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The effects of conspecific and heterospecific interactions on foraging and oviposition behaviours of two parasitoids of *Diaphorina citri*

Meghan A. Vankosky[a]* and Mark S. Hoddle[a,b]

[a]Department of Entomology, University of California, Riverside, CA, USA; [b]Center for Invasive Species Research, University of California, Riverside, CA, USA

ABSTRACT

Direct observation was used to construct activity budgets for *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae) and *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), foraging for nymphs of Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae). Two populations of *D. aligarhensis* were used in experiments: a biparental population from Pakistan and a uniparental population from Taiwan. Parasitoids were provisioned with 20 *D. citri* nymphs, second through fifth instar; female parasitoids from all three populations foraged alone, in conspecific pairs, or in heterospecific pairs. Walking occupied approximately 40% of the activity budget of *D. aligarhensis* and ovipositor probing occupied 10–20%. In contrast, probing and walking each accounted for approximately 30% of the activity budget of *T. radiata*. The presence of conspecific females affected the activity budget of *D. aligarhensis* females, such that the focal female was observed walking less often and probing more frequently. Heterospecific females did not affect *D. aligarhensis* activity. These results suggest mutual interference may occur between *D. aligarhensis* females foraging on the same host patch. The behaviour of *T. radiata* was not significantly affected by the presence of conspecific or heterospecific competitors. These results are discussed with respect to the release of multiple species of natural enemies in the classical biological control programme targeting *D. citri* in California.

Introduction

Direct observation of insects can provide insight into their behaviour. This method can elucidate how insects partition their activity budget and identify behaviours related to foraging and reproduction. The factors that influence insect foraging decisions have been studied using direct observation in the field (e.g. Cisneros & Rosenheim, 1998), and by manipulating foraging conditions in the laboratory (e.g. Dorn, Mattiacci, Bellotti,

CONTACT Meghan A. Vankosky ✉ meghan.vankosky@canada.ca ▣ Department of Entomology, University of California, 900 University Avenue, Riverside, CA 92521, USA
*Present address: Agriculture and Agri-Food Canada – Saskatoon Research and Development Centre, 107 Science Place, Saskatoon, Saskatchewan, Canada S7N 0X2. Email: meghan.vankosky@canada.ca

& Dorn, 2003; Reitz, Funderburk, & Waring, 2006; Vankosky & VanLaerhoven, 2015). Direct observation of foraging natural enemies can identify factors that affect prey preference. For example, Reitz et al. (2006) used direct observation to demonstrate that prey species of different size varied in their activity levels which in turn affected the preference of the predator, *Orius insidiosus* (Say) (Hemiptera: Anthocoridae). The effects of various biotic and abiotic factors on the efficacy of insects with biological control potential (i.e. predators, omnivores, and parasitoids) can be evaluated using this technique (Neuenschwander, 2001; Vankosky & VanLaerhoven, 2015), especially with respect to time spent searching for, or in contact with, prey or hosts (Cisneros & Rosenheim, 1998; Rosenheim, Goeriz, & Thacher, 2004; Vankosky & VanLaerhoven, 2015).

Direct observation may also be used to understand the outcomes of competitive interactions between conspecific or heterospecific individuals (e.g. Dorn et al., 2003; Irvin, Hoddle, & Morgan, 2006), including mutual interference (Hassell & Varley, 1969). Mutual interference between conspecific females may reduce search efficiency as parasitoid density increases (Free, Beddington, & Lawton, 1977). Therefore, direct observations can be used to investigate potential effects of the density or number of parasitoids released onto host patches, and contribute to refined release protocols. Additionally, direct observation can be used to assess the outcomes of biological control programmes that utilise more than one species of natural enemy that attack the same pest. For example, it has been proposed that biological control programmes that utilise multiple natural enemy species could have lower than expected efficacy because of intraguild predation or antagonistic competitive interactions which reduce pest mortality (Briggs, 1993; Ehler & Hall, 1982; Ferguson & Stiling, 1996; Rosenheim, Kaya, Ehler, Marois, & Jaffee, 1995). Alternatively, use of multiple natural enemy species may result in increased levels of pest suppression (Ferguson & Stiling, 1996; Mohammadpour, Jalali, Michaud, Ziaaddini, & Hashemirad, 2014; Xu, Yang, & Wan, 2015) because of niche partitioning due to climate, habitat, or host stage preferences, which reduce adverse competition outcomes (Denoth, Frid, & Myers, 2002).

In southern California USA, two parasitoid species are being released in a classical biological control programme targeting the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (Bistline-East, Pandey, Kececi, & Hoddle, 2015; Hoddle & Pandey, 2014; Vankosky & Hoddle, 2016). *D. citri* vectors the bacterium *Candidatus* Liberibacter asiaticus (*C*Las), the causative agent of huanglongbing (HLB) (Bové, 2006). *C*Las has been detected in southern California (Kumagai et al., 2016) and poses a threat to California's ~$2 billion citrus industry (United States Department of Agriculture [USDA], 2015). Currently, *D. citri* and *C*Las are largely restricted to urban-grown citrus in southern California (Hornbaker & Kumagai, 2016). An objective of the classical biological control programme in California is to reduce densities of *D. citri* in urban citrus (Hoddle, 2012). The intended outcome is to reduce rates of *C*Las acquisition from infected trees in urban areas and subsequent transmission of bacteria to uninfected trees in commercial production areas. Vector density reduction is recommended for slowing the spread of *C*Las by *D. citri* (Bové, 2006).

