Two parasitoids of *Diaphorina citri* (Hemiptera: Liviidae) have shared, stage-specific preference for host nymphs that does not impact pest mortality rates

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Abstract

Assessing the oviposition preference and offspring performance of 2 parasitoid species being used to establish classical biological control of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) infesting citrus may provide important insight into their potential coexistence in California, USA. *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), an ectoparasitoid, preferred fourth and fifth instar *D. citri* nymphs for oviposition in both choice and no-choice experiments. Larval development and adult longevity of *T. radiata* offspring were positively correlated to female oviposition preference. Oviposition preferences of *T. radiata* were unaffected by conspecific and heterospecific competitors. *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae), an endoparasitoid, preferred third and fourth instar hosts in choice experiments and fourth instar hosts in no-choice experiments. Parasitoid larvae that developed in these instars performed better than larvae that developed in second and fifth instars. The oviposition preferences of *D. aligarhensis* were unaffected by competitors in choice arenas, but were affected in no-choice experiments. Populations of *T. radiata* and *D. aligarhensis* from Pakistan, where they exist in sympatry and used in the experiments reported here, demonstrated a shared preference for fourth instar *D. citri* nymphs, which has not been documented previously for populations of *D. aligarhensis* from other geographic regions. This shared preference did not affect *D. citri* mortality rates, but it may affect the ability of *D. aligarhensis* to establish in areas of California where *T. radiata* is currently present.

Key Words: *Diaphorencyrtus aligarhensis*; conspecific competition; heterospecific competition; longevity; oviposition preference; *Tamarixia radiata*

Resumen

La evaluación de la preferencia de oviposición y el desempeño de la progenie de 2 especies de parasitoides que se utilizan para establecer el control biológico clásico de *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) que infestan los cítricos puede proporcionar información importante sobre su posible coexistencia en California, EE. UU. *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), es un ectoparasitoide, prefiere las ninfas del cuarto y quinto estadio *D. citri* para la oviposición tanto en experimentos de elección como de no elección. El desarrollo de larvas y la longevidad de adultos de *T. radiata* se correlacionaron positivamente con la preferencia de oviposición de las hembras. Las preferencias de oviposición de *T. radiata* no se vieron afectadas por los competidores conespecíficos y heteroespecíficos. *Diaphorencyrtus aligarhensis* (Shafee, Alam y Agarwal) (Hymenoptera: Encyrtidae), es un endoparasitoide, que prefiere hospederos de tercer y cuarto estadio en experimentos de elección y hospederos de cuarto estadio en experimentos de no elección. Las larvas de los parasitoides que se desarrollaron en estos estadios funcionaron mejor que las larvas que se desarrollaron en los estadios segundo y quinto. Las preferencias de oviposición de *D. aligarhensis* no se vieron afectadas por los competidores en las arenas de elección, pero se vieron afectadas en los experimentos de no elección. Las poblaciones de *T. radiata* y *D. aligarhensis* de Pakistán (donde existen en simpatría) y se utilizaron en los experimentos informados aquí demostraron una preferencia compartida por las ninfas del cuarto estadio de *D. citri*, que no se ha documentado previamente para las poblaciones de *D. aligarhensis* de otras regiones geográficas. Esta preferencia compartida no afectó las tasas de mortalidad de *D. citri*, pero puede afectar la capacidad de *D. aligarhensis* para establecerse en áreas de California donde *T. radiata* ya está presente.

Palabras Clave: *Diaphorencyrtus aligarhensis*; competencia conespecífica; competencia heteroespecífica; longevidad; preferencia de oviposición; *Tamarixia radiata*

Highly specific preferences for host stages that are correlated with fitness benefits might explain the coexistence of multiple parasitoid species that use a single host species. One such system of interest pertains to the pestiferous Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), and its primary parasitoids, *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae) and *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae) (Vankosky & Hoddle 2016). Both parasitoid species have been released in California, USA, in order to establish a classical biological control program targeting *D. citri* (Hoddle 2012; Hoddle & Pandey 2014; Bistline-East et al. 2015; Vankosky & Hoddle 2016). The primary objective of this biological control program is to reduce well-established populations of *D. citri* in urban areas, and minimize rates of spread into areas where citrus is grown commercially (Hoddle 2012; Hoddle & Pandey 2014; Bistline-East et al. 2015; Vankosky & Hoddle 2016). The direct impacts of *D. citri* infestation on citrus trees are minimal (Hall et al. 2012; Grafton-

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Cardwell et al. 2013). However, *D. citri* vectors the phloem-dwelling bacterium *Candidatus* Liberibacter asiaticus (*C*Las), the putative causative agent of citrus greening disease, or huanglongbing, that impacts all citrus varieties and is lethal and incurable (Bové 2006).

The *D. citri*-*C*Las pathosystem has had a devastating impact on citrus production in Florida, USA, where it has been estimated that 80% of commercial orchards have *C*Las-infected trees (Mishchuk et al. 2017). In 15% of infested orchards, 100% of trees are infected with *C*Las, resulting in significant yield losses and increased production costs (Mishchuk et al. 2017). *Diaphorina citri*-*C*Las has been similarly problematic in citrus production areas in China (Chen 2017) and Brazil (Parra et al. 2016). In California, the first *C*Las infected tree was found in Los Angeles County in 2012, followed by 10 additional trees in 2015 (Kumagai et al. 2016; Hornbaker & Kumagai 2016). By May 2017, a total of 60 trees had been confirmed positive for *C*Las infection, and infected trees were found in Orange and Riverside Counties for the first time (Citrus Insider 2017a, b). Limiting *D. citri* population densities (i.e., vector reduction) may slow *C*Las dispersal into new areas, and subsequently, the acquisition and spread of this bacterium throughout urban and commercial citrus growing regions of California (Bové 2006; Hoddle 2012).

Foreign exploration in Punjab, Pakistan, part of the native range of *D. citri* with a climate similar to the major citrus production region in the San Joaquin Valley in California, identified 2 species of host specific parasitoids, *D. aligarhensis* and *T. radiata*, suitable for release in California (Hoddle 2012). In commercial citrus in Pakistan, parasitism by *D. aligarhensis* and *T. radiata* contribute to 18% and 26% mortality, respectively, in monitored *D. citri* populations (Khan et al. 2014). Populations of both species obtained from Pakistan are biparental arrhenotokous (unfertilized eggs yield male offspring) koinobionts (host development continues after parasitism) (Hoddle & Pandey 2014; Bistline-East et al. 2015).

In North America, the majority of information regarding *D. aligarhensis* (endoparasitoid) and *T. radiata* (ectoparasitoid) is the result of work conducted in Florida using populations of parasitoids obtained from China, Taiwan, and Vietnam (Hoy & Nguyen 2001; Rohrig 2010; Rohrig et al. 2011; Chen & Stansly 2014b). Arrhenotokous *T. radiata* from these populations prefer fourth and fifth instar *D. citri* for oviposition hosts (Hall et al. 2012; Sule et al. 2014). Individual female *T. radiata* can kill approximately 500 *D. citri* via parasitism and host feeding over the course of their lifetime (Chien et al. 1995; Skelley & Hoy 2004). *Diaphorencyrtus aligarhensis* populations released in Florida were thelytokous (unfertilized eggs result in female offspring) (Rohrig 2010) and preferred second and third instar *D. citri* hosts (Skelley & Hoy 2004; Rohrig 2010; Rohrig et al. 2011). This species also causes host mortality via parasitism and host feeding (Chien et al. 1995; Skelley & Hoy 2004). One or both of these parasitoid species have been used in biological control programs in Florida and Texas, USA (Skelley & Hoy 2004; Rohrig et al. 2012; Chen & Stansly 2014b), and in other citrus growing countries including Brazil, Réunion Island, Taiwan, and Mexico (Etienne & Aubert 1980; Chien & Chu 1996; Chen & Stansly 2014b; Parra et al. 2016), where they have had varying levels of success in establishing both permanent populations and reducing *D. citri* populations.

