specific natural enemies from Asian citrus psyllid's home range was initiated with the intent of suppressing psyllid populations in urban areas where Asian citrus psyllid populations are greatest. By reducing the number of psyllids capable of acquiring and transmitting CLas, 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 Asian citrus psyllid natural enemies, in particular parasitoids that attack Asian citrus psyllid nymphs. Punjab is purportedly within

Asian citrus psyllid'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 Asian citrus psyllid parasitoids could exist in Punjab, with up to nine species of primary parasitoids possibly attacking Asian citrus psyllid nymphs. Several species of parasitoid were reared from Asian citrus psyllid 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 Asian citrus psyllid biocontrol (Halbert and Manjunath 2004). These two parasitoids are being utilized in California in a classical biological control program targeting Asian citrus psyllid (Hoddle and Pandey 2014, Bistline-East et al. 2015).

One of the goals of the Asian citrus psyllid 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 in quarantine at UCR from parasitized Asian citrus psyllid nymphs collected in Pakistan 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, 2014; Bistline-East and Hoddle 2014). The conclusion from foreign exploration efforts in Pakistan was that the guild of nine unidentified parasitoid species documented by Hussain and Nath (1927) in Punjab does not exist (Hoddle et al. 2014). The estimate of nine parasitoid species attacking Asian citrus psyllid nymphs likely resulted from the mis-association of parasitoids with Asian citrus psyllid that emerged from other very small plant-feeding insects (e.g., parasitoids which emerged from leaf hopper or lace bug eggs) that coinfested citrus foliage with Asian citrus psyllid nymphs (Hoddle et al. 2014).

P. diaphorinae was originally described by Hayat (1972), based on an examination of four male and six female specimens collected in Punjab, 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 Asian citrus psyllid, there was interest in assessing the potential of this natural enemy for the classical biological control of Asian citrus psyllid in California.

P. diaphorinae recovered from an October 2011 collecting trip to Punjab Pakistan ($n = 3$ males, 7 females) 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. diaphorinae* individuals ($n = 6$ females). Because there is almost no information on the biology and ecology of *P. diaphorinae*, the assumption that this wasp is a primary Asian citrus psyllid 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 Asian citrus psyllid nymphs and Asian citrus psyllid 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 (Asian citrus psyllid, *T. radiata*, *D. aligarhensis*) were sourced from colonies maintained at the University of California Riverside Insectary and Quarantine Facility (UCR IQF). Asian citrus psyllid 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 Asian citrus psyllid colonies were confirmed to be CLas-free through PCR analysis of 30 individual psyllids and reared on *Citrus volkameriana* V. Ten. & Pasq. (Rutaceae). Small citrus cuttings containing parasitized Asian citrus psyllid 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). Asian citrus psyllid 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 by 45 by 45 cm, length by width by 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 Asian citrus psyllid nymphs in UCR IQF. Six *P. diaphorinae* females (no males) were reared from Asian citrus psyllid 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

Asian citrus psyllid 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-yr-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 by 15 by 30 cm (width by depth by 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 mo. Potted *C. volkameriana* were pruned regularly to promote flush growth necessary to stimulate Asian citrus psyllid oviposition (Hall et al. 2008), as well as to maintain plants at sizes suitable for cages.

To conduct *P. diaphorinae*-Asian citrus psyllid 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 Asian citrus psyllid 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 Asian citrus psyllid nymphs were taken directly from the Asian citrus psyllid colony and nymphs were not manipulated.

Asian citrus psyllid 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 Asian citrus psyllid nymphs were 5 and 13 d old, respectively. Mummies were taken from colonies of these primary Asian citrus psyllid 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., unparasitized Asian citrus psyllid nymphs or Asian citrus psyllid mummies containing developing *T. radiata* and *D. aligarhensis* larvae or pupae) could serve as hosts for *P. diaphorinae*. The original literature described *P. diaphorinae* as a primary parasitoid of Asian citrus psyllid (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 Asian citrus psyllid nymphs of mixed ages on *C. volkameriana* plants. These exposure experiments used small Asian citrus psyllid infested *C. volkameriana* seedlings in Cone-tainers in some replicates and larger Asian citrus psyllid infested *C. volkameriana* in sleeve cages for other exposure trials.

Four Cone-tainers with *C. volkameriana* seedlings each infested with 10 Asian citrus psyllid 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 ~150–200 second to fourth instar Asian citrus psyllid 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 Asian citrus psyllid 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 Asian citrus psyllid density and the available number of *P. diaphorinae* not in use in other trials. Female *P. diaphorinae* were recovered from exposure tests and stored in 200-μl microcentrifuge tubes with honey at 14°C until used again in additional exposure experiments. Asian citrus psyllid nymphs in both Cone-tainers and cages were monitored daily for *P. diaphorinae* emergence.