The first natural enemy species released in southern California was *Tamarixia radiata* (Waterston) (Hymenoptera: Eulophidae), a biparental, arrhenotokous parasitoid sourced from Punjab, Pakistan (Hoddle & Pandey, 2014). Over 3.5 million *T. radiata* have been released in southern California since 2011 and it is now considered established in the

region (Hoddle, Amrich, Hoddle, & Kistner, 2016). *T. radiata* is an ectoparasitoid that prefers to parasitise fourth and fifth instar *D. citri* nymphs (Hall, Richardson, Ammar, & Halbert, 2012; Sule, Muhamad, Omer, & Hee, 2014). Laboratory studies suggest that *T. radiata* inflicts considerable mortality on *D. citri* through parasitism and host feeding (Skelley & Hoy, 2004). *T. radiata* has a female biased sex ratio (up to 88% female [Chen & Stansly, 2014; Tang & Huang, 1991]) and has been utilised for *D. citri* biological control in several countries (e.g. Réunion Island, Mexico, and Brazil [Chen & Stansly, 2014; Etienne & Aubert, 1980; Parra, Alves, Diniz, & Vieira, 2016]), and other parts of the United States including Texas and Florida (Chen & Stansly, 2014; Skelley & Hoy, 2004).

In December 2014, release of a second parasitoid, *Diaphorencyrtus aligarhensis* (Shafee, Alam & Agarwal) (Hymenoptera: Encyrtidae), also sourced from Punjab, Pakistan (Hoddle, 2012), was initiated in California (Bistline-East et al., 2015). *D. aligarhensis* is an endoparasitoid that prefers to parasitise second and third instar *D. citri* nymphs (Rohrig, Shirk, Hall, & Stansly, 2011). Parasitisation and host feeding by *D. aligarhensis* kills *D. citri* nymphs (Skelley & Hoy, 2004). *D. aligarhensis* has contributed to population suppression of *D. citri* in Pakistan, Réunion Island and Taiwan (Chien & Chu, 1996; Etienne & Aubert, 1980; Khan, Arif, Hoddle, & Hoddle, 2014). It is the primary parasitoid attacking *D. citri* infesting limes in Saudi Arabia (Al-Ghamdi & Faragalla, 2000). Populations of *D. aligarhensis* are either biparental or uniparental (thelytokous) (Hoddle, 2012; Rohrig, 2010). Uniparental populations of *D. aligarhensis* failed to establish in Florida (Rohrig, Hall, Qureshi, & Stansly, 2012). In California, a biparental population of *D. aligarhensis* from Pakistan is being released (Bistline-East et al., 2015; Hoddle, 2012). Approximately 284,000 parasitoids have been released in southern California (Richard Dunn, Citrus Research Board, *personal communication*) and monitoring is ongoing to determine establishment. Our understanding of these two parasitoid species is based largely on studies of populations occurring naturally in Taiwan, Vietnam, and China (Chu & Chien, 1991; Tang & Huang, 1991) or collected from these regions and used elsewhere (e.g. Florida) in classical biological control programmes for *D. citri* (Rohrig, 2010; Rohrig et al., 2011, 2012; Skelley & Hoy, 2004).

In Pakistan the populations of *T. radiata* and *D. aligarhensis* being released in southern California account for 26% and 18% of *D. citri* mortality in citrus orchards, respectively (Khan et al., 2014). Because the two parasitoids are sympatric in citrus orchards in their native range, and prefer different nymph instars, we expect that they may be able to coexist in California citrus orchards. At the time this work was undertaken, there was no published research on the activity budgets of either *T. radiata* or *D. aligarhensis* or assessments of competitive interactions between conspecifics or heterospecifics when foraging for *D. citri* nymphs. Rohrig (2010) investigated heterospecific competition for oviposition hosts (i.e. superparasitism, multiparasitism) but did not directly observe female behaviour when heterospecific females foraged together. This represents a gap in understanding the behaviour of these parasitoids, especially in situations where both species are active simultaneously.

Consequently, the objectives of this work were to construct activity budgets for *D. aligarhensis* and *T. radiata* to investigate the effects of conspecific and heterospecific interactions on the behaviour of females of both species. In addition to the biparental *D. aligarhensis* population from Pakistan, we also observed the behaviour of uniparental

D. aligarhensis collected in Taiwan and released for *D. citri* biological control in Florida (Rohrig et al., 2011). This uniparental population of *D. aligarhensis* failed to establish in Florida (Rohrig et al., 2012). The two *D. aligarhensis* populations (i.e. biparental from Pakistan and uniparental from Taiwan) were studied to determine if differences exist between them in terms of observed behaviours, or in their responses to conspecific and heterospecific competitors foraging on the same patch of *D. citri* nymphs.