The primary objective of the experiments reported here was to investigate the relationship between female preference for oviposition hosts (*D. citri*) of different nymphal instars, and the subsequent offspring performance of *T. radiata* and *D. aligarhensis* from Pakistan using choice and no-choice experiments in the laboratory. Our second objective was to assess the effect of competitive interactions between female parasitoids (i.e., different parasitoid foraging scenarios) on oviposition preference and *D. citri* mortality. Collectively, these data may assist understanding of interspecific interactions between arrhenotokous *T. radiata* and *D. aligarhensis* foraging on *D. citri* patches. This information could assist with understanding how these 2 primary parasitoids of *D. citri* coexist in sympatry in their native range (i.e., Pakistan), and provide insight into possible complementarity in a new, non-native range (i.e., California).

Relatively little is known about the biology and behavior of *T. radiata* and *D. aligarhensis* populations originating from Pakistan, but differences between populations may have important implications on the success or failure of *D. citri* biological control in California. Importantly, *D. aligarhensis* from Pakistan are arrhenotokous (Hoddle 2012; Bistline-East et al. 2015), in contrast to thelytokous populations of *D. aligarhensis* from Taiwan and Vietnam released in Florida (Rohrig et al. 2012). Although populations of *T. radiata* from Pakistan and other parts of Asia do not differ in such an obvious manner, less obvious differences may exist, as has been observed between populations of other parasitoid species (e.g., *Encarsia formosa* Gehan (Hymenoptera: Aphelinidae) (Henter & van Lenteren 1996). The experimental design used to test competitive interactions between *T. radiata* and *D. aligarhensis* allowed us to compare the oviposition preferences of parasitoid populations from different regions of Asia.

Materials and Methods

INSECT AND PLANT MATERIAL

Tamarixia radiata used for choice and no-choice experiments were collected from colonies maintained at the California Department of Food and Agriculture rearing facility located at Mt. Rubidoux in Riverside, California. This population is arrhenotokous and source material for the colony was obtained from Punjab, Pakistan, and authorized for release in California (USDA-APHIS release permit P526P-09-02585) (Hoddle 2012). Arrhenotokous adult *D. aligarhensis* colonies were maintained in a greenhouse at the University of California, Riverside, Insectary and Quarantine Facility. Source adults for this population also were collected in Punjab, Pakistan, and authorized for release in California (USDA-APHIS release permit P526P-11-00103). Colonies of both species were established originally in 2011 (Hoddle 2012) and supplemented with additional field collections from Pakistan up to Apr 2013. Parasitoid colonies were maintained at the University of California, Riverside, Insectary and Quarantine Facility in isogenic lines to preserve genetic diversity (Hoddle & Hoddle 2013).

Female parasitoids used in preference experiments were 3 to 7 d old with no previous foraging experience. They were held as male/female pairs inside 2.0 mL microcentrifuge tubes, without citrus flush, from the date of emergence until their use in experiments. During that time, parasitoids were fed honey droplets and were maintained inside temperature-controlled incubators at 16 °C, and a 14:10 h (L:D) photoperiod. Twenty-four h before experiments began, male/female pairs were transferred to clean microcentrifuge tubes that contained 8 *D. citri* nymphs (2 nymphs from each of the second through fifth instars with no citrus flush), and allowed to host feed and mate for 6 h. Parasitoids were held in a rearing room during this host feeding period (24 °C, 40% RH, 14:10 h [L:D] photoperiod). At the end of 6 h, females were separated from males and held individually in microcentrifuge tubes, in temperature-controlled incubators (16 °C, 14:10 h [L:D] photoperiod), until experiments were initiated. Preliminary studies determined that a period of exposure to hosts prior to experiments increased the likelihood of oviposition events, and decreased the likelihood of host feeding events during the experimental period, because nutrients required for egg production were already obtained (Chen & Stansly 2014a).

Potted *Citrus volkameriana* V. Ten. & Pasq. (Rutaceae) plants used for experiments and pruned to stimulate flush growth necessary to support populations of *D. citri*, were grown in greenhouses at the University of California, Riverside. Plants were transferred to the University of California, Riverside, Insectary and Quarantine Facility for insect rearing and use in experiments when needed. Protocols for parasitoid rearing and plant propagation used during the course of these experiments are described in greater detail elsewhere (Bistline-East et al. 2015; Vankosky & Hoddle 2017a, b).

CHOICE EXPERIMENT

Choice experiments were used to investigate the oviposition preferences of female parasitoids for second through fifth instar *D. citri* nymphs. First instar *D. citri* nymphs were not attacked by either species of parasitoid in pilot experiments, and were subsequently excluded from all further experiments. Two populations of parasitoids were tested: arrhenotokous *T. radiata* and arrhenotokous *D. aligarhensis* (both sourced from Punjab, Pakistan). Three different foraging scenarios (n = 16 replicates per scenario) were observed for each population: (1) individual parasitoids foraging alone, (2) parasitoids foraging with a heterospecific competitor, and (3) parasitoids foraging with a conspecific competitor.

Experimental arenas consisted of glass rings, 1.0 cm tall and 2.5 cm in diameter. One end of the glass ring was sealed with fine no-see-um mesh (Skeeta, Bradenton, Florida) that permitted air transfer and prevented insect escape. The other end of the glass ring was closed with a square glass slide (3.0 × 3.0 cm; Fisher Scientific, Waltham, Massachusetts, USA), temporarily affixed to the ring using Museum Wax (Ready America Inc., Escondido, California), to prevent insect escape during oviposition studies. *Diaphorina citri* nymphs were transferred off of *C. volkameriana* flush into experimental arenas using a fine-tipped paintbrush (3/0 190 Finest Red Sable, M. S. Grumbacher, Philadelphia, Pennsylvania, USA). To facilitate observations, no citrus foliage was present in the arenas. As observed in previous studies, lack of foliage did not cause unexpected behaviors by parasitoids or *D. citri* nymphs (Vankosky & Hoddle 2017a). Five *D. citri* nymphs of each of the second through fifth instars were offered to female parasitoids as oviposition hosts (20 total nymphs per arena). The instar stage of *D. citri* nymphs was determined using a dissecting microscope (2.5× magnification) and confirmed according to images provided by Hall et al. (2012). Nymphs were allowed 10 min to acclimate and settle in the experimental arena before female parasitoids were introduced. Vankosky and Hoddle (2017a) provide additional details regarding experimental arenas.

Arenas containing *D. citri* nymphs and female parasitoids were sealed and maintained in the laboratory (23 ± 5 °C, 40% RH, 14:10 h [L:D] photoperiod) for 2 h, then female parasitoids were removed. Because *T. radiata* is an ectoparasitoid that deposits eggs on the ventral surface of the host near the hind legs (Hall et al. 2012), *D. citri* nymphs were immediately examined for parasitoid eggs. Nymphs with parasitoid eggs attached to the ventral surface were scored as parasitized, and the instar attacked was recorded.

