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Oviposition behaviour of *Tamarixia radiata*: effects of host density and exposure time

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Abstract. 1. The number of hosts attacked as a function of host density is considered to be an important characteristic of parasitoid behaviour and is used to estimate key parameters such as handling time and 'instantaneous rate of discovery'. However, little has been done to validate functional response models by direct observation of parasitoid oviposition behaviour.

2. *Tamarixia radiata* is the most promising parasitoid for biological control attacking *Diaphorina citri*. Mass rearing and augmentative release seen as a potential strategy for suppression of *D. citri* has been documented in abandoned citrus, residential areas, and organic groves. Nevertheless, parasitism rates in culture and in the field are only moderate, leading to questions about oviposition behaviour in response to host density.

3. Behaviours of gravid *T. radiata* females presented with susceptible host instars were categorised and documented by direct observation for 30 min and by camera recordings made over 12 h. Frequency of searching and antennating increased with host density during the 30 min. Probing rejection rates and search duration increased significantly with host density over 12 h. These factors resulted in significantly lower fecundity than expected, possibly due to host mark-mediated deterrence within the small searching area. Females took approximately 3.6–4.2 min to probe and parasitise a host regardless of host density and exposure duration. These results were markedly different from the 52.2 min estimated from the functional response equation.

4. Further experiments are required to assess the range and persistence of the putative host-marking pheromone, and to better understand the relationship between functional response parameters and actual behaviour.

Key words. Behaviour diagram, functional response, host marking, searching area.

Introduction

Functional response characterises the relationship between consumption by predators or parasitoids and host or prey density, respectively. The classic functional response model described by Holling (1959) included two parameters: 'instantaneous rate of discovery' and handling time, both assumed to be constant over a range of host densities. Surprisingly, the validity of these assumptions has not been tested by direct behavioural observations of parasitoid oviposition behaviour. Furthermore, the existence of other time-consuming behaviours has yet to be independently evaluated and incorporated into the model as suggested by Holling (1959).

Tamarixia radiata is the primary parasitoid of *Diaphorina citri*, vector of huanglongbing (HLB) or citrus greening disease. Published results on the functional response of *T. radiata* to its host are somewhat varied. Chen *et al.* (1995) reported a maximum fecundity per female of 25 eggs day^{-1} at a density of 40 hosts, and Sule *et al.* (2014) a maximum parasitism rate per female of 21.2 eggs day^{-1} at a density of 80 hosts. Chen *et al.* (2016a) also found that oviposition by *T. radiata* increased with increasing host densities, but only maximising at 11.2 eggs day^{-1} with 40 hosts per female, above which there was no further increase. Other aspects of the functional response with respect to *T. radiata* remain unclear, including the

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relationship between observed behaviour and calculated values for parameters such as handling time and searching coefficient using equations proposed by Holling (1959) and Rogers (1972) (Chen *et al.*, 2016a). Furthermore, other time-consuming activities such as grooming and resting that would alter the functional response model have not been quantified.

Experience in Florida and elsewhere has shown that *T. radiata* has the ability to establish and spread rapidly (Qureshi *et al.*, 2009). Yet the incidence of parasitism in the field averaged only 12% and 20% in Brazil and Florida, respectively (Qureshi *et al.*, 2009) and at best around 50% under mass-rearing conditions (Chen *et al.*, 2016b). The functional response has so far not proved consistent in predicting success of biological control (Fernández-Arhex & Corley, 2003). This could be due, in part, to discrepancies between calculated parameters and actual behaviour. Therefore, we used direct observation to study oviposition behaviour of *T. radiata* under different host densities. We further compared results from 30 min of direct observations with those from camera recordings made during 12 h to assess the effects of exposure time on critical functional response parameters.