Following exposure to Asian citrus psyllid nymphs, *P. diaphorinae* were sequentially exposed to *T. radiata* and *D. aligarhensis* mummies, and one additional replicate of unparasitized Asian citrus psyllid 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 Asian citrus psyllid 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°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 outcomes of exposure trials which produced male *P. diaphorinae* (which were mated with the original females collected in Pakistan; see Results), colonies of *P. diaphorinae* were established and maintained in acrylic sleeve cages (see above) containing potted *C. volkameriana* plants infested with Asian citrus psyllid nymphs parasitized by *D. aligarhensis* or *T. radiata* at 27 ± 2°C, 40% RH, and a photoperiod of 14:10 (L:D) h. 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 hyperparasitoid may only parasitize the pupal stage of *T. radiata* and *D. aligarhensis* (Fig. 1). Consequently, pupae of these primary Asian citrus psyllid parasitoids were exposed to *P. diaphorinae* in these studies.

T. radiata colony cages were created by exposing 100–200 fourth instar Asian citrus psyllid nymphs (11–13 d old at 29°C), a life stage preferred for parasitism by this parasitoid (Skelley and Hoy 2004), on potted *C. volkameriana* to ~10 male and 15 female *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 Asian citrus psyllid hosts (Fig. 1). Approximately 5 male and 10 female (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.

D. aligarhensis cages were prepared in a similar manner to *T. radiata*. Approximately 100–200 second to third instar Asian citrus psyllid nymphs (8–10 d old at 29°C), the preferred life stage for parasitism by *D. aligarhensis* (Rohrig et al. 2011), were exposed to 10 male and 5 female *D. aligarhensis* for 2–4 d. *D. aligarhensis* mummies were held for 10 d after initial exposure and matured to the pupal stage (Fig. 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.

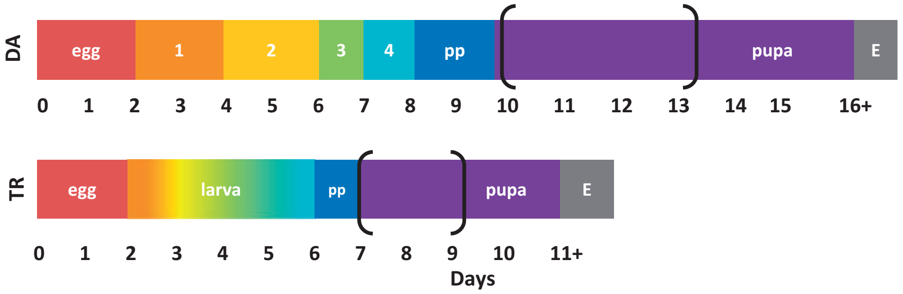

Fig. 1. Developmental timelines of *Diaphorencyrtus aligarhensis* ("DA") and *Tamarixia radiata* ("TR"). Numbered stages refer to larval instars; "pp" = prepupa; "E" = eclosion. Brackets indicate preferred window for *Psyllaphycus diaphorinae* oviposition.

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 ± 2°C, 40% RH, and a photoperiod of 14:10 [L:D] h) 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°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 at the 0.05 significance level. Tukey's HSD test with 0.05 significance level (analyzed in R) was used to determine which treatments differed significantly from one another.

Results

P. diaphorinae Development and Host Preference

P. diaphorinae did not successfully reproduce on unparasitized Asian citrus psyllid nymphs regardless of instar. However, *P. diaphorinae* parasitized immature *D. aligarhensis* and *T. radiata* developing inside Asian citrus psyllid nymphs (Table 1). *P. diaphorinae* exited Asian citrus psyllid mummies from a hole chewed in the posterior dorsal surface. This is the same position as exit holes produced by adult *D. aligarhensis* but opposite to those produced by emerging *T. radiata* which are located dorsally, but at the anterior end of the Asian citrus psyllid mummy. Initial exposure of unmated *P. diaphorinae* reared in UCR IQF from Asian citrus psyllid nymphs collected in Pakistan to *D. aligarhensis* resulted in 10 male *P. diaphorinae*, 5 dead mummies, and no adult *D. aligarhensis*. *T. radiata* exposure yielded 5 male *P. diaphorinae*, 3 male and 7 female *T. radiata*, and no 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 collected 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 2). Mean developmental time 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 slightly 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 both males ($P < 0.001$) and females ($P < 0.0001$) produced per cage was significantly elevated on *D. aligarhensis* compared to *T. radiata* (Fig. 2). 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* may be a better host for *P. diaphorinae* within the Asian citrus psyllid–*Tamarixia*–*Diaphorencyrtus* complex.

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). A one-way ANOVA indicated significant differences ($F = 3.83$; $\mathrm{df} = 3$; $P = 0.01$) between the four treatment types (solitary male, solitary female, paired male, and paired female).

Table 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 Asian citrus psyllid	Primary parasitoid	
Asian citrus psyllid nymphs[a]	50	0	46	0	3
T. radiata	15	5 males	0	10	0
D. aligarhensis	15	10 males	0	0	5

[a] One Asian citrus psyllid nymph could not be accounted for, so was not included in counts as either adult or dead.