Because *D. aligarhensis* is an endoparasitoid (Hall et al. 2012), dissection of *D. citri* nymphs was required to determine parasitization status. In *D. aligarhensis*-only treatments, nymphs were separated by instar, and isolated in 2.0 mL microcentrifuge tubes using a fine tipped paintbrush following parasitoid exposure. Microcentrifuge tubes contained *C. volkameriana* flush for nymphs to feed on and were held in a rearing room at 24 °C, 40% RH, and a 14:10 h (L:D) photoperiod for 48 h before being dissected and examined for eggs and first instar *D. aligarhensis* larvae (see Rohrig et al. 2011 for details regarding dissection methods). In treatments where *D. citri* nymphs were exposed to *T. radiata* and *D. aligarhensis* simultaneously, *D. citri* nymphs were initially examined for *T. radiata* eggs, then these eggs were removed from the nymphs that were then separated by instar, and isolated for 48 h as described above before being dissected and examined for *D. aligarhensis* eggs and larvae. All incidences of parasitism by both species were recorded.

NO-CHOICE EXPERIMENT

In no-choice experiments, 20 nymphs from 1 of 4 *D. citri* instars (second through fifth instar) were exposed to parasitoids under 6 foraging scenarios: (1) control without parasitoids, (2) 1 *D. aligarhensis* female, (3) 2 *D. aligarhensis* females, (4) 1 *T. radiata* female, (5) 2 *T. radiata* females, and (6) 1 female of both species released simultaneously. Foraging scenarios were replicated 12 times for each of the 4 *D. citri* instars, resulting in n = 288 replicates (i.e., experimental units: 4 instars × 6 foraging scenarios × 12 replicates = 288), for a total of 5,760 *D. citri* nymphs exposed to parasitoids.

Experiments were conducted using "cone-tainers" that consisted of 148 mL plastic vials inverted over 3.8 cm diameter cones that held soil and supported individual *C. volkameriana* seedlings (Bistline-East et al. 2015). Twenty *D. citri* nymphs of a single instar were transferred from stock colonies onto the flush of individual citrus seedlings using a fine-tipped paintbrush. Following transfer, nymphs were allowed 30 min to acclimate and settle on the citrus flush before female parasitoids were introduced. Cone-tainers were held in a rearing room at 24 °C, 40% RH, and a 14:10 h (L:D) photoperiod for 6 h, then the parasitoids were removed, the cone-tainers were sealed, left in the rearing room, and checked once per d for emergence of adult *D. citri* and adult parasitoids.

As adult *D. citri* emerged, they were removed from the cone-tainers, counted, and preserved. The number of *D. citri* adults that emerged from each cone-tainer was used to calculate *D. citri* mortality (i.e., due to parasitism, host feeding, and natural causes).

Adult parasitoids that emerged from each foraging scenario in the no-choice experiment were transferred to 2.0 mL microcentrifuge tubes provisioned with honey droplets (replenished every second d or as needed). Adult parasitoids were held in the same rearing room as the cone-tainers and checked daily until all adult parasitoids had died. The date of death and apparent cause of death of adult parasitoids were recorded. Causes of death included accidental entanglement in honey droplets, or natural causes (i.e., old age). The longevity (d) of each adult parasitoid that died a natural death was calculated.

After 20 d, seedlings in the cone-tainers were inspected for adult parasitoids a final time and the number of *D. citri* mummies from which adult parasitoids failed to emerge was recorded. Total parasitism of *D. citri* in each cone-tainer was calculated as the sum of emerged and unemerged parasitoids. Parasitoids sometimes failed to emerge from host mummies because parasitoid pupae died during development, because emerging adults were trapped under the host cuticle and were unable to emerge, or the *D. citri* nymph died before parasitoid development was complete. Thus, parasitism rates may have been underestimated.

STATISTICAL ANALYSES

The proportion (α_i) of *D. citri* nymphs of each instar selected as oviposition hosts by parasitoids in choice arenas was calculated using Equation 1 for Case 1 experiments (i.e., no host depletion), as described by Chesson (1983):

$$\alpha_i = \frac{(r_i/n_i)}{\sum_{j=2}^{m=5} r_j/n_j} \qquad \text{(Eq. 1)}$$

where *r* represents the number of hosts of type *i* used for oviposition, and *n* is the total number of hosts of type *i* that were initially available for oviposition. In the denominator of Equation 1, the proportion of each host type *j* in the arena used for oviposition is calculated, beginning with the second instar ($j = 2$) and ending at the fifth instar ($m = 5$). The experiment met the assumption of no host depletion because both *D. aligarhensis* and *T. radiata* multi-parasitize and superparasitize their hosts (Rohrig et al. 2011). Therefore, all nymphs remained viable oviposition hosts, regardless of prior parasitism. In addition, host feeding did not significantly reduce nymph availability during the short timeframe of the experiment, thus meeting the definition provided by Chesson (1983).

The effect of *D. citri* instar and foraging scenario (parasitoids alone, with conspecific competitors, and with heterospecific competitors) on oviposition preference was determined using repeated measures multivariate analyses of variance (MANOVA) for each parasitoid species using the general linear model (GLM) procedure (SAS Institute 2009). This analysis was used to compare "within treatment effects" associated with *D. citri* instar on parasitoid preference and the interaction of *D. citri* instar and foraging scenario on parasitoid preference.

The Sheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal & Rohlf 1995) was used to test for effects of the main factors (host instar, foraging scenario), and their interaction on parasitoid oviposition preference and *D. citri* mortality in the no-choice experiment, because both datasets failed to meet the assumptions required for parametric analysis. *H* values calculated by the Sheirer-Ray-Hare test were compared to $H_{critical}$ values from the χ^2 distribution for the corresponding numerator degrees of freedom (Zar 2010) to determine statistical significance (Sokal & Rohlf 1995). To investigate the effects of a significant interaction, the effect of the foraging scenario on mortality was determined for each of the 4 *D. citri* instars separately using 1-way Kruskal-Wallis tests (SAS Institute 2009). Significant Kruskal-Wallis tests were followed by a series of Mann-Whitney *U* tests, with Bonferroni adjustment of α, to separate means post hoc (SAS Institute 2009). The effects of host instar and foraging scenario on oviposition preference were determined separately for *D. aligarhensis* and *T. radiata*. Only 1 *T. radiata* adult and no *T. radiata* mummies were observed when second instar *D. citri* were the only available hosts; thus, this treatment was excluded from the analysis of *T. radiata* host preference. *Diaphorencyrtus aligarhensis* used all 4 host instars for oviposition; thus, all treatments were included in statistical analyses.

The date of adult parasitoid emergence from *D. citri* hosts was recorded and used to calculate parasitoid development time based on the date of nymph exposure to parasitism. The effect of *D. citri* instar on development of *D. aligarhensis* was determined using the Kruskal-Wallis 1-factor ANOVA equivalent, because this dataset failed to meet ANOVA assumptions (SAS Institute 2009). A series of paired comparisons were conducted using Mann-Whitney *U* tests to separate treatment means following significant test results (SAS Institute 2009), where the Bonferroni-corrected $\alpha = 0.0083$. The effect of *D. citri* instar on *T. radiata* development was determined using the GLM procedure to perform a 1-factor ANOVA with the PDIFF test specified in the LSMEANS statement used to separate treatment means post hoc (SAS Institute 2009). Results of the post hoc means separation tests were Bonferonni-adjusted to prevent Type I experimental error (SAS Institute 2009). Only 1 *T. radiata* adult emerged from second instar *D. citri* nymphs, so its development time could not be statistically compared to that of adult *T. radiata* that emerged from other instars.

