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RESPONSE OF THE LADYBIRD PARASITOID *Dinocampus coccinellae* TO TOXIC ALKALOIDS FROM THE SEVEN-SPOT LADYBIRD, *Coccinella septempunctata*

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Abstract—Electrophysiological and behavioral responses of the ladybird parasitoid *Dinocampus coccinellae* to volatiles from the seven-spot ladybird, *Coccinella septempunctata*, were investigated to identify semiochemicals involved in host location. Coupled gas chromatography–electroantennography (GC-EAG) with *D. coccinellae* located a small peak of prominent activity in an extract of volatiles from adult *C. septempunctata*. The active compound was identified by coupled GC-mass spectrometry and by comparison with an authentic sample as the free-base alkaloid precoccinelline, which forms part of the toxic defense of this ladybird. Behavioral studies in an olfactometer showed that *D. coccinellae* was significantly attracted to the volatile extract and also to the alkaloid. Myrrhine, a stereoisomer of precoccinelline found in low amounts in *C. septempunctata* and in other ladybird species, was shown to be electrophysiologically active and significantly attractive. Perception of ladybird alkaloids by *D. coccinellae* is a rare example of toxicants acting as aerially transmitted cues for interactions between the third and fourth trophic levels.

Key Words—Seven-spot ladybird, *Coccinella septempunctata*, Coleoptera, Coccinellidae, electroantennogram, behavior, *Dinocampus coccinellae*, Hymenoptera, Braconidae, alkaloid, precoccinelline, myrrhine, hippodamine, volatile, semiochemical.

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INTRODUCTION

Ladybird beetles or ladybugs (Coleoptera: Coccinellidae) are important predators contributing to the natural control of pest aphid populations. However, they are themselves attacked by a range of natural enemies (Majerus, 1994). General predation on ladybirds by vertebrates such as birds is largely prevented by highly toxic alkaloids contained in a reflex bleed released when the ladybird is attacked. The effectiveness of this chemical defense is enhanced by the associated bright warning (aposematic) coloration and corelease of characteristic volatile compounds, e.g., 2-isopropyl-3-methoxypyrazine, which serves as a protective allomone and also as an aggregation pheromone (Al Abassi et al., 1998). The wasp *Dinocampus coccinellae* Schrank (Hymenoptera: Braconidae) is the main parasitoid of a number of ladybird species and can cause substantial reductions in populations of the seven-spot ladybird, *Coccinella septempunctata* L. (Majerus, 1994; Triltsch, 1996). There is a close association with the host, involving oviposition by *D. coccinellae* into the adult ladybird body and subsequent larval development, which indicates a tolerance by the wasp to the toxic alkaloids. Furthermore, *D. coccinellae* is known to utilize a combination of visual and olfactory cues in short-range host location (Orr et al., 1992). Since the associated semiochemicals may provide a means of reducing the impact of *D. coccinellae* on populations of *C. septempunctata* currently being exploited in biological control programs, the antennal and behavioral responses of *D. coccinellae* to volatiles associated with *C. septempunctata* were investigated using coupled gas chromatography–electroantennography (GC-EAG) and olfactometer studies. Our hypothesis was that *D. coccinellae* would exploit 2-isopropyl-3-methoxypyrazine as a kairomone in host location, since this compound had previously been identified as an aggregation pheromone for adult *C. septempunctata* (Al Abassi et al., 1998).

METHODS AND MATERIALS

Insects. *C. septempunctata* adults were collected during July–August from the grounds of IACR-Rothamsted, Hertfordshire, U.K., and kept in ventilated polyethylene boxes at 20°C, with the aphid *Acyrthosiphon pisum* as a food source, prior to vacuum distillation. *D. coccinellae* for electrophysiological studies were obtained from parasitized *C. septempunctata* adults; they were maintained under the same conditions as the ladybirds and kept in Petri dishes lined with moist filter paper and provided with a mixture of honey and water. Parasitoids less than 3 days old were used. For olfactometer studies, parasitized *C. septempunctata* adults were sent by air mail to Sweden and emerging *D. coccinellae* were provided with a diet of whey-cheese (Mild Mesost) and water. Parasitoids more than 1 day old were used.

