

Deterrent effects of larval tracks on conspecific larvae in *Cycloneda limbifer*

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Abstract The response of *Cycloneda limbifer* Say (Coleoptera: Coccinellidae) larvae to conspecific larval tracks was investigated with the assistance of the automatic video tracking system EthoVision. Single larvae were monitored for 1 h in an arena with a choice of two substrates, one clean and one contaminated with the tracks of conspecific larvae. Fourth instar larvae of *C. limbifer* effectively differentiated clean substrates from substrates with fresh tracks of conspecific first instars. Larvae walked significantly further on clean than on contaminated substrates in each 20-min period of the 1 h trials. With the exception of the first 20-min period, residence times were also longer on clean than on contaminated substrates. The mean velocity of larvae on the substrates did not differ in any period of the test. The results indicate that a deterring pheromone in fresh tracks of *C. limbifer* larvae effectively deters conspecific larvae from staying on sites previously exposed to larvae. The mode of action of the semiochemical repellent secreted by larvae is complementary to a previously described semiochemical-mediated egg-spacing mechanism in the coccinellid *C. limbifer*. Active distribution of larvae has the potential to improve the initial distribution of offspring by females among aphid colonies of varying qualities and may facilitate survival during changes in the distribution of conspecific larvae in the course of the development.

Keywords Predators · *Cycloneda limbifer* · Coleoptera · Coccinellidae · Larvae · Avoidance · Detering pheromone · Deterrence · Spacing

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Introduction

Reliable data on the foraging behaviour of aphidophagous predators are necessary for evaluating their role in the regulation of aphid populations, and therefore for the eventual use of these predators in biological control. Detailed knowledge about the effects of factors that determine the density of aphidophagous insects in aphid colonies is essential for understanding the dynamics of predator-prey relationships.

The larvae and usually also the adults of many aphidophagous coccinellids are voracious predators. The high number of aphids consumed by the larvae presumably results from their relatively low nutritive value. In accord with this, Magro et al. (2002) found that the aphidophagous *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae) spend more time feeding than the coccidophagous *Cryptolaemus montrouzieri* Mulsant (Coleoptera: Coccinellidae). Aphidophagous species use olfactory cues of plant origin while searching for sites with suitable prey (Kesten 1969). Little is known about the influence of semiochemical attractants or deterrents on the active spacing of coccinellid larvae and adults. Although coccinellids frequently find aphids on plants at random, volatiles of the prey have been found to attract coccinellid larvae (Sengonca and Liu 1994; Hemptinne et al. 2000; Jamal and Brown 2001), adults (Obata 1986; Sengonca and Liu 1994) and induce egg laying (Evans and Dixon 1986). Larvae and adults of *Coccinella septempunctata* L. (Coleoptera: Coccinellidae) which find aphids attract starved individuals of the same instar or stage (Sengonca and Liu 1994). In larvae, the attraction steadily declined from first to fourth instar.

Elliott and Kieckhefer (2000) found that early in the season, coccinellids are capable of reducing high aphid densities in spring wheat fields. In another study, coccinellid adults effectively aggregated and caused an aphid population crash in a hop garden after the sudden decrease of the aphid population in a near-by field (Růžička et al. 1986). Evans and Youssef (1992) noted that the densities of aphidophagous species of coccinellids were strongly positively correlated with aphid densities among alfalfa fields scattered throughout a several km² area early during the growing season. Within a field, coccinellid larvae were found to be almost as efficient as adults in aggregating in prey patches, possibly by immigration from surrounding areas, although the aggregation of larvae required about twice as long as that of adults to reach peak density (Elliott and Kieckhefer 2000).

Hungry coccinellid larvae frequently cannibalize eggs, larvae, prepupae and pupae (Mills 1982; Osawa 1989; Agarwala 1991; Schellhorn and Andow 1999). Unrestricted oviposition of females attracted to an aphid colony could therefore cause not only the premature extinction of the resource, but also endanger preimaginal development of overcrowded competitors. As coccinellid females usually lay eggs in batches, single oviposition events can dramatically increase the density of larvae in small aphid colonies. Furthermore, coccinellid females are much more mobile than their larvae, so proper selection of oviposition sites is likely to be more important for the distribution of offspring than movement of larvae. On the other hand, if the presence of conspecifics effectively repels coccinellid larvae, the larvae could leave the original colony after an excessive oviposition before aphids completely disappear, and thereby avoid the danger of attack from starved conspecifics. Active avoidance of low quality resources by coccinellid larvae (as well as adults) is likely to contribute to the survival and fitness of these predators.