The LIFETEST procedure used the Kaplan-Meier method for nonparametric estimation to generate survivor functions (i.e., longevity) for adult parasitoids that emerged from each host instar (SAS Institute 2009). The slopes of the survivor functions were compared using the χ^2-distributed log-rank test (SAS Institute 2009) to determine the effect of host instar on the probability of adult survival over time. To avoid Type I Error, log-rank test results were adjusted using the Šidák correction and these adjusted *P*-values were compared to $\alpha = 0.05$ (SAS Institute 2009).

Results

In choice arenas, *D. aligarhensis* from Pakistan laid eggs in 28 of 48 replicate arenas, across all foraging scenarios (*D. aligarhensis* alone = 11 of 16 replicates [i.e., 11/16]; conspecific pair = 9/16; heterospecific pair = 8/16). *Tamarixia radiata* laid eggs in 40 of 48 arenas (*T. radiata* alone = 15/16; conspecific pair = 12/16; heterospecific pair = 13/16).

Both *D. aligarhensis* (Wilks' $\lambda = 0.13$; $F = 52.73$; df = 3,23; $P < 0.0001$) and *T. radiata* (Wilks' $\lambda = 0.00$; $F > 99.99$; df = 2,35; $P < 0.0001$) exhibited preferences for nymphs of specific *D. citri* instars for oviposition in choice experiments. *Diaphorencyrtus aligarhensis* laid more eggs in third and fourth instar *D. citri* nymphs than in second and fifth instar nymphs (Fig. 1). *Tamarixia radiata* preferred fifth instar *D. citri* nymphs (Fig. 1). There was no significant effect of the interaction between host instar and foraging scenario (i.e., foraging alone, or in hetero- or conspecific pairs) on oviposition host preference for *D. aligarhensis* (Wilks' $\lambda = 0.87$; $F = 0.58$; df = 6,46; $P = 0.7476$) or *T. radiata* (Wilks' $\lambda = 0.98$; $F = 0.33$; df = 2,36; $P = 0.7229$).

The mortality of *D. citri* nymphs in no-choice experiments was affected by foraging scenario ($H = 26.98$; df = 4,264; $P < 0.001$), *D. citri* instar ($H = 19.34$; df = 3,264; $P < 0.001$), and by the interaction of these factors ($H = 37.03$; df = 15,264; $P < 0.005$). As a result of the interaction effect, *D. citri* mortality was affected by foraging scenario for second ($H = 16.44$; df = 5; $P = 0.0057$), third ($H = 17.93$; df = 5; $P = 0.0030$), and fifth ($H = 25.68$; df = 5; $P = 0.0001$) instar *D. citri* nymphs, but not for fourth instar *D. citri* ($H = 11.02$; df = 5; $P = 0.0511$) (Fig. 2). Mortality of second instar *D. citri* nymphs was greater when nymphs were exposed to pairs of *D. aligarhensis* compared to pairs of *T. radiata* (Fig. 2). Mortality of third instar *D. citri* nymphs was greater when exposed to individual *D. aligarhensis* than to pairs of *T. radiata* or to no parasitoids (Fig. 2). Mortality of fifth instar *D. citri* nymphs was generally greater when exposed to *T. radiata* (alone or in competitive scenarios) than when exposed to *D. aligarhensis* (Fig. 2). Mortality exceeded 50% when heterospecific females foraged simultaneously in no-choice experiments for all *D. citri* instars.

Parasitism in the no-choice experiment was used as a proxy for female preference. Parasitism of *D. citri* by *D. aligarhensis* was determined and compared across 3 foraging scenarios (i.e., *D. aligarhensis* alone, in conspecific pairs, and in heterospecific pairs). Results of the Sheirer-Ray-Hare Kruskal-Wallis test indicated that parasitism was affected by *D. citri* instar ($H = 14.93$; df = 3,132; $P < 0.005$), foraging scenario ($H = 7.69$; df = 2,132; $P < 0.05$; Fig. 3), and the interaction of these factors ($H = 18.05$; df = 6,132; $P < 0.05$). The interaction was driven by parasitism of fourth instar hosts. Specifically, when female *D. aligarhensis* foraged alone, 5 ± 1 *D. aligarhensis* F1 progeny emerged from fourth instar *D. citri* nymphs (Fig. 3). In contrast, approximately 1 F1 adult emerged from fourth instar hosts when *D. aligarhensis* females foraged in hetero- and conspecific pairs (Fig. 3).

Parasitism of *D. citri* nymphs by *T. radiata* was affected by host instar ($H = 56.60$; df = 2,99; $P < 0.001$), but not by foraging scenario ($H = 0.94$; df = 2,99; $P > 0.05$) or the interaction of those factors (*H* =

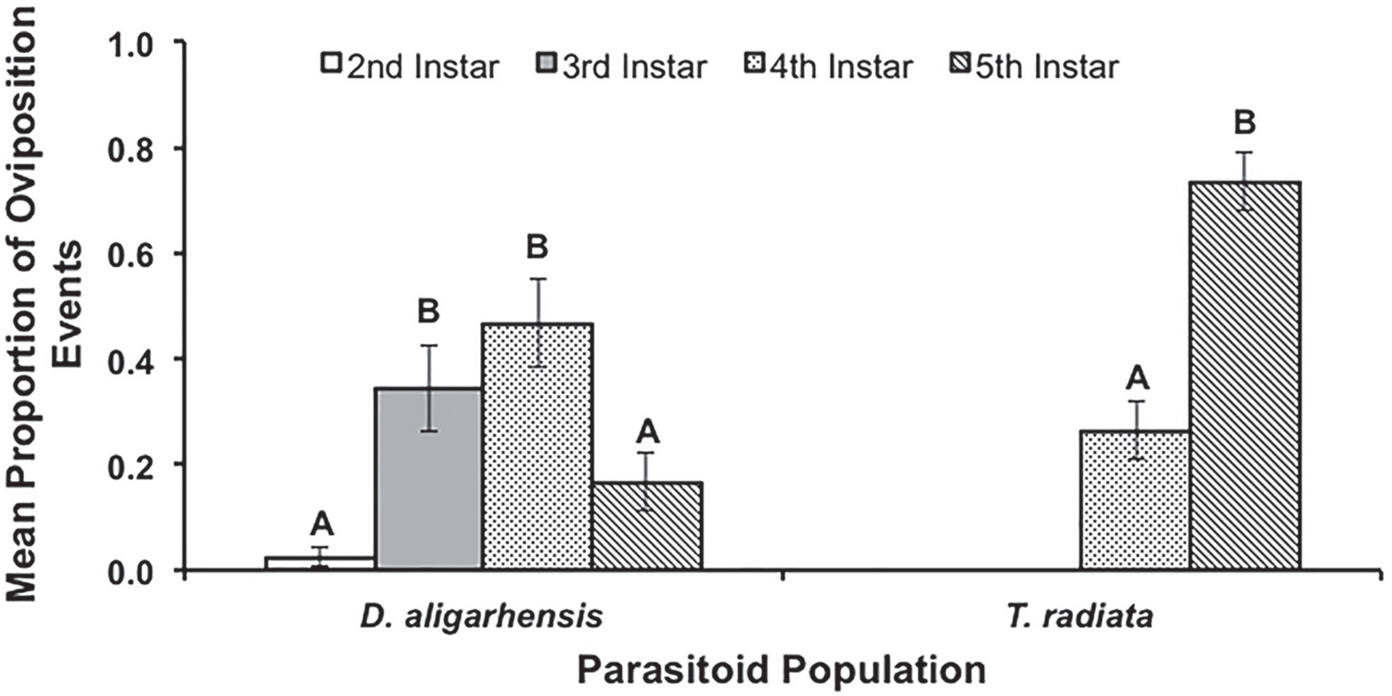

Fig. 1. Mean (± SE) proportion of oviposition events (α_i), indicating preference of female parasitoids for second through fifth instar *Diaphorina citri* nymphs in choice arenas. For each parasitoid species, means with the same letters are not significantly different ($P > 0.05$).