Isolation of Volatiles. Adult *C. septempunctata* (ca. 1000, not sexed) were cooled with liquid nitrogen and extracted with freshly distilled chloroform (2 × 200 ml) for 24 hr (48 hr in total) at 25°C. The combined extracts were dried using anhydrous magnesium sulfate, filtered, and evaporated to ca. 5 ml. Volatiles were distilled under vacuum (0.03 torr) for 21 hr at 25°C as described previously (Pickett and Griffiths, 1980), and the distillate concentrated under a stream of nitrogen to 100 μl (10 ladybird equivalents/μl) and stored in a tightly capped microvial at −20°C.

Gas Chromatography (GC). The vacuum distilled volatiles were analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a cold on-column injector, a flame ionization detector (FID), and a 50-m × 0.32-mm-ID HP-1 bonded-phase fused-silica capillary column. The oven temperature was maintained at 40°C for 2 min and then programmed at 10°/min to 250°C. The carrier gas was hydrogen.

Electrophysiology. Electroantennogram (EAG) recordings from recently emerged *D. coccinellae* were made using Ag–AgCl glass electrodes filled with saline solution [composition as in Maddrell (1969) but without glucose]. The insect was anesthetized by chilling and an antenna was excised and suspended between the two electrodes. The tip of the terminal process of the antenna was removed to ensure a good contact. The signals were passed through a high-impedance amplifier (UN-06, Syntech) and analyzed using a customized software package (Syntech).

Stimulus Delivery. The delivery system, which employed a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). The stimulus (2-sec duration) was delivered into a purified airstream (1 liter/min) flowing continuously over the preparation. Samples (10 μl) of the standard solutions of test compounds were applied to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the paper strip was placed in the cartridge. The control stimulus was hexane (10 μl). Fresh cartridges were prepared immediately prior to each stimulation. Five individual *D. coccinellae* were used.

Coupled Gas Chromatography–Electroantennography (GC-EAG). The coupled GC-electrophysiology system, in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams, 1990). Separation of the vacuum distillate volatiles was achieved on an AI 93 GC equipped with a cold on-column injector and an FID. Two columns were used, a 50-m × 0.32-mm-ID HP-1 column and a 30-m × 0.32-mm-ID HP-Wax column. For the HP-1 column, the oven temperature was maintained at 40°C for 2 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. For the HP-Wax column, the oven temperature was maintained at 40°C for 1 min and then programmed at 10°/min to 220°C. The carrier gas was hydrogen. The outputs from the EAG amplifier

and the FID were monitored simultaneously and analyzed using the software package.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). A capillary GC column (50 m × 0.32 mm ID HP-1) fitted with an on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°/min to 250°C. Tentative identification by GC-MS was confirmed by peak enhancement with authentic samples (Pickett, 1990).

Chemicals. 2-Isopropyl-3-methoxypyrazine (97%) was purchased from the Aldrich Chemical Company (Gillingham, United Kingdom). Coccinelline, i.e., the *N*-oxide of precoccinelline, the major toxic component of the reflex bleed of adult *C. septempunctata*, was isolated by acid–base extraction of a chloroform extract (extraction method as above, from ca. 900 adults), followed by liquid chromatography over neutral alumina (BDH, Brockmann Grade 1), eluting with chloroform and 99 : 1 chloroform–methanol (Pasteels et al., 1973). Precoccinelline (59 mg, 93% pure by GC) was obtained by catalytic hydrogenation [Adams' catalyst (PtO_2), methanol, 25°C] of coccinelline (80 mg) (Tursch et al., 1971). Myrrhine (10 mg, 90% pure by GC) was prepared from coccinelline (40 mg) via the Polonovski reaction (Tursch et al., 1975). Precoccinelline and myrrhine were then further purified (>99% by GC) by repeated liquid chromatography over neutral alumina (BDH, Brockmann grade 1), using 99 : 1 chloroform–methanol as the eluant. For all alkaloids, identity was confirmed by comparison of the IR, MS, ^{1}H NMR, and ^{13}C NMR spectra with literature values (Mueller et al., 1984; Lebrun et al., 1999). A sample of hippodamine (5 mg, >99% by GC) was provided by Professor D. Daloze (see Acknowledgments). Solutions for electrophysiological studies (10^{-5}–10^{-8} g/10 μl) were prepared in redistilled hexane, and for behavioral studies (10^{-6} g/μl) in redistilled diethyl ether.