Females of phytophagous coccinellids avoid egg laying on plants where many eggs are present (Ohgushi and Sawada 1985). In aphidophagous species, physical encounters of females with conspecific larvae and adults have been reported in a number of studies to inhibit oviposition (Hemptinne and Dixon 1991; Hemptinne et al. 1992; Hemptinne et al. 1993). As in the case of chrysopids (Růžička 1994), an oviposition-detering pheromone

has been found in the tracks left by conspecific (Růžička 1997; Doumbia et al. 1998) and heterospecific (Růžička 2001, 2006; Magro et al. 2006) coccinellid larvae. As observed for chrysopid larvae (Růžička 1994), coccinellid larvae mark their tracks with minute amounts of oviposition-detering abdominal secretions that also enable firm adhesion of the abdominal disc to the oviposition surface (Laubertie et al. 2006). As physical contact with conspecific individuals is undoubtedly less frequent than encounters with their tracks, strong effects of an oviposition-detering pheromone are likely to be especially important for the spacing of coccinellids (Oliver et al. 2006), chrysopids (Růžička 1994, 1998) and other predators of aphids (Růžička and Havelka 1998). Furthermore, the detection of tracks of small instars is probably more important than of larvae in more advanced developmental stages because young larvae are able to destroy a higher quantity of prey before reaching pupation than older larvae. For this reason, attention has been focused only on the effects of tracks of first instars (Růžička 1994, 1998, 2001). Tracks of *Cycloneda limbifer* Say (Coleoptera: Coccinellidae) larvae effectively deterred females of conspecific and heterospecific coccinellids from oviposition (Růžička 2001, 2003, 2006).

The distribution of coccinellid eggs among different prey resources by females may not always be adequate to the quality of resources. If coccinellid larvae are also capable of detecting and effectively avoiding sites with tracks of conspecific larvae then the initial spacing of the progeny by females could be further improved through the same cue. Deterrent effects of larval tracks on other larvae are likely to optimize the distribution of larvae among aphid colonies through time. Such a strategy would allow adjustment to gradual changes in the suitability of prey resources and so avoid, or minimise, undesirable exposure of pre-imaginal stages to dangerous competitors. The first study to test this hypothesis, however, reported that larvae of *C. septempunctata* were able to respond to tracks left by themselves but not to tracks of other larvae (Marks 1977). Other attempts to find deterrent effects of larval tracks on conspecific larvae have been unsuccessful (Ferran and Dixon 1993). Afterwards, repellent effects of larval tracks on the larvae were not investigated.

In this study, we tested whether tracks of larvae of the coccinellid *C. limbifer* can deter conspecific larvae. In the absence of prey, movement of the larvae was monitored in an experimental arena with a choice of two substrates. One substrate was clean and the other was contaminated with the tracks of conspecific larvae. In order to minimise the inaccuracies inherent to direct human observation, we used EthoVision, an automatic video tracking, motion analysis and behaviour recognition system (Noldus et al. 2002) to monitor and quantify the behaviour of larvae.

Materials and methods

Insects

A stock culture of *C. limbifer* (Cuba origin) was kept for approximately 100 generations on *Aphis fabae* Scopoli and *Aphis craccivora* Koch (Hemiptera: Aphididae) in the laboratory at $23 \pm 2^\circ\text{C}$ and with a light regime of 18 L:6 D. The two aphid species were reared on *Vicia faba* L.

Preparation of substrates contaminated with larval tracks

Tracks of first instar *C. limbifer* larvae were collected on transparent plastic substrates cut from 3M write-on transparency film (AF4300) normally used for overhead projection.

Each substrate was a 40×200 mm strip folded every 10 mm to a total length of 130 mm. Fourteen substrates destined for contamination with larval tracks were placed in pairs on the bottom of seven Petri dishes. Inner rims of 19.5 cm diameter Petri dishes were painted with a Fluon (polytetrafluoroethylene dispersion in water, Sigma-Aldrich Chemie GmbH), to prevent escape of larvae. Two substrates were simultaneously exposed to 40 first instars for 4 h in each dish. First instar larvae (16–22 h old) were used for contamination of plastic strips. No aphids were given to the larvae prior to their use for contamination. Tracks used in subsequent trials were less than 6 h old.