Materials and methods

Colonies

Rearing methods generally followed those described in Skelley and Hoy (2004). *Murraya paniculata* was grown from seed and transplanted into 3.92-litre Air-Pots™ (TerraHydro, Carmichael, California) filled with potting media composed of 40% Canadian sphagnum peat plus bark, vermiculite, perlite, dolomitic limestone, and a wetting agent (Fafard 4P Professional Growing Mix, Sun Gro Horticulture, Agawam, Massachusetts). Plants were grown in a clear single-wall polycarbonate greenhouse maintained at $T = 12–33$ °C, RH = 52–97% monitored by a HOBO® RH/Temp/Light/External Data Logger-H08–004-02 (Onset Computer Corporation, Bourne, Massachusetts). The greenhouse was provided with fan and pad evaporative cooling protected by a filtered screen room, supplemental propane heat, and air-curtained anteroom covered with insect screen mesh (United Greenhouse Systems, Edgerton, Wisconsi). Each selected plant had at least eight shoots of new growth, with each new shoot about 3 cm in length and the terminal bud not fully open. Plants were sprayed as needed 4 days or more before use with 1% M-pede insecticidal soap (Dow AgroSciences LLC, Indianapolis, Indiana) to control unwanted psyllids and other pests.

Diaphorina citri were reared in BugDorm™-2400 Insect Rearing Tents (75 × 75 × 115 cm; MegaView Science Co. Ltd., Taichung, Taiwan) inside an air-conditioned glass greenhouse maintained at 22–32 °C, 50–80% RH (HOBO® RH/ Temp/ Light/ External Data Logger- H08–004-02). Nine flushing *M. paniculata* plants were placed into each cage, and approximately 1500 *D. citri* were released and held for 72 h to oviposit. Plants were then moved to a similar clean cage for about 10 days until eggs hatched and nymphs developed to the fourth instar.

Six *M. paniculata* plants infested with fourth nymphal instar psyllids were transferred to a ventilated wooden frame cage with polyacrylic sides inside a 4 × 4 m air-conditioned polycarbonate greenhouse maintained at 26 ± 1 °C, RH 55–85%. In all, 120 female and 60 male *T. radiata* were released inside it for 7 days and the progeny were collected from day 8 until no more were found (around day 22) (Chen *et al.*, 2016b).

At 6 days after *T. radiata* were released into the *D. citri* colony, small (2 cm) portions of shoots containing one parasitised nymph with the wasp close to pupal stage were excised and placed individually into a glass tube 75 mm long × 12 mm diameter (Fisher Scientific, Hampton, New Hampshire). Tubes were checked frequently, and wasps removed immediately upon emergence (Chen & Stansly, 2014) to a Petri dish with approximately 40, fourth-instar *D. citri* nymphs, and held for 72 h with host nymphs changed every 24 h.

Behaviour studies based on 30 min of direct observation

A young *M. paniculata* shoot with five leaflets was attached to a sterile polystyrene Petri dish (product no. 0875712; Fisher Scientific, Pittsburgh, Pennsylvania) of diameter 14 cm (area 154 cm^2) using double-sided Scotch cellophane tape (product no. MMS000039327; Scotch, St. Paul, Minnesota). Six host densities were prepared consisting of 10, 20, 30, 40, 50, and 60 fourth-instar *D. citri* nymphs. Nymphs were divided among the five leaflets, and randomly distributed on each leaflet. A 3-day-old *T. radiata* female was released into this arena for 10 min to adjust to the environment. The wasp was then tracked using a stereoscopic microscope for 30 min and all behaviours recorded using OBSERVER XT 11.0 (2013) behaviour coding and analysis software (Noldus, Wageningen, the Netherlands). Behaviours recorded included antennation, grooming, resting, host searching, probing, oviposition (verified later by carefully inverting the host with a probe), and host feeding. Duration and frequency of each behaviour were recorded. There were 10 replications at each host density.

Behaviour studies based on 12 h of recordings

The observation arena was prepared in a similar way as described earlier. A young *M. paniculata* shoot with three leaflets was attached to a sterile polystyrene Petri dish (product no. 0875713A, Fisher Scientific) of diameter 5.3 cm (area 67 cm^2) using double-sided Scotch cellophane tape. The different size Petri dish was required to accommodate the focal range of the camera. Three host densities using fourth-instar *D. citri* nymphs were prepared: 10, 30, and 60, with the fourth-instar nymphs evenly divided among the three leaflets, and randomly distributed on each leaflet. A 3-day-old *T. radiata* female was released into this arena for 10 min to adjust to the environment. Then she was tracked and recorded for 12 h using a Dino-Lite Edge Digital Microscope attached to the computer (The Microscope Store, LLC, Roanoke, Virginia). There were six replicates at each host density. Recordings were checked by fast-forwarding every 10 s, and the number of hosts encountered and parasitised, searching duration, non-searching (grooming and resting) duration, and handling (probing and parasitising) duration were noted. Additionally, the first 30 min of

each recording were monitored completely and tracked with OBSERVER software to compare with results from the 30-min study done in the larger (154 cm^2) arena.