Using data obtained from direct observation of foraging parasitoids, we tested three predictions. First, we predicted that the activity budgets for both parasitoid species would be dominated by behaviours directly related to their fitness (i.e. oviposition; Wajnberg, 2006). Second, we predicted that female parasitoids would follow a predictable or standardised series of behaviours leading to oviposition as prescribed behavioural pathways have been quantified for other female parasitoids (Joyce, Hoddle, Bellows, & González, 2001; Zappalà & Hoy, 2004). Third, we expected female parasitoids to alter their foraging and oviposition behaviour in response to perceived conspecific or heterospecific competition, as reported for other encyrtid and eulophid parasitoids (i.e. Dorn et al., 2003; Dorn, Mattiacci, Bellotti, & Dorn, 2001; Urbaneja, Llácer, Garrido, & Jacas, 2003). The results of studies designed to test these predictions are reported here for two species of *D. citri* parasitoid, *T. radiata* and the uniparental (Taiwan) and biparental (Pakistan) populations of *D. aligarhensis*.

Materials and methods

Insect sources

Diaphorina citri used in experiments were obtained from a colony maintained in the insectary and quarantine facility (IQF) at the University of California, Riverside (UCR) on one of its host plants, *Citrus volkameriana* V. Ten. & Pasq. (Sapindales: Rutaceae). Plants were pruned to promote flush growth and placed inside cages constructed of clear 'U'-shaped plastic risers (SW Plastics, F2191, Riverside, CA) and no-see-um mesh (Skeeta, Bradenton, FL), as described by Bistline-East et al. (2015). Flushing citrus plants inside riser cages were inoculated with adult *D. citri* from a *C*Las-free colony, originally obtained via field collection in southern California and moved to IQF under California Department of Food and Agriculture (CDFA) permit No. 2870 (Bistline-East et al., 2015). Nymphs were left to develop to stages appropriate for parasitism at which time they were inoculated with adult parasitoids collected from colonies maintained in IQF or by the CDFA (see below for details on parasitoids). For the experiment described herein, flushing *C. volkameriana* plants were set up twice weekly (i.e. Tuesday and Thursday) to ensure constant availability of *D. citri* nymphs of appropriate developmental stages for presentation to parasitoids. All cages set up and used for experiments were held in a climate-controlled rearing room (29°C, 40% RH, 14:10 L:D photoperiod) in IQF.

The ectoparasitoid, *T. radiata* (biparental, imported into IQF UCR from Pakistan under USDA-APHIS permit P526P-09-02585), was obtained from the CDFA rearing facility at Mt. Rubidoux, Riverside, CA. The endoparasitoid, *D. aligarhensis*, was collected from one of two colonies maintained in two separate IQF greenhouses at UCR. The biparental *D. aligarhensis* colony was established with specimens collected from Pakistan between March 2011 and June 2012 and moved to IQF UCR under USDA-APHIS

permit P526P-11-00103 (Hoddle, 2012). Parasitoids from a second, uniparental population of *D. aligarhensis* originally collected in Taiwan, were obtained from Dr Eric Rohrig, Florida Department of Agriculture and Consumer Services, Gainesville, FL in December 2015 (material moved to IQF UCR under USDA-APHIS permit P526P-15-01734). Specimens obtained from Florida were used to initiate a colony used for experiments conducted in IQF UCR.

All female parasitoids observed were 3–7-d old and biparental parasitoids were mated. Before experimental observations were made, male/female parasitoid pairs (biparental Pakistani populations) and individual female *D. aligarhensis* (uniparental Taiwanese population) were held in 2 mL microcentrifuge vials provisioned with honey droplets to provide a carbohydrate source and kept in cold storage (16°C, 14:10 L:D photoperiod). Twenty-four hours prior to the initiation of behavioural observations, parasitoids were moved to clean vials for 6 h during which they were allowed to mate (if biparental) and host feed. Eight *D. citri* nymphs (two each from second to fifth instar) were provided and vials were held beside the microscope in the room where observations took place the next day. Oviposition may have occurred during this period. Preliminary observations indicated that the host-feeding period increased the likelihood of oviposition inside test arenas. After 6 h, individual females (without males for biparental *D. aligarhensis*) were transferred into clean vials provisioned with honey and returned to cold storage until the following day when observational experiments were run (approximately 18–24 h after initial host exposure).

Experimental arenas and observations

Foraging behaviour of female parasitoids was observed in arenas using a dissecting microscope under varying magnification (1.6x to 2.5x) as needed to confirm specific behaviours. Arenas consisted of glass rings, 2.5 cm in diameter and 1.0 cm high. One end of the ring was sealed with no-see-um mesh using hot glue. The other end of the glass ring was closed during observations using a square glass slide adhered to its surface using Museum Wax (Ready America Inc., Escondido, CA). Immediately before the observation period began, 20 *D. citri* nymphs (five each of the second, third, fourth, and fifth instars) were transferred to the arena using a fine paintbrush (3/0 190 Finest Red Sable, M. Grumbacher, USA). First instar nymphs were ignored by female parasitoids in pilot studies and thus were excluded from the experimental arenas. No plant material was placed inside the arenas. Preliminary observations showed that neither flush preferred for feeding by nymphs nor excised sections of mature leaves could be sufficiently secured inside the arena to prevent nymphs and parasitoids from hiding, which interfered with visual observations. In arenas without plant material, nymphs settled within 5–10 min and once settled, >90% of nymphs remained stationary until parasitoid inspection or attack, which stimulated short bouts of ambulation. After *D. citri* nymphs were settled, parasitoids were transferred into test arenas by gently tapping the microcentrifuge tube until the parasitoid dropped into the arena. Test arenas were then sealed to prevent escape of parasitoids and *D. citri* nymphs.