Olfactometry. Behavioral assays using parthenogenetic *D. coccinellae* adults were carried out in a two-way Perspex olfactometer as described previously (Al Abassi et al., 1998). This comprised a weak airstream being directed towards the center of the olfactometer from two drawn-out arms to which volatile sources were applied at the inlets. For each experiment, one *D. coccinellae* was introduced into the center of the chamber and its position noted every 2 min for 20 min. Each experiment was replicated 8–10 times and the results analysed by paired *t* test; the number of visits into the treatment arm was compared with visits to the control arm. If the insect did not move between observations, the experiment was terminated and the data discarded. Stimuli comprised: (a) a single intact *C. septempunctata* adult; (b) the vacuum distillation extract, applied in a 0.5-μl microcap (i.e., 5 ladybird equivalents); (c) the test compounds (Figure 1) 2-isopropyl-3-methoxypyrazine (I), precoccinelline (II), myrrhine (III), and

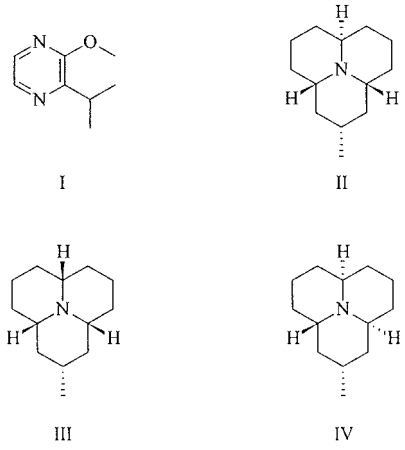

FIG. 1. Structures of compounds I–IV.

hippodamine (IV), diluted to $10^{-6}/\mu$l and applied individually in 0.5 μl microcaps, giving a stimulus approximately equivalent to the level of II found in the ladybird vacuum distillate.

RESULTS

In the olfactometer, adult *D. coccinellae* were not significantly attracted to a single intact *C. septempunctata* adult but showed strong attraction to the vacuum distillation extract (5 ladybird equivalents) (Table 1). Coupled GC-EAG of this extract with *D. coccinellae*, using nonpolar (HP-1) and polar (HP-Wax) capillary columns, showed that 2-isopropyl-3-methoxypyrazine had no electrophysiological activity (I, Figure 2). However, a small peak with a long retention time on both columns was associated with prominent electrophysiological activity (II, Figure 2). This compound was tentatively identified by coupled GC-MS and comparison with published mass spectra (e.g., Tursch et al., 1975; Mueller et al., 1984) as a 2-methylperhydro-9b-azaphenylene alkaloid (M^+ 193, Figure 3). Peak enhancement on coinjection with an authentic sample confirmed the identity of the compound as the free-base precoccinelline (II), a known defense alkaloid of *C. septempunctata* having high toxicity. A minor component of the volatile extract also showed significant electrophysiological activity (III, Figures 2 and 4), and GC-MS analysis gave a spectrum virtually identical to that of II (M^+ 193), suggesting that this component was a diastereoisomer. Coinjection with an authentic sample confirmed the identity of this peak as the ladybird alkaloid myrrhine (III). EAG dose–response curves from *D. coccinellae* confirmed that the pyrazine I was not active, even at high concentrations, while

TABLE 1. RESPONSES OF *Dinocampus coccinellae* IN THE OLFACTOMETER

Stimulus	Observations (mean N)[a]			Replicates (N)
	Treated arm	Control arm[b]	P	
C. septempunctata				
Single adult	5.6 ± 3.7	4.4 ± 3.7	NS[c]	8
Vacuum distillate[d]	5.8 ± 1.8	2.9 ± 1.5	<0.01	10
2-Isopropyl-3-methoxypyrazine[e]	4.8 ± 1.2	5.2 ± 1.2	NS	10
Precoccinelline[e]	5.2 ± 2.5	2.4 ± 1.7	<0.05	10
Myrrhine[e]	5.3 ± 1.4	1.2 ± 1.8	<0.01	10
Hippodamine[e]	4.5 ± 2.0	3.8 ± 2.6	NS	10