4.64; df = 4,99; $P > 0.05$). Only 1 F1 adult emerged from the 36 cone-tainers with second instar *D. citri* nymphs, and less than 1 F1 adult, on average, emerged from cone-tainers with third instar *D. citri* nymphs, regardless of foraging scenario (Fig. 4). In contrast, the mean emergence of *T. radiata* adults was 3 or more in no-choice arenas when fourth and fifth instar hosts were available for attack (Fig. 4).

Development time (egg to adult emergence) of 171 F1 *D. aligarhensis* was calculated for parasitoids that emerged from second (n = 59), third (n = 34), fourth (n = 61), and fifth (n = 17) instar *D. citri* nymphs. Host instar selected for oviposition affected the development time of *D. aligarhensis* larvae ($H = 76.66$; df = 3; $P < 0.0001$), such that development was slowest in second instar hosts and fastest in fourth instar hosts (Fig. 5A). *Tamarixia radiata* development time was calculated for 269 parasitoids that emerged from second (n = 1), third (n = 6), fourth (n = 109), and fifth (n = 153) instar *D. citri* nymphs. Host instar selected for oviposition affected development of *T. radiata* larvae ($F = 18.45$; df = 3,267; $P < 0.0001$), such that larvae developed slowest in second and third instar hosts and fastest in fourth and fifth instar hosts (Fig. 5B).

Of the F1 adult parasitoids that emerged from the no-choice experiment, 166 *D. aligarhensis* and 253 *T. radiata* died a natural death. The probability of adult *D. aligarhensis* survival over time was affected by the instar of the *D. citri* host at the time of oviposition (log-rank $\chi^2 = 11.41$; df = 3; $P = 0.0097$; Fig. 6A). The survival probability of F1 *D. aligarhensis* progeny from fourth instar hosts was greater than those of *D. aligarhensis* from second ($\chi^2 = 10.25$; df = 1; Šidák-adjusted $P = 0.0082$) and third instar hosts ($\chi^2 = 8.11$; df = 1; Šidák-adjusted $P = 0.0261$; Fig. 6A). The probability of F1 *T. radiata* survival over time also was affected by host instar at time of oviposition (log-rank $\chi^2 = 17.27$; df = 3; $P = 0.0006$; Fig. 6B). However, with the Šidák adjustment of P, no significant pairwise comparisons were observed because sample sizes between instars varied considerably. Although not significantly different, the survival probability of *T. radiata* adults that emerged from fourth and fifth instar hosts was generally greater than that of adults emerging from second and third instar hosts.

Discussion

When *D. aligarhensis* were limited to only 1 host stage for oviposition, female preference depended on both the host instar and the presence of competitors. Females demonstrated a strong preference for fourth instar hosts when foraging alone, and no strong preference for third instar hosts in any foraging scenario. Previous work has shown that *D. aligarhensis* is more likely to be impacted by competitive interactions than *T. radiata* (Vankosky & Hoddle 2017a, b). This observation appears to be exacerbated when only 1 host stage is available for oviposition. The mean number of adult parasitoids that emerged from fourth instar hosts was greater than that which emerged from any other host instar. In the no-choice experiment, adult parasitoid emergence was used as a proxy for female preference to avoid destructive sampling and allow measurement of offspring performance. Thus, apparent differences in preference in the no-choice experiment may be due to underestimation of parasitism due to the experimental design.

In choice arenas, arrhenotokous female *D. aligarhensis* from Pakistan preferred third and fourth instar *D. citri* nymphs for oviposition, followed by fifth and then second instar hosts. The preference of *D. aligarhensis* for fourth instar *D. citri* nymphs has not been recorded previously. This result contradicts work by Rohrig (2010) that demonstrated that thelytokous *D. aligarhensis* released in Florida preferred second and third instar hosts relative to fourth and fifth instar hosts. This result may be due to slight differences in the experimental designs used to test preference (i.e., number of hosts available, duration of host exposure to parasitism) (Rohrig 2010). However, differences between *D. aligarhensis* populations exist (i.e., arrhenotokous vs. thelytokous reproduction) that may explain varying host stage preferences (Hoddle 2012; Rohrig et al. 2012).