The activity of the parasitoid(s) inside each arena was visualised and recorded once every 60 s (using iPhone 4s timer) for 1 h (after Irvin et al., 2006), starting 5 min after parasitoids were placed into arenas. Observations were made in real time because constant

adjustment of the microscope focus was required to ensure accurate behavioural observations of parasitoids (Vankosky & VanLaerhoven, 2015). Behaviours were recorded directly into an Excel spreadsheet.

Table 1 describes the 6 behaviours most frequently observed and recorded during the 60-min observation periods. For the endoparasitoid *D. aligarhensis*, dissection is required to confirm successful oviposition events (Rohrig, 2010). For the ectoparasitoid *T. radiata*, *D. citri* nymphs must be flipped over to observe eggs on the ventral surface. Thus, to avoid destructive sampling or disturbing foraging parasitoids, we recorded 'ovipositor probing' events (hereafter, referred to as 'probing'), and not oviposition events.

For each of the three parasitoid populations, behaviour was observed under three different foraging scenarios. First, a single female foraged alone in the arena. Second, two females from the same population foraged simultaneously (conspecific pair). Third, a single female from each parasitoid species (*T. radiata* and *D. aligarhensis*) foraged together in a single arena (heterospecific pair). In heterospecific pairings, observations were recorded for both species, as the females are morphologically distinct and easily separable. Consequently, behaviours for both *T. radiata* and *D. aligarhensis* females in heterospecific pairings were recorded simultaneously. In conspecific pairings, one female was randomly designated as the focal female when the first observation was recorded; thereafter, this female was identified based on minor morphological differences (i.e. body size or colour patterns), location in the arena, or current behaviour. For example, once the focal female was probing or host feeding, she remained with that host for several minutes and movement away from the host was monitored in the time between recordings.

For each foraging scenario described above, a total of 16 replicates were made for a grand total of 48 replicates across all experimental scenarios. Replicates were performed during daylight hours and were spread evenly throughout the day to account for possible variation in insect behaviour (Cisneros & Rosenheim, 1998; Vankosky & VanLaerhoven, 2015). A single observer performed all replicates of the experiment to maintain observational consistency between replicates (Vankosky & VanLaerhoven, 2015).

Table 1. Description of the foraging behaviours displayed by female parasitoids of *D. citri* in arenas provisioned with 20 *D. citri* nymphs (five each of second through fifth instar nymphs).

Behavior	Description
Walking	Movement of the parasitoid around the experimental arena, with or without antennal drumming on the surface of the arena, with occasional contact with the host, but with no apparent host recognition. Contact with competitors occasionally occurred while walking
Resting	Parasitoid stood motionless, or with only very slight antennal movement, at a single location in the arena (i.e. no ambulation)
Grooming	Female parasitoid engaged in grooming (cleaning) of the legs and/or antennae, often using her legs or mouthparts
Antennation	Inspecting *D. citri* nymphs with antennae
Probing	Adoption of the 'oviposition stance' by the female while in contact with *D. citri* nymphs that may or may not have resulted in an egg being laid. Female *D. aligarhensis* (endoparasitoid) stood on the host and probed and then inserted the ovipositor into the host's dorsal surface. Egg deposition by *D. aligarhensis* must be determined by dissecting nymphs (Rohrig, 2010). Female *T. radiata* (ectoparasitoid) adopted a similar oviposition stance, but grasped the side of the host and probed the ventral surface of the host to deposit an egg on the external surface of the host near its posterior legs or to create a wound for host feeding
Host feeding	Contact observed between the host and the mouthparts of the parasitoid at an ovipositor-induced wound. Feeding on honeydew may also occur, but none was present in the arenas

Statistical analyses

The activity budgets of female parasitoids were expressed as the proportion of the 60 total observations during which females performed each of the 6 behaviours. Our first prediction was that the activity budgets of female parasitoids would be devoted to probing, resulting in an unbalanced activity budget. We tested this prediction using the nonparametric Kruskal–Wallis analysis of variance (ANOVA) equivalent (Cisneros & Rosenheim, 1998; Vankosky & VanLaerhoven, 2015), separately for the three parasitoid populations. For each population, 64 replicates were included in the analysis and the level of significance (α) was 0.05. Significant Kruskal–Wallis results were subsequently explored using 15 paired Mann–Whitney U tests, where $\alpha = 0.003$ (Bonferroni adjustment of $\alpha = 0.05$ to control type I error) (SAS Institute, 2009).

Our second prediction was that the behaviour of foraging females would follow predictable pathways. To assess this prediction, we constructed ethograms to visualise the behaviour of females foraging without competitors ($n = 16$). The total number of transitions between all possible pairs of behaviours was determined using raw observation data, which were recorded in sequence. These values were used to construct ethograms. Transitions that occurred fewer than seven times are not shown (after Joyce et al., 2001).