Choice trials

Prior the choice test, each fourth instar larva of *C. limbifer* was kept separately for 4 h without aphids in a Petri dish, 9 cm in diameter. Individual fourth instars were monitored in a glass arena 40–48 h after moulting. New arena with new corrugated substrates was prepared for each test. The arena consisted of an 18.5 cm diameter clean Petri dish with 2 cm wide Fluon paint on the inner rim. One clean and one contaminated substrate were placed in the arena, inside slightly larger rectangular zones, each measuring 53×145 mm. The larva was thus free to forage and occupy the substrate of its choice in each trial. Each substrate was fixed in its rectangular zone with two narrow strips of transparent adhesive tape. No aphids were present during the test. Videotracking of a larva began 30 s after introducing it into the centre of the arena and lasted 1 h. One test was conducted at a time and each larva was used only once. The test was replicated 14 times, with the position (left/right) of substrates (clean and contaminated) being alternated between replications.

Video tracking, data acquisition and statistical analysis

The behaviour of each larva was recorded and analysed using the computerized video tracking system EthoVision (Noldus Information Technology, Wageningen, The Netherlands). The system consists of a video monitor, personal computer with a frame grabber (Targa Plus, TrueVision), and colour CCD camera equipped with a zoom lens fixed above the centre of the arena. The computer was placed outside the room. This setup allowed no one to be present in the room during the experiment, thus avoiding disturbance of the natural behaviour of the larva by an observer during the test. The experiments were carried out in a photographic darkroom illuminated by a series of fluorescent tubes (Narva LS 65 W-1 coolwhite) attached to the ceiling. The light was diffused by hanging a thin white cloth under the lamps. The light intensity measured in the arena was 800 Lux. The temperature in the room during experiments was kept at 26°C. The experimental setup employed in this study follows Růžička and Zemek (2003).

The location of the larva in the arena was determined automatically by the EthoVision software using a gray scaling method for object detection. The arena was subdivided electronically into a 'cues' (=contaminated) area and a 'control' (=clean) area. Data obtained with digitized paths of an individual larva were used to calculate the following parameters: (1) total distance walked, (2) mean velocity, (3) number of visits to each substrate and (4) total time spent on each substrate. For detailed definitions and algorithms of parameter calculations see Noldus Information Technology (1997). In addition to calculations incorporating data from the entire duration of the test, we also analysed the data in three 20-min periods separately. Data were analysed using Wilcoxon paired sample tests

(Siegel and Castellan 1988). All P values are based on two-tailed tests and values less than 0.05 were considered significant.

Results

During 1 h of the choice test, fourth instars of *C. limbifer* walked significantly ($P = 0.0004$) further on the clean substrate (mean \pm SE = 339.4 ± 60.1 cm) than on the contaminated substrate (158.9 ± 29.6 cm). Larvae also visited more often ($P = 0.0327$) the clean substrate (48.1 ± 7.3 times) than the contaminated one (36.8 ± 5 times). Although the larvae spent on average more time (1413.9 ± 127.9 s) on the clean than on the contaminated substrate (900.6 ± 128.2 s), the difference was not statistically significant ($P = 0.0906$). The mean velocity of larvae on the clean (0.25 ± 0.04 cm/s) and on the contaminated (0.21 ± 0.04 cm/s) substrate did not differ ($P = 0.5016$).

When the three consecutive 20-min periods of the test were analysed separately (Fig. 1), *C. limbifer* fourth instars walked significantly further in each period on the clean than on the contaminated substrate ($P = 0.0295$, $P = 0.0006$ and $P = 0.0052$). With the exception of the first period, the larvae also spent more time on the clean than on the contaminated substrate ($P = 0.6257$, $P = 0.0040$ and $P = 0.0494$). In the middle period of the test, visits of larvae were significantly more frequent on the clean than on the contaminated substrate ($P = 0.6355$, $P = 0.0122$, $P = 0.4143$). The mean velocity on substrates did not differ in any period ($P = 0.1563$, $P = 0.2676$ and $P = 0.9032$). Thus, the differences between distance walked, residence time and number of visits of larvae on the clean and on the contaminated substrate were always greatest in the middle period.