[a] Cumulative counts over 20 min (± SD).
[b] Control = solvent (diethyl ether).
[c] NS = not significantly different at $P = 0.05$ (paired t test).
[d] Five ladybird equivalents.
[e] Compounds tested at 10^{-6} g/μl (0.5 μl applied).

precoccinelline (II) and myrrhine (III), and the other known ladybird alkaloid hippodamine (IV), showed similarly high activities (Figure 5). In olfactometer assays, *D. coccinellae* was significantly attracted to the alkaloids II and III but not to the pyrazine I or the alkaloid IV (Table 1).

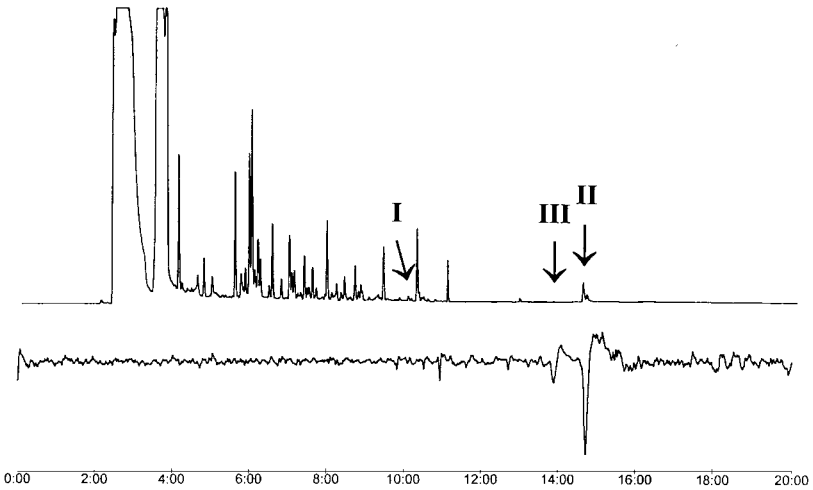

FIG. 2. Coupled GC-EAG with female *Dinocampus coccinellae*. Upper trace: GC of volatiles from a vacuum distillate of adult *Coccinella septempunctata* (HP-1 column); lower trace: EAG response. I = 2-isopropyl-3-methoxypyrazine; II = precoccinelline; III = myrrhine.

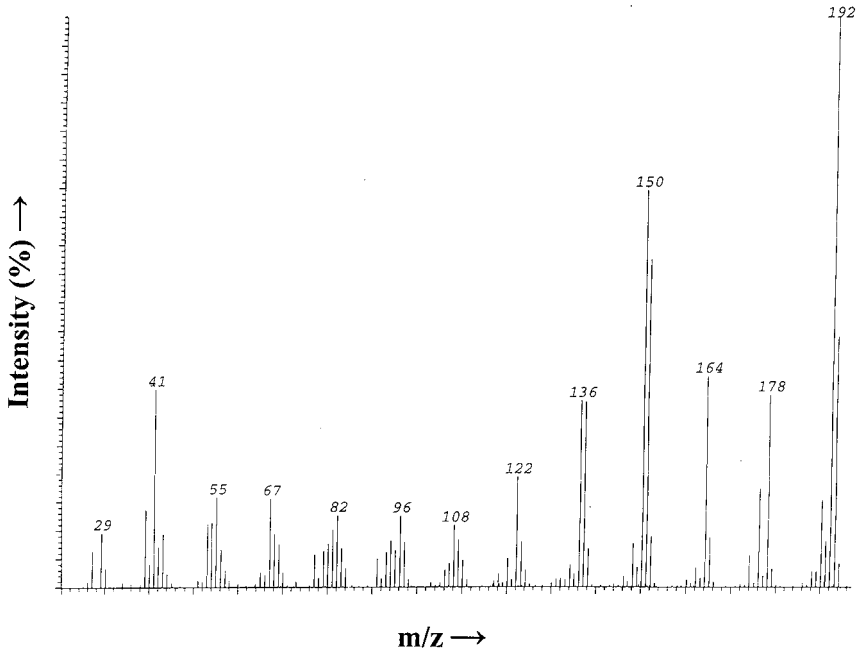

FIG. 3. Mass spectrum from peak II in Figure 2.