Finally, we predicted that females foraging with conspecific and heterospecific females would exhibit different behaviour compared to females foraging without potential competitors present. We assessed this prediction, separately for each population, using multivariate analysis of variance (MANOVA) (Cisneros & Rosenheim, 1998; Foster, Barkus, & Yavorsky, 2006; Reitz et al., 2006). Before analysis, the proportions (as calculated above) were transformed by first expressing each proportion as a percentage and then using: ln((%time +1)/ (100 – (%time +1))) (Cisneros & Rosenheim, 1998; Reitz et al., 2006). The analysis was conducted using the general linear model (GLM) procedure with the MANOVA statement included in the model script (SAS Institute, 2009). The MANOVA was run multiple times, with one behaviour in the repertoire randomly excluded each time to meet the assumption of independence (Cisneros & Rosenheim, 1998). For each parasitoid population, the results for each run were consistent. Therefore, the values reported below are for the MANOVA analysis that included all six behaviours. Significant MANOVA results ($p < .05$) indicated that certain behaviours in the repertoire were affected by the presence of a potential competitor. These behaviours were further explored using ANOVA and means comparison tests (GLM procedure) to identify differences between parasitoids foraging without competitors, with heterospecific competitors, and with conspecific competitors (Foster et al., 2006; SAS Institute, 2009). The Bonferroni correction was applied to $\alpha = 0.05$ to compare for type I error due to multiple comparisons (SAS Institute, 2009).

Results

Activity budget and ethograms (predictions 1 and 2)

All three parasitoid populations had unbalanced activity budgets (biparental *D. aligarhensis*: $H = 236.40$, $df = 5$, $p < .0001$; uniparental *D. aligarhensis*: $H = 187.41$, $df = 5$, $p < .0001$; *T. radiata*: $H = 208.57$, $df = 5$, $p < .0001$). Female *D. aligarhensis* from both populations were walking when 45% of the observations were recorded; the second and third most frequently observed behaviours for both *D. aligarhensis* populations were probing and

antennation (Figure 1). In comparison, *T. radiata* females walked in less than one-third of the total observations and equal proportions of the observations consisted of walking and probing (Figure 1). Resting was the least observed behaviour for all three populations.

There were no predictable behavioural pathways observed for any of the three parasitoid populations (Figure 2). Rather, each behaviour was equally likely to transition to any of the other observed behaviours. A notable exception was that the only behaviour that consistently transitioned into host feeding was probing. For example, probing transitioned to host feeding 22 and 43 times for biparental and uniparental *D. aligarhensis*, respectively, and 13 times for *T. radiata*. Observed bi- and uniparental *D. aligarhensis* and *T. radiata* transitioned to host feeding five times or less from each of the other behaviours (antennating, walking, resting, and grooming). In *D. aligarhensis* populations, host feeding transitioned to probing (biparental = 7 times; uniparental = 23 times), walking (biparental = 11 times; uniparental = 8 times), and antennating (biparental = 6 times; uniparental = 14 times) with the most frequency (Figure 2). When *T. radiata* stopped host feeding, their subsequent behaviours included walking (9 times), probing (4 times), or antennating (5 times) (Figure 2).

Effects of competition

The overall MANOVA analysis indicted that activity of biparental female *D. aligarhensis* was affected by the presence of competitors ($\lambda = 0.53$, $F_{12,80} = 2.52$, $p = .0073$). Behaviours affected by competitors included walking ($F_{2,45} = 4.65$, $p = .0146$), antennation ($F_{2,45} = 3.26$, $p = .0467$; not significant when further analysed with Bonferroni-corrected ANOVA to separate treatment means), and probing ($F_{2,45} = 5.57$, $p = .0069$). Focal
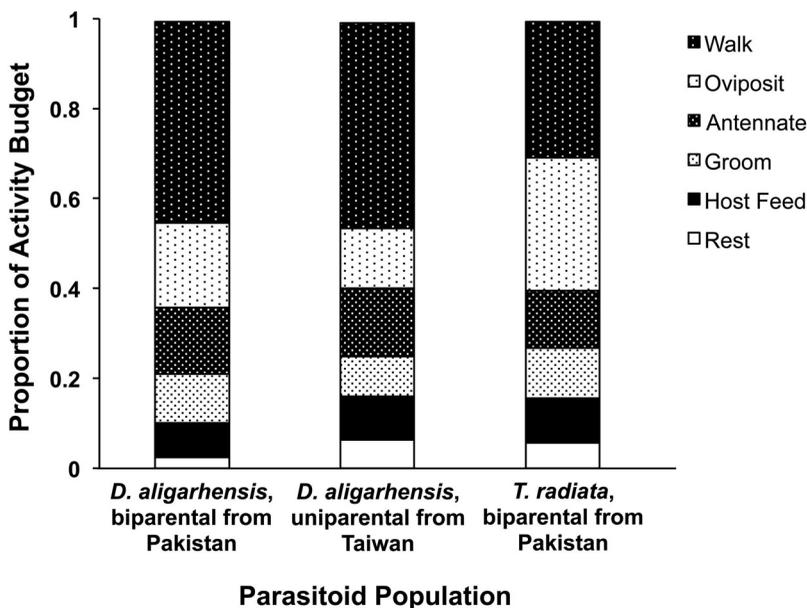