Statistical analysis

The number of hosts parasitised (N_p), parasitisation duration per host (T_p), searching duration (T_s), and other behaviour durations, including time interval between two ovipositions (OI) among host densities, were compared using an ANOVA with means separated by Fisher's least significant difference ($P < 0.05$).

Frequencies of all behaviours at each host density were visualised using behaviour diagrams (Rohrig, 2010). Frequency of each behaviour at the six host density levels was tested using regression analysis. Fractions of all behaviours among six host densities were compared using a multinomial χ^2 test ($P < 0.05$). Probing rejection rates leading to activities other than oviposition were compared using Kruskal–Wallis test due to non-normal distribution. The relationship between searching duration and exposure time was compared using linear regression analysis assuming a positive correlation. Data were analysed with JMP software (SAS Institute Inc., 2013).

Results

Host density effects on behaviour frequency based on 30 min of observation

Behaviours at six different densities showed similar patterns (Figures S1–S5, Appendix S1). As an example, wasps presented with 40 hosts executed 756 behavioural events observed over the 30-min observation period (Fig. 1). Of the 264 host-searching events, 211 (80%) led to antennating of a nymph. Ninety-nine (33.6%) of the 295 antennations led to host probing, five to host feeding, and two to honeydew feeding; the rest (64.1%) resulted in rejection and searching again for hosts. Twenty-one out of the 99 (21.2%) probing events led to successful oviposition and 78.8% led to rejection, followed either by host searching or antennating. Sixty-four grooming events were observed, of which 70.3% led to host searching. The remaining behaviour diagrams at each host density are available as Figures S1–S5, Appendix S1.

Searching frequency increased from 19.7 ± 1.2 (mean $\pm$ SEM) at density 10 to 36.2 ± 4.3 at density 60, with significant differences among host densities [$F = 10.05$, $\text{d.f.}_{(\text{model, error})} = 1, 58$, $P = 0.0024$]. Antennation frequency also varied significantly among densities [$F = 9.31$, $\text{d.f.}_{(\text{model, error})} = 1, 58$, $P = 0.034$], increasing from 23.6 ± 2.2 to 40.9 ± 5.5 over densities 10–60. In contrast, frequency of probing (mean 10.7 ± 0.76), grooming (mean 5.88 ± 0.37), resting (0.5 ± 0.13), host feeding (0.38 ± 0.08) honeydew feeding (0.2 ± 0.07) and oviposition (1.48 ± 0.2) showed no significant effect of host density (Table S1, Appendix S1).

The multinomial χ^2 test showed that fractions of frequencies of individual behaviours over the frequency of all behaviours observed were not independent of host density ($\chi^2 = 69.43$, d.f. = 36, $P = 0.0007$). The largest deviations from expected fractions under the null hypothesis of independence were seen in the probing fraction ($\chi_{\text{probing}}{}^2 = 28.1$, 40.5% of the total χ^2 statistic), which showed an overall decreasing trend from 0.21 to 0.12 as host density increased from 10 to 60. This decrease was offset by modest increases in the observed fractions of searching events (0.31 at density 10, 0.37 at density 60) and antennating events (0.37 at density 10, 0.42 at density 60). Fractions of other behaviours remained relatively constant.