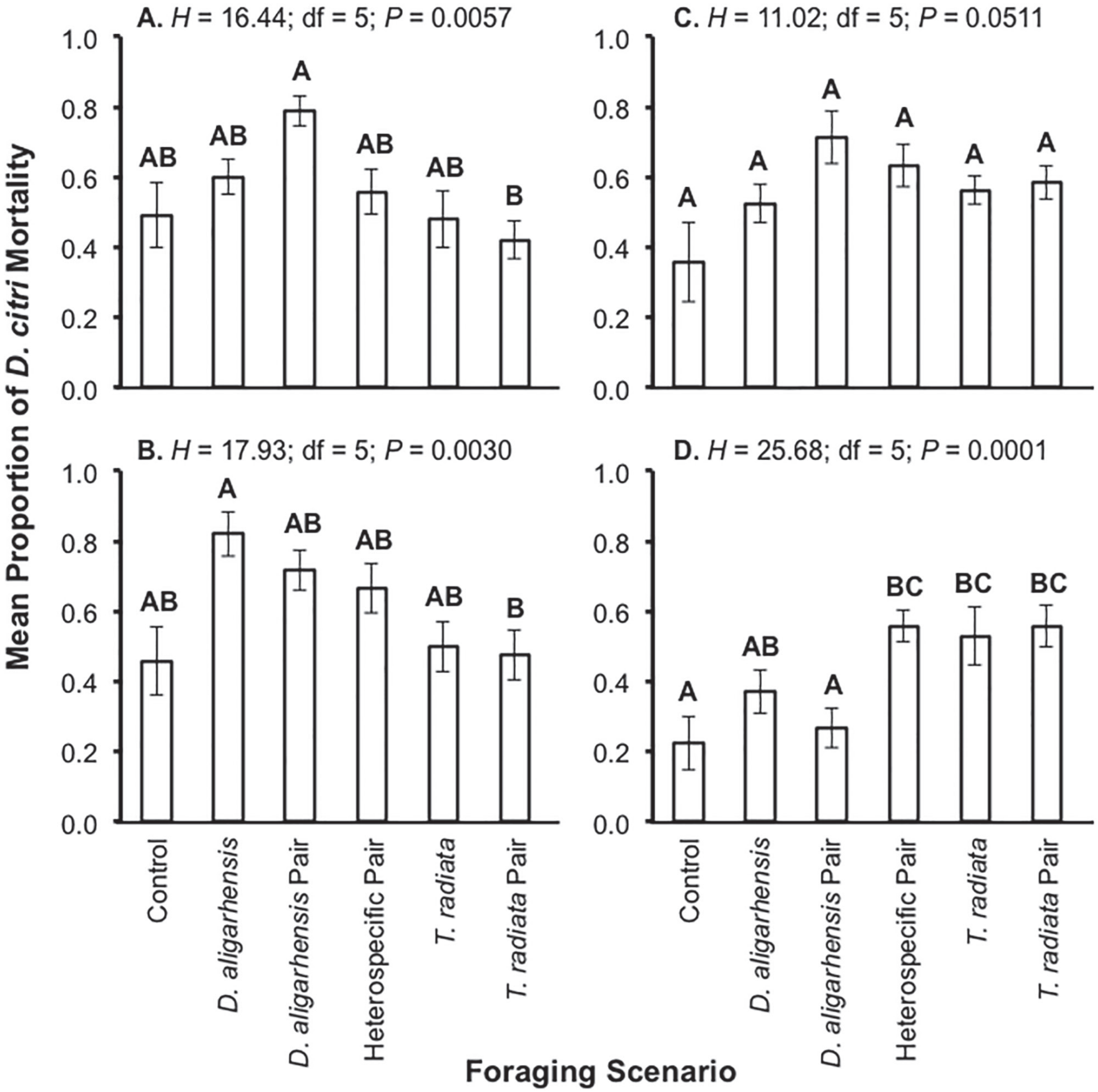

Fig. 2. The effect of foraging scenario on mean (± SE) *Diaphorina citri* mortality when (A) second, (B) third, (C) fourth, and (D) fifth instar *Diaphorina citri* nymphs were exposed to female parasitoids in no-choice experiments. In each panel, means with the same letters are not significantly different ($P > 0.05$).

Selecting third and fourth instar *D. citri* nymphs for oviposition may provide a selective advantage to *D. aligarhensis*. First, early instar *D. citri* are less likely to survive long enough to support *D. aligarhensis* development, because these nymphs tend to be more susceptible to predation and more vulnerable to catastrophic changes in abiotic conditions (Aubert 1987; Kistner et al. 2016). For example, Kistner et al. (2016) reported that *D. citri* eggs were especially prone to predation and that early instar nymphs frequently disappeared, reducing the size of study cohorts by more than half during the first 3 instars.

Second, the development time of *D. aligarhensis* larvae is faster in late-instar hosts, which may reduce mortality risks associated with abiotic conditions, intraguild predation (e.g., intraguild predation is associated with > 95% mortality of *T. radiata* larvae; Michaud 2004), or hyperparasitism, to which *D. aligarhensis* is particularly vulnerable (Hoddle et al. 2013; Bistline-East & Hoddle 2014). Rohrig et al. (2011) observed a 25% decrease in development time of *D. aligarhensis* larvae in fourth instar hosts (the less preferred host stage of thelytokous *D. aligarhensis*) compared to larvae that developed in second and third instar *D. citri* nymphs (the preferred host stage of thelytokous *D. aligarhensis*). No differences in longevity or fecundity of female parasitoids that developed from eggs laid in second, third, or fourth instar hosts were observed (Rohrig et al. 2011). In the no-choice experiment

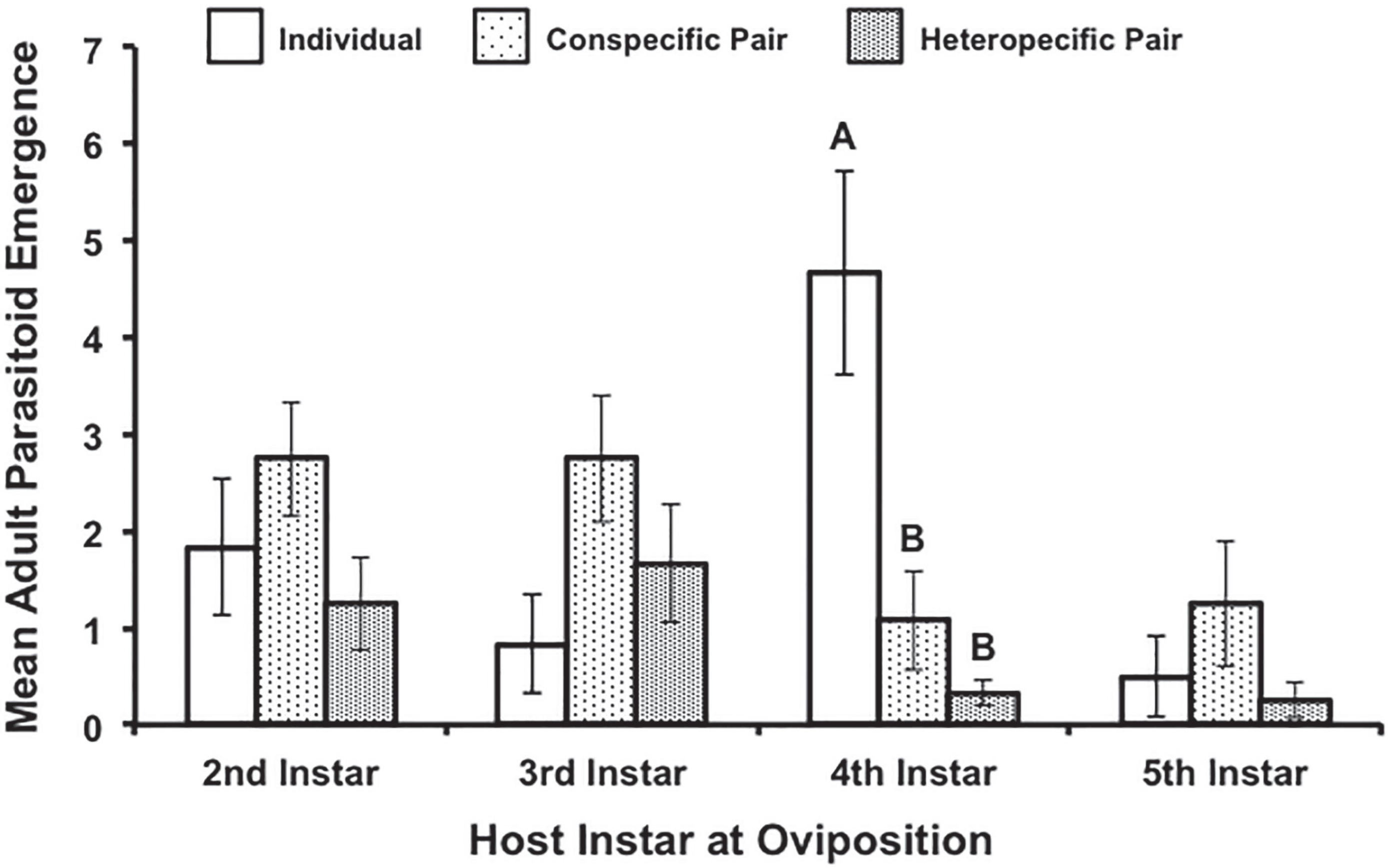

Fig. 3. Mean (± SE) emergence of adult *Diaphorencyrtus aligarhensis* from second through fifth instar *Diaphorina citri* nymphs in no-choice experiments when females foraged alone or with hetero- and conspecific competitors. Foraging scenario only affected parasitoid emergence when *D. aligarhensis* foraged for fourth instar *D. citri* nymphs (means with the same letters are not significantly different; *P* > 0.05).

