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The Immediate Source of the Oviposition-Deterring Pheromone Produced by Larvae of *Adalia bipunctata* (L.) (Coleoptera, Coccinellidae)

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As is the case for other insects ovipositing on or in resources that are limited in time and/or space, the two-spot ladybird beetle, Adalia bipunctata *(L.) produces an oviposition-deterring pheromone (ODP), which is produced by the larval stages. Foraging larvae touch the substrate with their tarsi and the anal disk on the tenth abdominal segment. The aim of this paper was to determine whether the ODP produced by larvae was deposited by the tarsi or the anal disk. Fourth instar larvae either had their anal disk and tarsi, or anal disk, or tarsi coated with a water-soluble mounting medium. Larvae so treated were allowed to walk on filter paper that was subsequently presented to gravid females. The tracks of larvae that had both their tarsi and anal disk masked did not inhibit oviposition. However, the tracks of larvae that had only their tarsi masked significantly inhibited oviposition but those of larvae that had only their anal disk masked did not. It is concluded that the ODP is deposited on the substrate by the anal disk on the tenth abdominal segment of larvae.*

KEY WORDS: oviposition-deterring pheromone; larvae; anal disk; tarsi; coccinellidae.

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INTRODUCTION

As soon as larvae of the two-spot ladybird, *Adalia bipunctata* L., hatch and begin foraging for aphid prey they deposit an oviposition-deterring pheromone (ODP) (Doumbia *et al*., 1998). Conspecific females refrain from ovipositing near aphid colonies contaminated with this pheromone (Hemptinne *et al*., 1992; Doumbia *et al*., 1998; Fréchette *et al*., 2003), which consists mainly of alkanes (Hemptinne *et al*., 2001). In the field, aphidophagous ladybirds tend to start laying eggs in aphid colonies quite early in the colony's development and cease laying eggs as soon as colonies are marked by foraging first instar larvae. Colonies of aphids are therefore only suitable for oviposition between these two events, that is during short time intervals referred to as egg windows (Dixon, 1997). This reproductive strategy probably evolved in response to constraints associated with the ephemeral nature of aphid colonies. Aphid colonies typically last for 6–8 weeks while ladybird larvae take from 4 to 5 weeks to complete their development (Dixon, 1998, 2000). Field observations, laboratory studies and a mathematical simulation indicate that the optimum time for oviposition is during the egg window (Hemptinne *et al*., 1992; Kindlmann and Dixon, 1993). If oviposition occurs before the egg window, when aphid density is low, the larvae are likely to die of starvation before they can find and catch their first prey. Eggs laid after the egg window are highly likely to be eaten by conspecific ladybird larvae or fail to complete their development before the aphids become scarce and the starving larvae resort to eating one another. Thus, selection is likely to favour those ladybirds that are able to detect and avoid ovipositing in aphid colonies contaminated with ODP. Interestingly, although the chemical composition of this ODP has been studied the structures associated with the deposition of this pheromone are not fully known.

Other insects, which exploit resources that are limited in time and/or space, also produce ODPs. For example, females that lay eggs in or on flowers, seeds or fruit, as well as parasitoids and predators of aphids have been found to deposit ODPs on or within hosts they have recently exploited (Gabel and Thiery, 1992; Godfray, 1994; Ruzicka, 1994, 1996, 2002, 2003; Städler *et al*., 1994; Dempster, 1997; Doumbia *et al*., 1998; Quiring *et al*., 1998; Ruzicka and Havelka, 1998; Ferguson *et al*., 1999; Anbutsu and Togashi, 2001; Nufio and Papaj, 2001). In most species, the ODP is secreted and deposited by females although in a few systems both the egg (review in Nufio and Papaj, 2001) and larval stages have been found to be the sources of the ODPs (Ruzicka, 1994; Doumbia *et al*., 1998).