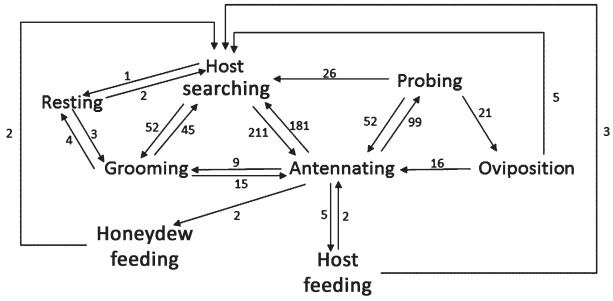

Fig. 1. Behaviour diagram for a 3-day-old mated female *Tamarixia radiata* at a density of 40. Numbers refer to frequency of the behaviour over a 30-min observation period ($N = 10$). Arrows leading to a specific behaviour indicate that behaviour is derived from others.

Host density effect on behaviour durations

No significant host density effect was observed on N_p and T_p during the 30-min observation time. Mean T_p was estimated at 3.65 ± 0.24 min (0.06 ± 0.004 h) [$F = 0.52$, $\text{d.f.}_{(\text{model, error})} = 5, 42$, $P = 0.76$], and mean N_p at 2.18 ± 0.15 parasitised hosts [$F = 0.55$, $\text{d.f.}_{(\text{model, error})} = 5, 54$, $P = 0.74$].

Oviposition interval ranged from 3.68 ± 0.72 to 5.57 ± 0.90 min, with a mean estimate of 4.85 ± 0.41 min and no significant effect of host density [$F = 0.49$, $\text{d.f.}_{(\text{model, error})} = 5, 58$, $P = 0.78$]. Searching duration (including antennation) and resting duration were not significantly influenced by host densities [$F = 1.28$, $\text{d.f.}_{(\text{model, error})} = 5, 54$, $P = 0.28$; $F = 1.7273$, $\text{d.f.}_{(\text{model, error})} = 5, 54$, $P = 0.14$, for searching and resting respectively], and the mean durations were 10.71 ± 0.52, and 10.54 ± 4.71 min, for searching and resting, respectively. Host density did affect grooming duration [$F = 18.31$; $\text{d.f.}_{(\text{model, error})} = 5, 54$; $P < 0.0001$], with the mean increasing from 3.75 ± 0.93 to 15.67 ± 1.31 min as host density increased from 10 to 60. There was no significant host density effect on probing rejection rate ($57.0 \pm 0.04\%$, $\chi^2 = 1.85$, d.f. = 5, $P = 0.87$).

Behaviour based on 12 h recordings

Number of hosts parasitised, time spent parasitising and interval between ovipositions did not vary significantly with host density. N_p increased from 5.3 ± 0.42 to 6.67 ± 0.76 to 7.17 ± 0.60 at increasing host densities of 10, 30, and 50, respectively [$F = 2.41$; $\text{d.f.}_{(\text{model, error})} = 2, 15$; $P = 0.12$]. T_p averaged 0.07 h over 12 h ($F = 0.35$, d.f. = 2, 15; $P = 0.71$) and OI values were 1.9 ± 0.23, 1.64 ± 0.17 and 1.66 ± 0.13 h for 10,

30, and 50 hosts, respectively (mean = 1.75 ± 0.1, $\chi^2 = 2.37$, d.f. = 2, $P = 0.31$).

The mean percentage of time spent searching varied significantly, with ranges of 21.7–43.5% with 10 hosts, 11–22.8% with 30 hosts, and 36.4–89.4% with 50 hosts ($F = 3.83$, d.f. = 1,70, $P = 0.05$; $F = 15.62$, d.f. = 1,70, $P = 0.0002$; and $F = 6.54$, d.f. = 1,70, $P = 0.02$, respectively). The probing rejection rate increased significantly with increasing host density ($F = 9.55$, d.f. = 1,16, $P = 0.008$), from 60.2 ± 7.1% to 70.0 ± 4.2% to 80.5 ± 3.4% at densities of 10, 30, and 50 respectively.

The mean number of hosts parasitised (N_p) during the first 30 min in the 67-cm^2 area averaged 1.4 ± 0.24, with no significant differences among host densities ($F = 3.66$, d.f. = 2, 15, $P = 0.06$). This result was at variance with an N_p of 2.18 ± 0.15 reported earlier for the 30-min study in the larger arena. The mean rejection rate was 67.3 ± 6.2% for the first 30 min of the 12 h study, also with no host density effect ($\chi^2 = 1.28$, d.f. = 2, $P = 0.53$). This result was roughly consistent with a rejection rate of 57.0 ± 0.04% for the 30-min study.