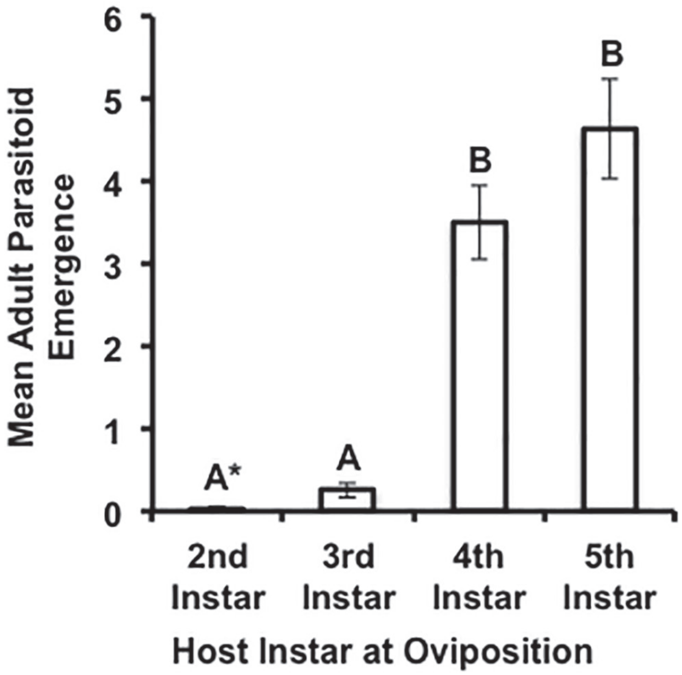

Fig. 4. Mean (± SE) adult *Tamarixia radiata* emergence from second through fifth instar *Diaphorina citri* nymphs in no-choice experiments. Means with the same letters are not significantly different (*P* > 0.05). *Only 1 F1 *Tamarixia radiata* emerged from second instar *D. citri* nymphs (mean [± SE] = 0.028 ± 0.028 F1 adults).

described here, *D. aligarhensis* larvae developed fastest when eggs were laid in fourth instar hosts. Larvae developed approximately 3 d faster in fourth than in second instar *D. citri* nymphs (14% decrease in development time), and 2 d faster in fourth instar hosts than in third instar hosts (10% decrease in development time). Larvae that developed from eggs laid in fifth instar *D. citri* developed approximately 2 d faster than larvae from eggs laid in second instar hosts (9.5% decrease in development time). The F1 *D. aligarhensis* adults that developed in fourth instar *D. citri* nymphs had the greatest probability of survival throughout their lifetime. Shortened development time and increased adult longevity are positive indicators of offspring performance (Thompson 1988) that are expected to positively correlate to female oviposition preferences (Jaenike 1978; Thompson 1988). Thus, the preference of female arrhenotokous *D. aligarhensis* for oviposition hosts appears to be positively correlated with offspring performance. In comparison, offspring performance of the thelytokous *D. aligarhensis* studied by Rohrig et al. (2011) was not correlated (negatively or positively) with female preference.

Tamarixia radiata sourced from Pakistan rejected second and third instar *D. citri* nymphs in choice arenas and demonstrated an oviposition preference for fifth instar hosts. In no-choice arenas, 41 and 57% of the total F1 adults emerged from fourth and fifth instar *D. citri*, respectively. Only 2% of F1 *T. radiata* adults emerged from second and third instar hosts. The preference of *T. radiata* for fourth and fifth instar *D. citri* nymphs corresponded to reduced development time of *T. radiata* larvae and longer lifespans of adult progeny. Our results agree with those of similar choice and no-choice experiments conducted with populations of *T. radiata* from China, Taiwan,

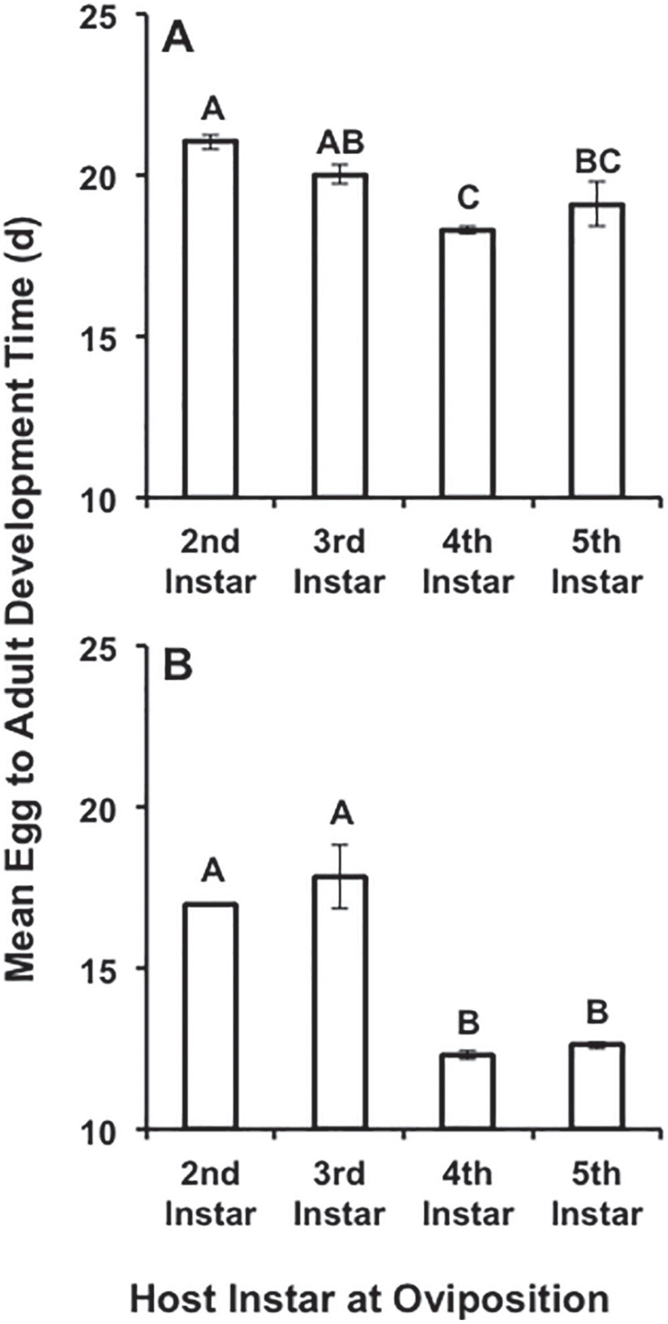

Fig. 5. Mean (± SE) development time of parasitoid eggs to adult emergence of (A) *Diaphorencyrtus aligarhensis* and (B) *Tamarixia radiata* that developed on different *Diaphorina citri* instars in no-choice experiments. Treatment means with the same letters are not significantly different ($P > 0.05$).

and Vietnam (Chu & Chien 1991; Hall et al. 2012; Chen & Stansly 2014b; Sule et al. 2014), indicating that *T. radiata* populations released in California and Florida do not differ in oviposition preference, unlike *D. aligarhensis* populations. Previous studies showed that female *T. radiata* that successfully emerged from fifth instar hosts were larger, and had greater fecundity and longevity when compared to females that emerged from smaller third and fourth instar *D. citri* (Chu & Chien 1991; Hall et al. 2012; Chen & Stansly 2014b).

The preference of *T. radiata* for late instar *D. citri* hosts is likely related to host size. Sule et al. (2014) noted that first, second, and third instar *D. citri* nymphs were generally too small to support *T. radiata* development, even though parasitized *D. citri* nymphs can continue to develop after being parasitized by this parasitoid. Specifically, Chien et al. (1991) reported that just 33% of *T. radiata* larvae developing on third instar hosts survived to adulthood. This result suggests that parasitoid larvae consume third instar host nymphs faster than the host can grow, and this constrains parasitoid development. Choosing to lay eggs in third instar *D. citri* represents a significant risk for female *T. radiata*, whereas larger bodied fourth and fifth instar *D. citri* nymphs consistently yield fitness gains.