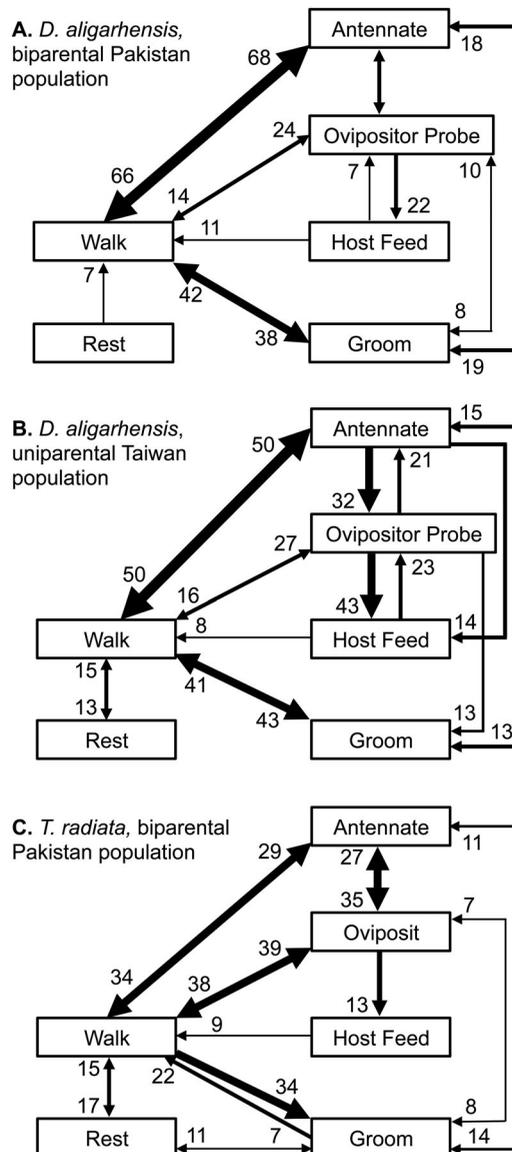**Figure 1.** The activity budgets of three populations of *Diaphorina citri* parasitoids.

Figure 2. Ethograms for three populations of *Diaphorina citri* parasitoids, showing the number of behavioural transitions made by female parasitoids foraging without competitors present. Arrows point from the initial to the subsequent behaviour; double-headed arrows represent behaviours that transitioned interchangeably. Transitions that occurred less than seven times are not shown (after Joyce et al., 2001).

females were observed walking less when foraging with a conspecific female than when foraging alone (Figure 3). In contrast, focal females were observed probing more often when foraging with a conspecific female relative to females foraging alone or with heterospecific females (Figure 3). The presence or absence of competitors did not affect the number of times females were observed resting ($F_{2,45} = 1.02$, $p = .3696$), grooming ($F_{2,45} = 2.67$, $p = .0805$), or host feeding ($F_{2,45} = 0.46$, $p = .6332$).
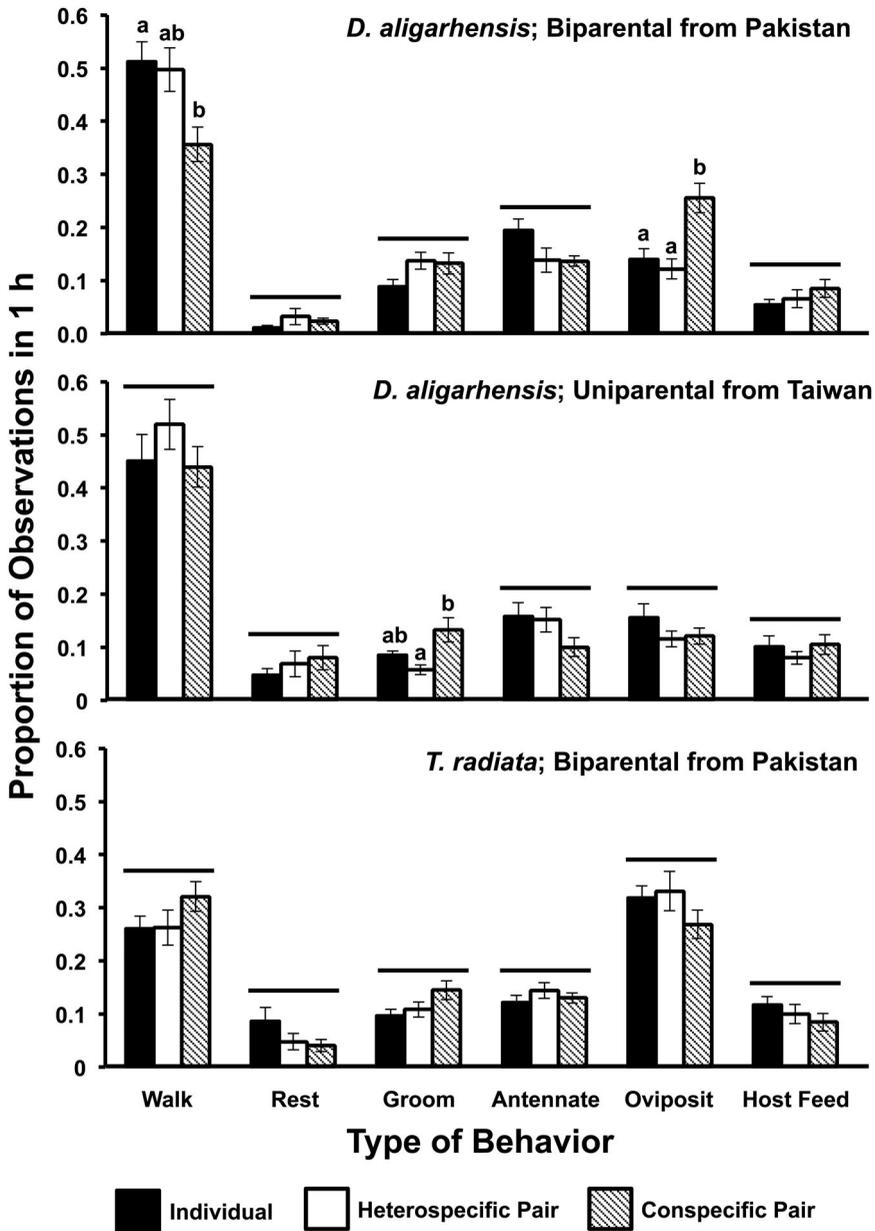
Figure 3. The effects of competition on the activity budgets of two species of *Diaphorina citri* parasitoids (uniparental and biparental *Diaphorencyrtus aligarhensis* and biparental *Tamarixia radiata*); where competition did not affect the prevalence of behaviours in the activity budget, a horizontal line is shown (MANOVA, $p > .05$). For behaviours affected by competition (MANOVA, $p < .05$), means with the same letters are not significantly different (ANOVA, $p > .05$).