Ladybird beetles larvae have been found to deposit an ODP as they forage (Doumbia *et al*., 1998). However, the source of the pheromone and how it is deposited on the substrate is unknown. Close observation reveals that foraging larvae only touch the substrate on which they walk with the under-surface of their tarsi and periodically with the anal disk on the tip of the tenth abdominal segment. The anal disk functions as a pygopodium and makes it easier for larvae of coccinellids to forage on the smooth surfaces of leaves (Spiegler, 1962; Hodek, 1973). Both the tarsi and anal disk are possible sources of the oviposition-deterring pheromone present in larval tracks. The aim of this paper is to determine whether either of these structures is the immediate source of ODP.

MATERIAL AND METHODS

Ladybird Culture

The two-spot ladybird, *Adalia bipunctata* (L.), used in this study came from a laboratory stock culture. Adults of this stock culture were reared at $18 \pm 1^{\circ}$C, LD 16:8, in 5-litre plastic boxes, which contained a piece of corrugated filter paper on which the females laid eggs. Three times a week the ladybirds were fed an excess of pea aphids, *Acyrthosiphon pisum* Harris. Two stems of broad bean, *Vicia faba* L., were added to each box to improve the survival of the aphids.

Ladybirds Used in The Experiments

The ladybirds used in the experiments were obtained by incubating eggs from the stock culture in 175 cm^3 plastic boxes under the same conditions as described above. The larvae were fed 3 times a week with an excess of pea aphids until pupation. Freshly emerged adults were isolated within 24 h of their emergence from pupae. When their cuticles had hardened, they were sexed and couples, consisting of a male and a female, were placed in a 90 mm Petri dish containing a piece of corrugated paper and kept at $20 \pm 1^{\circ}$C, LD 16:8. Each day, the ladybirds were transferred to clean Petri dishes and fed an excess of pea aphids. Eggs were counted and removed daily. Ladybirds selected for the experiments were between 15 and 50 day old and had laid at least one batch of eggs daily over the previous 5 days. In order to standardize hunger and oviposition drive, females were deprived of food and a male for 16 h prior to the beginning of the experiment.

Experiment 1: Is the ODP Deposited by Those Parts of a Larva's Body That Touch the Substrate?

To test the hypothesis that the ODP comes from those parts of a foraging larva that touch the substrate, fourth instar larvae had their tarsi and the anal disk on their tenth abdominal segment masked. Recently moulted fourth instar larvae of *A. bipunctata* were first immobilised by placing them on a cold substrate and their tarsi and anal disk were coated with Aquatex®, a water-soluble mounting medium used in microscopy. These larvae were then kept at 6°C in a refrigerator for 2 min and then at room temperature for about an hour to allow the Aquatex® to dry. Aquatex® was used because it is non-toxic to the larvae and dries rapidly. In particular, it does not affect locomotion as larvae treated with this product walked on average the same distance over filter paper in 2 min as non-treated larvae ($\bar{x}_{\text{treated}} = \bar{x}_{\text{control}} = 42.5$ cm; $n_1 = n_2 = 10$, $t = 0.0000$; 18 df; $P = 1.0000$). The oviposition response of gravid females to the presence of tracks produced by "masked" and "unmasked" larvae was compared.

There were four treatments. (a) Exposure to clean filter paper—Contol 1. (b) Exposure to filter paper contaminated with the tracks of masked larvae. Their tracks were obtained by placing Whatman® filter paper in the lid of a 90 mm diameter Petri dish containing 5 masked larvae and an excess of pea aphids. The filter paper was removed after 24 h, and brushed to remove faeces, live aphids and aphid remains. (c) Exposure to filter paper bearing tracks of previously cold immobilised larvae—Control 2. Fourth instar larvae were cold immobilized and kept in a refrigerator as previously described, but their tarsi and anal disk were not masked prior to recording their tracks. The objective of this was to control for the possible effect of cold immobilization on the potential to produce ODP. (d) Exposure to filter paper contaminated with the tracks of normal larvae, collected as in (b).

At the beginning of this experiment each female was placed in a Petri dish with a small section of bean stem and about 50 pea aphids of mixed instar. These females were randomly allocated to one of four treatments: exposure to either (a) clean filter paper—Contol 1, (b) filter paper bearing the tracks of masked larvae, (c) filter paper bearing tracks of previously cold immobilised larvae—Control 2 or (d) filter paper bearing the tracks of normal larvae. There were 25 replicates of each treatment.