The overlap in preference of fourth instar *D. citri* nymphs observed here could result in reduced parasitism rates in host patches where both parasitoid species forage concurrently. To assess this possibility, the choice and no-choice experiments reported here included scenarios where *T. radiata* and *D. aligarhensis* foraged in conspecific and heterospecific pairs. The preference of *D. aligarhensis* was not affected in choice arenas in the presence of hetero- or conspecific females. In competitive situations in no-choice arenas, parasitism of *D. citri* nymphs increased when 2 *D. aligarhensis* foraged simultaneously and decreased when heterospecific females foraged together. An exception was observed for fourth instar *D. citri* nymphs that were parasitized most when *D. aligarhensis* females foraged alone, and least when both species foraged together. This probably occurred because *T. radiata* was deprived of its most preferred host stage (i.e., fifth instar), forcing more *T. radiata* oviposition on fourth instar nymphs. In studies of parasitoid behavior in choice foraging arenas, Vankosky and Hoddle (2017a) reported that the behavior of *T. radiata* females was unaffected in competitive foraging scenarios. In the competitive scenarios reported here, the preferences of *T. radiata* for oviposition hosts was unaffected by the presence of potential competitors, as reported previously (Vankosky & Hoddle 2017a).

Importantly, mortality rates of *D. citri* nymphs were not adversely affected when multiple species of parasitoid foraged together in the experiments reported here. In no-choice experiments, mortality of second, third, and fourth instar *D. citri* ranged from 40 to 80% in all treatments with parasitoids. The mortality of fifth instar *D. citri* was less than 40% when *T. radiata* were absent, probably because this is a less preferred host stage for *D. aligarhensis* and possibly because large *D. citri* nymphs can deter female *D. aligarhensis* from ovipositing by shaking or “twitching” their bodies (Rohrig 2010). In comparison to *D. aligarhensis*, *T. radiata* use venom to immobilize their oviposition hosts (Chien et al. 1991), thus facilitating parasitism of fifth instar *D. citri* nymphs.

Although parasitoids foraging in field conditions are unlikely to encounter host cohorts of uniform developmental stage as in no-choice arenas, these results do have potential implications for the classical biological control program targeting *D. citri* in California. First, the results reported here agree with previous work indicating that *T. radiata* is a strong inter-specific competitor, and is relatively unaffected by the presence of foraging *D. aligarhensis* females (Rohrig 2010; Vankosky & Hoddle 2017a, b). Thus, the establishment of *D. aligarhensis* is unlikely to adversely affect parasitism of *D. citri* by *T. radiata*, which is widely established in California (Hoddle et al. 2016). Second, the establishment of *D. aligarhensis* in California may be limited to areas where *T. radiata* is absent or exists in low-density populations. The low levels of parasitism by *D. aligarhensis* in the presence of *T. radiata* observed in laboratory studies may not be sufficient for *D. aligarhensis* populations to become established in areas already inhabited by *T. radiata*. The relationship between these parasitoids may have contributed to the lack of establishment of *D. aligarhensis* in Florida (Rohrig et al. 2012). Field experiments

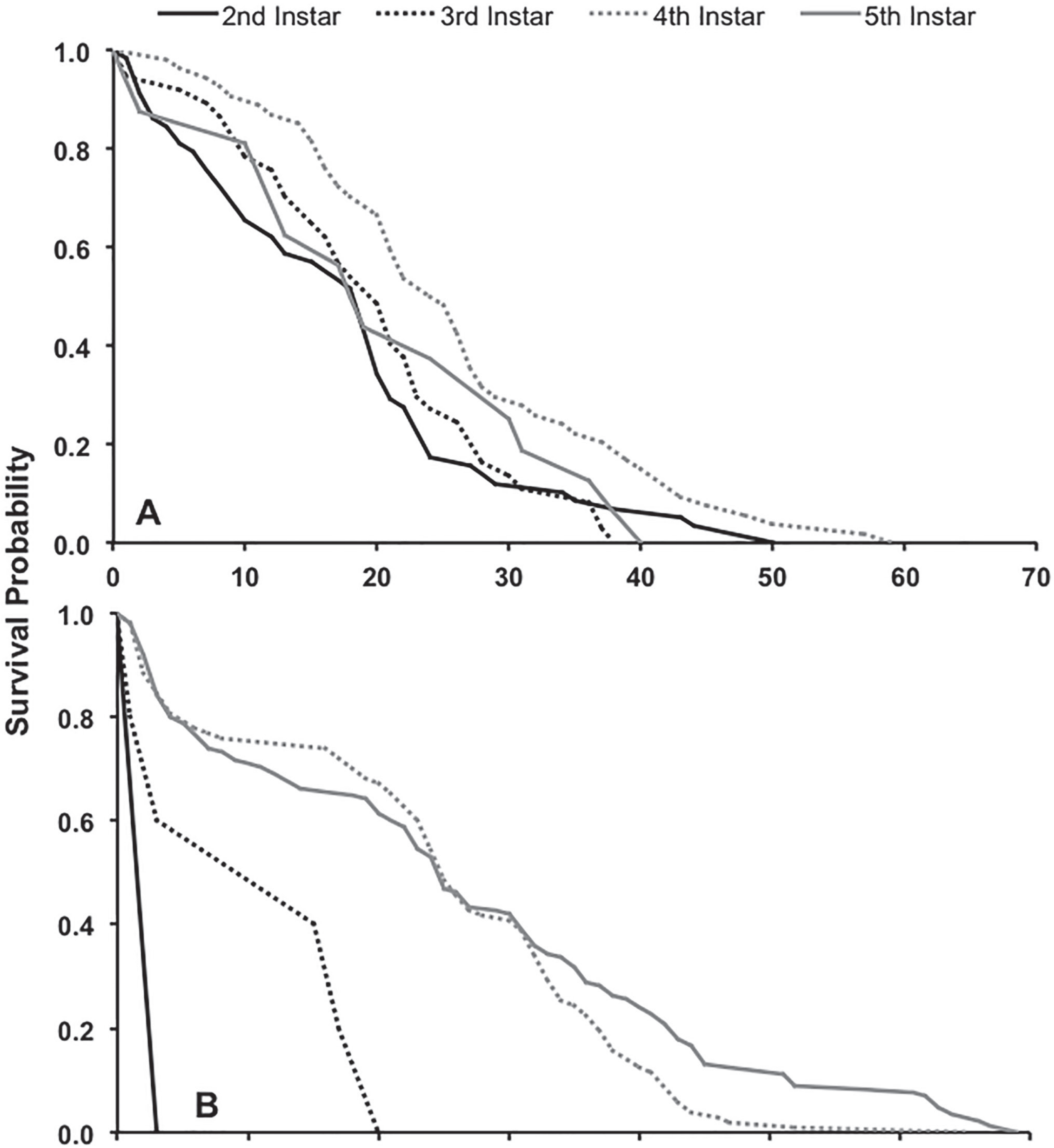

Fig. 6. The survival probability of adult (A) *Diaphorencyrtus aligarhensis* and (B) *Tamarixia radiata* that emerged from second, third, fourth, and fifth instar *Diaphorina citri* nymphs in no-choice experiments.

in citrus production areas in a variety of growing conditions (i.e., hot, dry interior desert areas vs. cooler, humid coastal zones) in California are needed to assess whether varying environmental conditions affect the likelihood of both parasitoid species coexisting in sympatry, on parasitoid reproductive success, and on parasitism rates.

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