Table 2. *Psyllaphycus diaphorinae* emergence on *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* over 20 generations while maintained in colony

Host	Mean host age (d)	Emergence numbers			Mean development time (d)[a]	
		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

[a] Mean developmental time of *P. diaphorinae* ± standard error (SE).

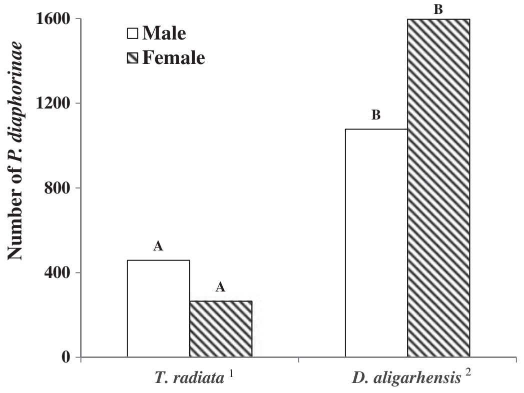

Fig. 2. Total number of *Psyllaphycus diaphorinae* emerged from *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* in colony. Different letters above bars indicate significant differences yielded from Wilcoxon rank-sum test ($P < 0.0001$). [1]Emergence on *T. radiata*: $n = 458$ (male), $n = 265$ (female); sex ratio 37% female. [2]Emergence on *D. aligarhensis*: $n = 1{,}077$ (male), $n = 1{,}596$ (female); sex ratio 60% female.

Fig. 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 ($P = 0.02$).

However, the only significant difference in longevity when pairs of treatments were compared was between solitary males and paired females ($P = 0.02$; Fig. 3)

Discussion

Hussain and Nath (1927) stated that a probable guild of up to nine primary parasitoids of Asian citrus psyllid 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 Asian citrus psyllid mummies. One of these unnamed species reared by Hussain and Nath (1927) may have been *P. diaphorinae*. To date, four species likely comprising a portion of these original nine have been experimentally tested and conclusively determined to be hyperparasitoids of primary parasitoids (*T. radiata* and *D. aligarhensis*) attacking Asian citrus psyllid nymphs (Hoddle et al. 2013, 2014; Bistline-East and Hoddle 2014). After a series of no choice exposure trials on Asian citrus psyllid, *T. radiata*, and *D. aligarhensis*, *P. diaphorinae* can likewise be eliminated as a potential primary parasitoid of Asian citrus psyllid (Hayat 1972, Triapitsyn et al. 2013). *P. diaphorinae* failed to reproduce on multiple instars of unparasitized Asian citrus psyllid nymphs but successfully reproduced on both *T. radiata* and *D. aligarhensis* pupae, indicating that it is almost certainly an obligate hyperparasitoid in the Asian citrus psyllid–*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 several hyperparasitoid species 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 d pupal stage at 26°C [Rohrig et al. 2011], versus *T. radiata* which have a 4 d pupal stage at 25°C [Mann and Stelinski 2010]) (Fig. 1) simply allows for more time to be located and utilized by hyperparasitoids. However, this demonstrated preference, as well as similar documented preferences of other hyperparasitoids, could potentially explain why *D. aligarhensis* is not as successful as *T. radiata* in parasitizing Asian citrus

Table 3. Hyperparasitoids reared from Asian citrus psyllid mummies parasitized by *Tamarixia radiata* and *Diaphorencyrtus aligarhensis* in their native range

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

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

psyllid 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 that hyperparasitoids in greenhouses 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, disrupt biological control 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 Asian citrus psyllid 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 Asian citrus psyllid natural enemies from China, Taiwan, and Pakistan indicated that *D. aligarhensis* can experience hyperparasitism rates of over 40% (Table 3); because of this high frequency of hyperparasitism, there remains the possibility of accidental introduction of *P. diaphorinae* or other hyperparasitoids into California. Although such an occurrence would threaten populations of introduced *D. aligarhensis*, and to a lesser extent *T. radiata*, and likely negatively impact the Asian citrus psyllid biological control program, *P. diaphorinae* is unlikely to impact native California parasitoid species. When exposed to colonies of *Bactericera cockerelli* Šulc (Hemiptera: Triozidae) parasitized by *Tamarixia triozae* (Burks) (Hymenoptera: Eulophidae), a species closely related to *T. radiata* and commonly occurring in both agricultural fields and urban gardens across California, *P. diaphorinae* consistently failed to reproduce on *T. triozae*, suggesting it may have some host specificity (A.B.E., unpublished data).

While hyperparasitoid species from 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) may have the potential to attack primary parasitoids (especially *D. aligarhensis*) released for the classical biological control of Asian citrus psyllid, should host-shift occur (Sullivan and Völkl 1999). Surveys for hyperparasitoids (both native and accidentally introduced) attacking *D. aligarhensis* and *T. radiata* in California are therefore warranted as releases of these two species continue for classical biological control of Asian citrus psyllid.

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