DISCUSSION

Although *D. coccinellae* is known to be attracted by *C. septempunctata* (Orr et al., 1992), in this study, single intact ladybirds did not elicit statistically significant attraction. However, in the search for electrophysiologically and behaviorally active material, the volatile sample obtained by extraction and vacuum distillation of *C. septempunctata* gave highly significant responses. This difference may be due to a concentration effect, but it is more likely that the initial extraction in chloroform accessed compounds normally released only on damage, i.e., in the reflex bleed produced during predator attack. *D. coccinellae* showed no EAG or behavioral responses to 2-isopropyl-3-methoxypyrazine (I), despite such activity being anticipated under the hypothesis initiating this study. Surprisingly, the electrophysiologically active components of the vacuum distillation extract were found to comprise the free-base alkaloids precoccinelline (II) and myrrhine (III). When tested at biologically appropriate levels in the olfactometer, both compounds were significantly attractive to *D. coccinellae* and thus confer the kairomonal activity of the *C. septempunctata* volatiles.

Precoccinelline (II), myrrhine (III), and hippodamine (IV) are natu-

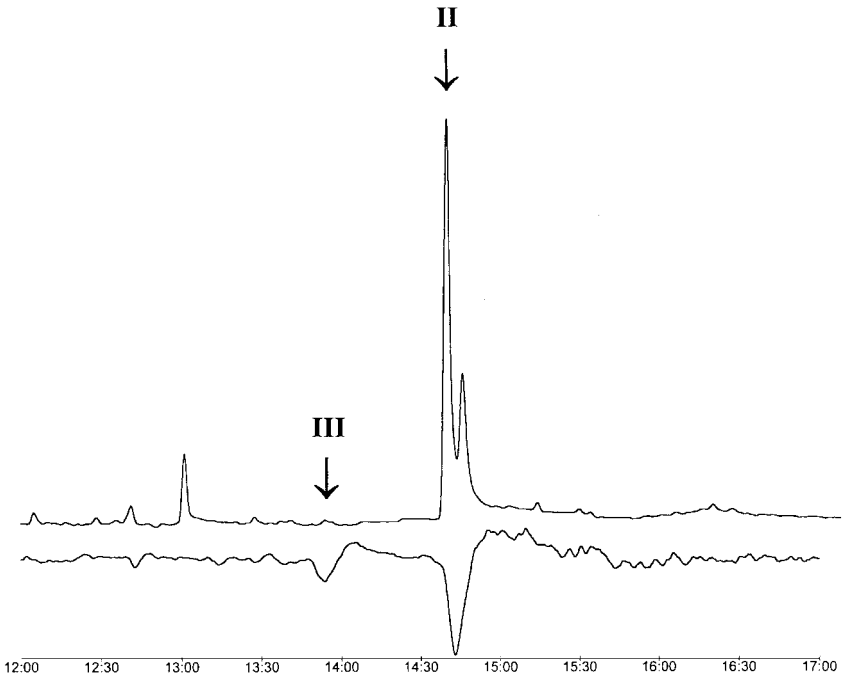

FIG. 4. Expansion of trace from Figure 2 (12–17 min), showing peak for myrrhine (III).

rally occurring 2-methylperhydro-9b-azaphenylene alkaloids that appear to be restricted mainly to ladybirds; of the six possible diastereoisomers, only these three have so far been isolated (Daloze et al., 1994/5; King and Meinwald, 1996). They are also utilized in a similar defense role by cantharid beetles and some amphibious vertebrates (Moore and Brown, 1978; Daly et al., 1993). Their limited occurrence in nature and their importance in the family Coccinellidae suggest that, for a specialist parasitoid such as *D. coccinellae*, these compounds would be reliable indicators for locating suitable hosts (Vet et al., 1991). Contrary to expectations, *D. coccinellae* was not significantly attracted to hippodamine (IV), the free-base defense alkaloid produced by another host species, the convergent ladybird, *Hippodamia convergens* Guerin. However, it was attracted to myrrhine (III), the minor EAG active component in the *C. septempunctata* volatiles; this compound is the major defense alkaloid produced by the eighteen-spot ladybird, *Myrrha octodecimguttata* L., a species that *D. coccinellae* has not been observed to parasitize (Richerson, 1970; Hodek and Honek, 1996; Majerus, 1997). Although further behavioral and ecological studies are necessary to elucidate the issues involved, the identification of toxic alkaloids as volatile