Discussion

The suitability of an aphid colony for the development of aphidophagous larvae depends not only on the actual number of aphids, but also on other factors that influence the future development of the colony. The prey/predator ratio, species and stages of competitors can strongly influence the growth potential of colonies.

In this study, we found that coccinellid larvae respond to the tracks left by conspecific larvae by avoiding staying on contaminated substrates. This could indicate that larvae continuously counterbalance spatial variation in densities of repellent tracks by moving from sites with higher to lower track densities. In the field, the ratios between attractant and deterrent semiochemical cues probably determine the mobility and foraging of coccinellid larvae.

Active searching of coccinellid larvae for less exploited prey resource is likely to reduce potentially hazardous exposure of developmental stages to cannibalism or attacks from heterospecific predators. Older instars walk longer and search more effectively for superior quality colonies than younger ones. Thus, older coccinellid larvae in particular should be reluctant to stay in colonies with numerous early instars because these resources may be prematurely depleted and therefore become hazardous for immobile prepupae and pupae. If last instars occasionally pupate outside aphid colonies while searching for better resources, the prepupae and pupae may also be cannibalised by younger conspecific larvae less than in colonies with numerous conspecific larvae.

Results of all previous attempts to assess the repellent effects of coccinellid larval tracks on conspecific larvae have been inconclusive. Coccinellid larvae tracks left on plants have

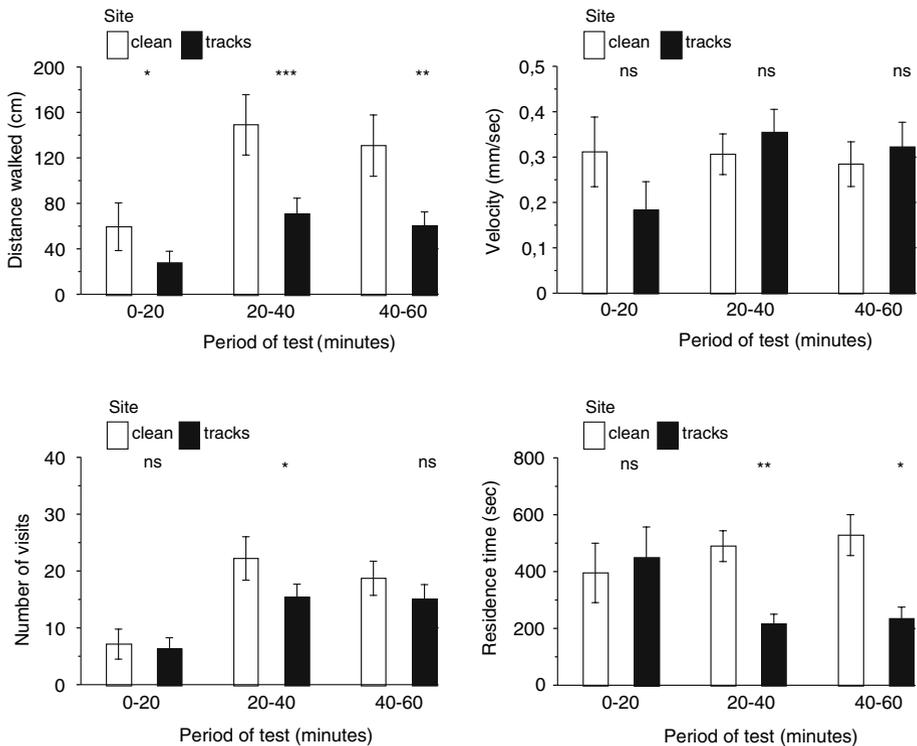


Fig. 1 Effects of conspecific tracks of *Cycloneda limbifer* first instars on the behaviour of fourth instars. Results (mean \pm SE of choice trials between a clean substrate and a substrate contaminated with fresh tracks of conspecific first instars in three subsequent intervals are shown. (Wilcoxon paired sample tests: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns—not significant $P > 0.05$.)

been reported to deter only the individual that left the track but not other larvae (Marks 1977). Repellent effects of larval tracks on conspecific larvae have not been found later (Ferran and Dixon 1993). Unfortunately, the search for effects of a deterring pheromone left by larvae on foraging conspecific larvae has probably been neglected, in part due to the discovery of intra- (Růžička 1997; Dombia et al. 1998; Yasuda et al. 2000; Oliver et al. 2006) and interspecific (Růžička 2001, 2006) oviposition-detering effects of larval tracks on females of aphidophagous coccinellids, a topic which has since received considerable attention. Furthermore, in comparison with adults, the relatively short walking range and maladaptation of larvae to starvation was also thought to limit their potential for active dispersal and searching for more convenient feeding sites.