The activity budget of *D. aligarhensis* from the uniparental population was also affected by competition ($\lambda = 0.60$, $F_{12,80} = 1.98$, $p = .0373$). Grooming was influenced by the presence of competitors ($F_{2,45} = 6.11$, $p = .0045$), such that the focal female was observed grooming more often in the presence of conspecific females than in the presence of

heterospecific females (Figure 3). The presence or absence of competitors did not affect the number of times that females were observed walking ($F_{2,45} = 0.74$, $p = .4830$), resting ($F_{2,45} = 0.28$, $p = .7554$), antennating ($F_{2,45} = 2.36$, $p = .1064$), probing ($F_{2,45} = 0.01$, $p = .9868$), or host feeding ($F_{2,45} = 0.07$, $p = .9292$).

Potential competitors (hetero- and conspecific) did not affect the activity budget of *T. radiata* ($\lambda = 0.76$, $F_{12,80} = 0.96$, $p = .4925$; Figure 3).

Discussion

The success of biological control programmes that utilise multiple species of natural enemy is a subject of debate (Denoth et al., 2002; Ehler & Hall, 1982; Ferguson & Stiling, 1996; Keller, 1984; Myers, Higgins, & Kovacs, 1989). Mathematical models have been developed that emphasise different parameters that might drive the success or failure of biological control programmes that use more than one natural enemy species when targeting a single pest species (reviewed by Hassell, 2000). Predictions made from competitive interaction models should be tested with a combination of laboratory and field experiments. Direct observation of parasitoid behaviour, as reported here, can be used to estimate aspects of activity budgets that may change in response to interactions with either heterospecific or conspecific females competing for hosts in a patch. This information may provide insight into possible levels of pest suppression that could be expected during varying competition scenarios (Mansfield, 2016; Mohamad, Wajnberg, Monge, & Goubault, 2015), including mutual interference between conspecific females (Free et al., 1977; Hassell & Varley, 1969).

In experimental arenas, we observed that walking dominated the activity budget of female *D. aligarhensis* from both the uniparental and biparental populations (>40% of total observations). Females from both *D. aligarhensis* populations devoted nearly equal proportions of their activity budget to walking. Thus, a high proportion of time spent walking may be characteristic of *D. aligarhensis*. During walking bouts, females were often observed walking over immobile hosts without inspecting them. Frequent walking might have been a response to the artificial conditions in which the experiment was conducted, as parasitoids were unable to leave experimental arenas, and were therefore unable to abandon host patches. To our knowledge, time allocation and patch leaving rules of *D. aligarhensis* have not been studied. To fully understand this aspect of *D. aligarhensis* behaviour, its foraging behaviour in a more complex spatial 'landscape' (i.e. on a small plant with multiple host patches) should be observed to determine its possible time allocation rules for processing, utilising, and abandoning host patches (e.g. giving up time, or the fixed number rule, see Wajnberg, 2006).

The second and third most prevalent behaviours in the *D. aligarhensis* repertoire were ovipositor probing and antennation, which accounted for 10–20% of the activity budget for females from both the uniparental and biparental populations. Both of these behaviours occurred in direct contact with the host. In contrast to *D. aligarhensis*, probing accounted for nearly 30% of the activity budget of female *T. radiata*. Based on this observation, *T. radiata* is likely to contribute to greater host mortality than *D. aligarhensis*, which would also result in numerical superiority in terms of breeding success. This observation agrees with fieldwork conducted in Pakistan where *T. radiata* accounted for 26% of *D. citri* mortality while *D. aligarhensis* accounted for 18% (Khan et al., 2014). Laboratory

based assessments found that female *T. radiata* parasitised 36% and host fed on 57% of available hosts (Skelley & Hoy, 2004). In contrast, *D. aligarhensis* parasitised 7% of available hosts while host feeding on 66% of hosts under similar experimental conditions (Skelley & Hoy, 2004).