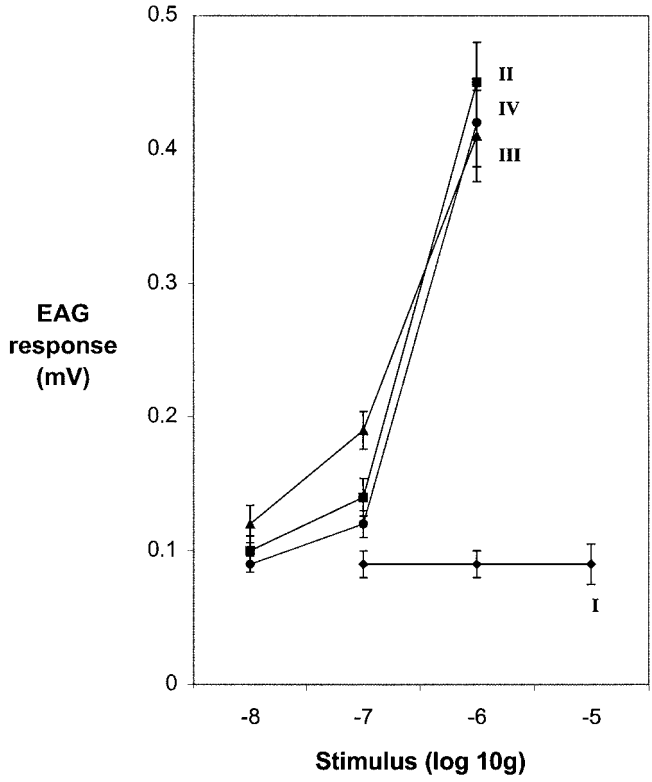

FIG. 5. EAG dose–response of *Dinocampus coccinellae* to 2-isopropyl-3-methoxypyrazine (I), precoccinelline (II), myrrhine (III) and hippodamine (IV). The points on each graph are the means of five preparations ± SE.

kairomones for *D. coccinellae* will form the basis for such work in these laboratories and, it is hoped, elsewhere.

Seminal studies on the defensive chemistry of ladybirds suggested that, of the six possible diastereoisomers, precoccinelline (II) and hippodamine (IV) are unique to *C. septempunctata* and *H. convergens*, respectively (Pasteels et al., 1973), which implies stereochemical control in their biosynthesis. However, with the onset of more advanced analytical techniques, investigations have revealed that such control is not as effective as previously thought, with mixtures of II and IV being found in both species (Daloze et al., 1994/5), although the presence of myrrhine (III) in *C. septempunctata* was not reported. Moreover, hippodamine (IV) was not found in the study of *C. septempunctata* volatiles described here.

The chemical ecology of multitrophic interactions such as those between

plants, aphids and aphid parasitoids has been widely studied with a view to exploiting parasitoids in biological control of pest aphid populations (Wadhams et al., 1999). In contrast, the chemical ecology of interactions between plants, aphids, ladybirds, and their parasitoids has received relatively little attention. The results of this study demonstrate the role of volatile semiochemicals in mediating trophic interactions between the third and fourth levels and represent a rare example of toxicants to which an animal has adapted acting directly as volatile kairomones.

For the development of aphid control programs that involve manipulation of ladybirds, methods of manipulating their natural enemies may also be needed. The significant attraction of *D. coccinellae* to such specific cues as the ladybird alkaloids suggests that there is potential for development of control strategies for this particular natural enemy, thereby enhancing the beneficial role of ladybirds through increased populations. However, as host location is likely to involve a mixture of olfactory and visual cues (Orr et al., 1992), further studies are required to assess the significance of the latter in this process.

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