In the laboratory, larval tracks inhibit oviposition in the first 9 h of an experiment. The inhibition then progressively declines presumably because females cannot refrain from laying eggs indefinitely (Hemptinne *et al.*, 1992). Therefore, the effects of the four treatments were assessed by comparing the proportions of females that had laid eggs during the first 9 h

period using χ^2 tests. This test was applicable since the average expected value was always bigger than 6.0 (Zar, 1996).

Experiment 2: Is the ODP Deposited by the Tarsi or Anal Disk?

The aim of this experiment was to determine which of these two structures is the source of ODP.

At the beginning of this experiment each female was placed in a Petri dish with a piece of bean stem and about 50 aphids of mixed instar. These females were randomly subjected to one of two treatments: (1) filter paper with tracks of larvae whose anal disk had been masked with Aquatex® as described above and (2) clean filter paper. There were 20 replicates of each treatment. As above, the proportions of ladybirds that had laid during the first 9-h period was noted.

The above experiment was then repeated but in this case the females were randomly exposed to one of two treatments: (1) filter paper with tracks of larvae whose tarsi had been masked with Aquatex® and (2) clean filter paper. There were 19 replicates of each treatment. As above, the proportions of ladybirds that had laid eggs during the first 9-h period was noted. The results were analysed using χ^2 tests.

RESULTS

Experiment 1: Is the ODP Deposited by Those Parts of a Larva's Body That Touch the Substrate When Foraging?

The proportions of females that laid eggs were significantly different among the four treatments during the first 9-h period. (Table I). The response of females to clean filter paper (Control 1) and filter paper bearing the tracks of masked larvae was similar ($\chi^2 = 0.44$, $P > 0.05$), as was the response of females to filter paper either bearing the tracks of previously cold immobilised larvae (Control 2) or normal larvae ($\chi^2 = 0.32$, $P > 0.05$). This indicates that filter paper walked on by larvae with masked tarsi and anal disk was not contaminated with ODP while that walked on by previously cold immobilised larvae was contaminated.

The oviposition responses of females on filter paper contaminated with the tracks of masked larvae and on clean paper were pooled. Similarly filter paper walked on by normal larvae and those previously cold immobilised are likely to have been contaminated with ODP, so the oviposition responses to these filter papers were also pooled. Analysis of these two sets of results indicates that proportionally more females laid eggs on

Table 1. The numbers of Females that did or did not Lay Eggs During the first 9 h Period in Petri Dishes with a) Clean Filter Paper (Control 1), or Filter Paper Previously Walked on by Larvae that b) had Masked Tarsi and Anal disk, or c) had Previously been Cold Immobilised (Control 2), or d) Normal Larvae

	Number of females	
	Laying eggs	Not laying eggs
a) Control 1	20	5
b) Masked larvae	18	7
c) Control 2	10	15
d) Normal larvae	12	13
χ^2 **test**	11.33; $P < 0.05$	

uncontaminated filter paper than on contaminated filter paper (Table II). That is, the method of the ODP deposition appears to be the tarsi and/or the anal disk of the larvae.

Experiment 2: Is the Source of ODP the Tarsi or Anal Disk of Larvae?

The proportion of females that laid eggs in the presence of filter paper previously walked on by larvae with masked anal disks was not significantly different from the control (Table III). However, the proportion of females that laid eggs in the presence of filter papers previously walked on by larvae that had only their tarsi masked was significantly less than that in the control. After 9 h, significantly more females had laid eggs in the presence of clean filter paper (Table III). This indicate that the anal disk is the most likely source of the ODP.