Discussion

Tamarixia radiata females took approximately 3.6–4.2 min to probe and parasitise a host (T_p) with no significant effect of host density seen in either study. This result is consistent with a basic assumption of the classic functional response model described by Holling (1959). The OI averaged 4.85 min during the 30-min observation period, and 1.75 h over 12 h. This result is not surprising as the wasps could hardly lay an egg every 5 min for 12 h. Nevertheless, OI accounted for a similar 16.2% and 14.3% of total exposure times for 30 min and 12 h, respectively.

Searching and antennating durations also did not change with host density, in contrast to greater frequency of these activities with more abundant hosts. Thus, more total time was spent searching and antennating at high host density, leaving less time for other activities. A likely candidate for less time spent at higher host densities was probing, the frequency of which, relative to all other activities, decreased from 21% to 12% over the range 10–60 hosts, although the absolute frequency of probing did not increase significantly with host density. In contrast, grooming duration increased significantly with increasing host density, probably in response to increased amounts of honeydew.

An average of five nymphs were probed over the course of 30-min observations, of which a mean 2.18 were parasitised and the remaining 57% were rejected with no effect of host density on rejection rate. This result contrasted with probing rejection rates over 12 h which increased significantly from 60.2% to 70.0% to 80.5% at host densities of 10, 30, and 50, respectively. The resulting maximum fecundity of 7.17 eggs was at variance with the 11.4 reported by Chen *et al.* (2016a) at a density of 50 per female for a 14-h light cycle in a Petri dish with searching area of 147 cm^2.

Given that all conditions were similar, the lower fecundity seen in the 12-h experiment could be due to the smaller (67 cm^2) searching area. To further evaluate this hypothesis, we compared the first 30 min of the 12-h recording with the 30-min observations done in the larger (154 cm^2) arena. Sure enough, probing rejection rate in the small arena averaged 67.3% for the first 30 min with no density effects. This result was markedly greater than 57.0% seen in the larger arena during the 30-min observation experiment. This finding further confirmed the hypothesis that rejection rates increased and thus fecundity decreased with searching area.

Another apparent effect of small arena size was the observation that the proportion of time spent searching was density-dependent, ranging from 21.7% to 43.5%, with 10 hosts, compared with 36.4–89.4% with 50 hosts. No such density effect on searching time was seen in the larger arena. We know that the rate of parasitisation decreases over a prolonged exposure time (Chen *et al.*, 2016b), presumably as the supply of mature eggs is exhausted. Nevertheless, we would expect about 9.2 more eggs to be laid between the first 30 min (2.18 eggs) and a total of 11.4 eggs from the previous study in the 157-cm^2 arena. Instead, we found only an increase of about 5.8 eggs from an initial 1.4 eggs during the first 30 min to a final of 7.17 eggs after 12 h in the 67-cm^2 arena.

The negative effect of searching area on fecundity, as well as increased searching time and rejection rate are all consistent with potential patch effects of a host mark left by the parasitising *T. radiata* female to deter further oviposition (Chen *et al.*, 2016c). Such a 'patch' mark would exert the strongest effect on surrounding hosts under conditions of high host density and limited searching area. In functional response terms, this outcome would be a consequence of reduced attack rate at high host density caused by host marking. This mechanism could also be responsible for the low incidence of parasitism observed in mass rearing and in the field. Further work is needed to verify this hypothesis.

This study also revealed discordance between the observed time of 3.6–4.2 min (average 3.9 min) used by the female *T. radiata* to parasitise her host and a handling time of 0.87 h (52.2 min) calculated using the functional response equation (Chen *et al.*, 2016a). Clearly, behavioural predictions based on a classical understanding of functional response cannot be taken at face value. The implications of these and other results obtained with *T. radiata* are further explored in a companion paper to this study (Chen & Stansly, 2017, unpublished).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12470

Appendix S1. Behaviour diagrams and frequency analysis at different host densities.

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