Magro et al. (2002) showed that when aphids were present, adults of the coccinellid *A. bipunctata* spent 1.6 times longer walking than third instars. Shortages of aphids and/or deterrent cues are likely to increase this difference even more. In our study, the larvae of *C. limbifer* were monitored for twice as long as adults because preliminary tests in the absence of aphids showed that the mean velocity of the fourth instars was approximately half the mean velocity of conspecific females (Růžička and Zemek 2003).

Monitoring of residential times of fourth instar larvae of *C. limbifer* given the choice between moving on clean or contaminated substrates revealed that coccinellid larvae can

assess the presence of conspecific tracks as well as effectively avoid staying on substrates with tracks of other larvae. Our results indicate that coccinellid larvae might be able to optimize their distribution between aphid colonies not only in accordance with prey density (Elliott and Kieckhefer 2000), but also in accord with the number of conspecific larvae present by responding to the density of their tracks. The benefits of larvae being able to distribute themselves effectively using a deterring pheromone could be particularly evident when resources are near one another (e.g., on the same plant). Deterring pheromone in tracks of conspecific coccinellid larvae probably induce active spacing at the larval stage more effectively than some other deterrent cues.

Higher number of visits on clean substrates than on those contaminated with tracks from conspecifics may indicate an ability of coccinellid larvae to detect a conspecific deterring pheromone over a short distance. However, monitored larvae may also have disseminated a deterring pheromone from substrates with tracks of first instars to clean glass around these substrates. The closer to the strip with tracks of first instars, the higher contamination of originally clean glass can be expected. Thus, the contaminated glass could deter a fourth instar approaching the strip.

In this study, the lowest difference between distances walked on clean and contaminated substrates was recorded in the first period. This probably indicates that larvae needed to acclimatise to the new environment with different substrates after the previous stay in smaller dishes. The differences between the average distance walked and average residence periods of larvae on clean and contaminated substrates were greatest during the middle period of the test and had a tendency to decline in the last period. One explanation for this pattern is that as larvae spread deterring pheromone from the contaminated to the clean substrate and add their own tracks on both substrates over the course of the trial, the signal of the repellent is gradually lost in growing 'noise' of these tracks. This would result in blurring the results in the last period. We have previously observed a similar decrease in responses of coccinellid females to larval tracks (Růžička and Zemek 2003) when neither conspecific nor heterospecific tracks of first instars had significant effects on the behaviour of *C. limbifer* females in the final period of tests. An additional contamination of both substrates with faeces of monitored individuals might have caused also a decline in the response of adults as well as larvae. The decrease of the response of larvae to a deterring pheromone in the course of the choice test may also be due to adaptation or starvation.

Although we did not study the effects of larval tracks on the behaviour of the same individual, it is unlikely that an individual *C. limbifer* larva would not recognise its own tracks. A comparison of the effects of tracks left by the same individual and sibling and nonsibling conspecifics might, however, prove interesting. The intensities of deterrent responses of particular instar larvae to tracks of different instars possibly also vary due to different amounts of deterring pheromone in tracks between instar stages (Růžička 2001). More research into the deterrent effects of larval tracks on larvae of other coccinellid species and on larvae of other predators of aphids is required.

Our results indicate that grown up coccinellid larvae may be able to disperse away from potentially unsuitable aphid colonies by responding to the changing density of larval tracks and hence actively contribute to a decline in the rate of cannibalism. We suggest that the occurrence of immature heterospecific competitors in an aphid colony might be for some species of aphidophagous coccinellids even more hazardous than the presence of conspecific larva. Thus, the interspecific effects of larval tracks on active spacing of aphidophagous larvae deserve further attention.

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