In the experimental arenas described here, none of the three parasitoid populations had predictable or consistent behavioural pathways. This was contrary to our expectations, and may be indicative of the artificial experimental conditions. Other encyrtid parasitoids follow prescribed behavioural pathways that end in oviposition (Joyce et al., 2001; Zappalà & Hoy, 2004). For example, *Coccidoxenoides peregrinus* (Timberlake) (Hymenoptera: Encyrtidae) most frequently followed a 'search, antennate, probe, and oviposit' pathway (Joyce et al., 2001). The oviposition behaviour of another encyrtid, *Ageniaspis citricola* Logvinovskaya (Hymenoptera: Encyrtidae) also followed a strict order of behavioural events that led to oviposition (Zappalà & Hoy, 2004). In contrast to these encyrtids, *D. aligarhensis* probed hosts following both antennation and walking with similar frequency. *D. aligarhensis* frequently host fed after probing, as this action penetrated the host's cuticle, thereby providing access to haemolymph for consumption (Jervis & Kidd, 1986). Host feeding by *D. aligarhensis* occasionally transitioned back to probing, perhaps because the wound made by the first penetration did not release sufficient haemolymph or facilitate host feeding.

The activity budgets of *D. aligarhensis* from uniparental and biparental populations changed when conspecific or heterospecific competitors were present. Between the two *D. aligarhensis* populations, only three behaviours were affected: walking, grooming, and probing. The presence of a potential conspecific competitor altered the activity budget of female *D. aligarhensis* more than the presence of potential heterospecific competitors, indicative of mutual interference. Competition between *T. radiata* and *D. aligarhensis* may occur less often because their preferred host stages for oviposition differ (i.e. *T. radiata* prefer older *D. citri* nymphs than *D. aligarhensis*; Hall et al., 2012; Rohrig et al., 2011; Sule et al., 2014). Therefore, it is possible that *D. aligarhensis* reduces its oviposition activity in the presence of conspecific competitors because both females may target the same hosts in close proximity when host patches are small. Contrary to our expectation, focal biparental *D. aligarhensis* were observed probing more often with conspecific females than when alone or with *T. radiata*, suggesting that female *D. aligarhensis* may increase probing (leading to either host feeding or oviposition) in the presence of conspecifics. It would be interesting to determine whether offspring sex ratio is affected and conforms to the predictions of local mate competition under varying levels of female-female competition (Hamilton, 1967). In comparison to *D. aligarhensis*, the activity budget of *T. radiata* was not altered when females experienced hetero- or conspecific interactions.

The implications of interactions between conspecific and heterospecific individuals vary between different parasitoids species, which has led to a wide variety of behavioural responses and strategies when interactions occur. For example, *Aenasius vexans* Kerrich (Hymenoptera: Encyrtidae) and *Acerophagus coccois* Smith (Hymenoptera: Encyrtidae), parasitoids of cassava mealybug (*Phenacoccus herreni* Cox & Williams; Hemiptera: Pseudococcidae), adopt different strategies when foraging together on the same host patch. This results in parasitisation of different numbers of hosts and altered time budgets with respect to oviposition (Dorn et al., 2001). In a study of conspecific competition

between female *Enoggera nassaui* (Girault) (Hymenoptera: Pteromalidae), the first female to lay an egg, designated the 'owner', was less aggressive than the 'intruder' which arrived at the host patch second (Mansfield, 2016). Females of this species spent about 11 min per host on oviposition behaviours including drumming, probing, host feeding, and ovipositor insertion when foraging alone (Mansfield, 2016). When foraging together, the 'owner' required about 10 min to complete oviposition while the 'intruder' required only about 7 min (Mansfield, 2016). Female *E. nassaui* foraging alone or together parasitised the same number of hosts, but significantly altered their strategies when conspecific competitors were present (Mansfield, 2016). In our study system, the presence of heterospecific competitors did not significantly alter the behaviour of either of *D. citri*'s primary parasitoids in a manner measurable by this experiment. Our results complement those of Rohrig (2010) who observed equal rates of parasitism by *D. aligarhensis* and *T. radiata* when females foraged in heterospecific pairs, unobserved, for 8 h. We observed no outward acts of aggression between either hetero- or conspecific female pairs, although rarely (<1% of total observations) one female would interrupt the behaviour of the other.

Based on our observations, we believe it is unlikely that simultaneous release of both *T. radiata* and *D. aligarhensis* will have a negative impact on the foraging behaviours of either parasitoid should they simultaneously exploit patches of *D. citri* nymphs. Our observations suggest that interactions between conspecific *D. aligarhensis* may alter parasitoid behaviour more than interactions between heterospecific females. This work focused on adult–adult competition and the potential for intrinsic competition between larval parasitoids was not examined.

Irvin et al. (2006) demonstrated that both intrinsic and extrinsic competition should be assessed in order to best understand the effect of hetero- and conspecific competition between different species of natural enemy used in biological control programmes targeting a specific pest. It has been reported that larval *T. radiata*, from populations originating in Taiwan, outcompeted uniparental larval *D. aligarhensis* (originating from China), unless *D. aligarhensis* eggs were laid at least 5-d before *T. radiata* parasitised the host (Rohrig, 2010). Although similar work addressing larval competition between populations of *T. radiata* and *D. aligarhensis* (biparental) originating from Pakistan that are being used in the classical biological control programme targeting *D. citri* have not been completed, intrinsic competition between these two species may be expected. The potential for intrinsic larval competition warrants study to ascertain what effects it may have on the establishment, spread, and impact of *D. aligarhensis* when used for classical biological control of *D. citri* in areas where *T. radiata* is already established or is being released concurrently with *D. aligarhensis*.

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Disclosure statement

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