DISCUSSION

In the laboratory, females of the two-spot ladybird, *A. bipunctata*, refrain from laying eggs when they are kept in a Petri dish lined with filter

Table II. The numbers of Females that did or did not Lay Eggs During the first 9 h Period in Petri Dishes Lined with a) Uncontaminated Filter Paper (Treatments a and b in Table 1), or b) Filter Paper Contaminated with Larval Tracks (Treatments c and d in Table 1)

	Number of females	
	Laying eggs	Not laying eggs
a) uncontaminated	38	12
b) contaminated	22	28
χ^2 **test**	10.67; $P < 0.01$	

Table III. The Numbers of Females that did and did not Lay Eggs During the First 9 h Period in Petri Dishes Lined Either with a) Clean Filter Paper or b) Filter Paper Previously Walked on by Larvae with Masked Anal Disks, and c) Clean Filter Paper or d) Filter Paper Previously Walked on by Larvae with Masked Tarsi

	Number of females		
	Laying eggs	Not laying eggs	χ^2 tests
a) clean paper	18	2	
b) larvae with masked anal disk	15	5	0.003; P > 0.05
χ^2 test	1.56; P > 0.05		
c) clean paper	17	2	
d) larvae with masked tarsi	7	12	5.78; P < 0.05
χ^2 test	11.31; P < 0.001		

paper previously walked on by conspecific larvae. This is attributed to an oviposition-deterring pheromone (ODP) present in the tracks of larvae (Hemptinne *et al.*, 1992; Doumbia *et al.*, 1998.). When confined in Petri dishes in the presence of an ODP, *A. bipunctata* females refrain from ovipositing for at least 9 h (Hemptinne *et al.*, 1992). Similarly, in the field females are reluctant to lay eggs on aphid infested bean plants bearing the tracks of conspecific larvae. The females in this case eat some aphids and then quickly fly or walk away, presumably in search of a more suitable oviposition site (Fréchette *et al.*, 2003).

In this discussion pheromone is used in the sense of Karlson and Lüscher (1959) because larvae secrete in their habitat a substance that upon being detected by conspecific females triggers a specific reaction. The ODP of the two-spot ladybird is also a signal according to Nufio and Papaj (2001). In this case, however, chemical communication takes place between individuals belonging to two different age groups. Therefore, as pointed out by Nufio and Papaj (2001), it is not easy to distinguish between cues deposited by larvae searching for aphids on plants from a real signal that evolved to convey information to conspecifics.

Our results indicate that the source of the oviposition-deterring pheromone is the anal disk of larvae. Substrates walked on by larvae that had only their tarsi coated inhibited oviposition, but not those walked on by larvae that had only their anal disk covered with Aquatex. These results confirm that ladybird larvae produce an ODP and that the source is the anal disk. Observing the oviposition behaviour of another aphidophagous predator, the lacewing *Chrysopa oculata* Say, on substrates walked on by conspecific larvae or marked with droplets of an abdominal secretion of third instar larvae Ruzicka (1994) deduced that egg laying was probably inhibited by substances "produced somewhere on or near the abdominal tip" of larvae.

The tracks left by larvae of the two-spot ladybird contain more than 40 substances, the majority of which are alkanes (Hemptinne *et al*., 2001). The primary function of most, if not all, of these compounds is probably in the mechanism that enables the tarsi and anal disk ladybird larvae to adhere to smooth plant cuticle (Spiegler, 1962; Kosaki and Yamaoka, 1996). As it is the case for other Coleoptera, tubular hairs of ladybird tarsal pads are likely to function as adhesive organs because they are moistened by some glandular secretion (Wigglesworth, 1972; Strong *et al*., 1984). If this is the case, this study suggests a way forward to pinpoint the molecules constituting the ODP among the chemical complexity of the larval tracks: compare the chromatograms of tracks deposited by normal larvae and those with masked tarsi.

The anal disk of two-spot ladybird larvae is particularly well developed. It is seen as an adaptation to living in trees, because it improves their ability to adhere to smooth plant cuticle (Hodek, 1973; Dixon, 2000). That is, they are more likely to remain in the vicinity of their prey because they are less likely to be dislodged from tree leaves by wind or after encounters with competitors or predators. Species living on herbaceous plants, such as *Coccinella septempunctata* L., do not have a conspicuous anal disk and readily fall from plants when disturbed. Contrary to the two-spot ladybird, the seven-spot ladybird does not appear to produce an ODP (Hemptinne *et al*., 1993). The fact that the anal disk is more developed in some species than in others raises the question whether the size of the disk is more associated with the habitat in which they live or the production of ODP.

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