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ORIGINAL PAPER

Recognition of foreign oviposition-marking pheromone in a multi-trophic context

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Abstract Both phytophagous and parasitic insects deposit oviposition-marking pheromones (OMPs) following oviposition that function to inform conspecifics of a previously utilized host of reduced suitability. The blueberry maggot fly, *Rhagoletis mendax* Curran (Diptera: Tephritidae), deposits eggs individually into blueberries and then marks the fruit surface with an OMP which reduces acceptance of fruit for oviposition by conspecifics. *Diachasma alloeum* (Muesebeck) (Hymenoptera: Braconidae) is a parasitic wasp attacking larval *R. mendax* which also deposits an OMP, signaling conspecifics of a wasp-occupied host. Behavioral studies were conducted testing the hypothesis that the OMP of the parasitic wasp modifies the oviposition behavior of its host fly. In this study, we show that the OMP of *D. alloeum* is recognized by *R. mendax*, and female flies will reject wasp-marked fruit for oviposition. Thus, we present a rare demonstration of pheromonal recognition between animals occupying different taxonomic orders and trophic levels. This chemical eavesdropping may enhance the ability of the fly to avoid fruit unsuitable for larval development.

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Introduction

Infochemicals mediate behavioral and physiological interactions among animals and plants and are well studied in insects. Oviposition-marking pheromones (OMPs) are deposited by many parasitic and phytophagous insects immediately following egg-laying and inform conspecifics of a previously utilized host of reduced suitability. The resulting effect is reduced time spent on a marked resource as well as reduced probability of oviposition (Roitberg and Prokopy 1987; Nufio and Papaj 2001). These signals likely evolved as a mechanism for avoiding superparasitism, reducing competition for limited host resources (plant or animal) among brood of conspecific organisms (Prokopy 1981a, b; Nufio and Papaj 2001). The benefits of producing and recognizing these signals are dependent on the fitness gain of the marker and the receiver. Females that mark fruit are protecting their reproductive investment from conspecifics and also avoiding multiple self-ovipositions into the same fruit. Recognition of the signal by conspecifics benefits them because they avoid a food resource that is already occupied and that would reduce survival of their offspring (Hoffmeister and Roitberg 2002). Also, recognition of OMPs refines the host selection process over the use of host plant characteristics alone, including plant volatiles, by providing a mechanism for determining occupancy (and therefore potential quality) of the host. The existence and function of OMPs has been widely described among numerous and wide-ranging insect taxa including the

Coleoptera, Diptera, Hymenoptera, Lepidoptera, and Neuroptera (Prokopy 1981a; van Lenteren 1981; Roitberg and Prokopy 1987; Landolt and Averill 1999).

OMPs have been studied widely within the tephritid family of true fruit flies (reviewed by Prokopy 1981a, b; Nufio and Papaj 2001). Their function has been confirmed in at least ten species within the genus *Rhagoletis* (Katsoyannos 1975; Averill and Prokopy 1981; Prokopy 1981a), including the blueberry maggot fly, *Rhagoletis mendax* Curran (Prokopy et al. 1987). The chemical identity of most OMPs is undetermined (Hurter et al. 1987), but in general, the duration of OMP persistence is shorter than the time required for completion of larval development (Prokopy 1981a; Averill and Prokopy 1987).

The ecology of infochemical (kairomones and allomones) production and use between organisms occupying different trophic levels (plant, herbivore, and natural enemy) has been summarized primarily from the perspective of host location by a natural enemy (Vet and Dicke 1992). Previous studies have shown that natural enemies, such as parasitic Hymenoptera, utilize the OMPs of their hosts. Female parasitic wasps, *Utetes canaliculatus* (Gahan), exploit the OMP of *Rhagoletis pomonella* (Walsh) as a kairomone to identify host-infested fruit (Prokopy and Webster 1978). The egg parasitoid, *Halicoptera rosae* Burks, exhibits increased searching time on fruit infested by *Rhagoletis basiola* Osten-Sacken, which increases parasitization rates (Roitberg and Lalonde 1991; Hoffmeister et al. 2000). Similarly, the parasitoid, *Halticoptera laevigata* Thomson, searches longer and probes more frequently on fruit marked by its host, *Myoleja lucida* Fallén than on unmarked fruit (Hoffmeister and Gienapp 1999).

Both *R. mendax* and *Diachasma alloeum* females are host specialists (Payne and Berlocher 1995; Glas and Vet 1983) and invest time to inspect the outer surface of a blueberry fruit prior to ovipositing. The sequence of events is as follows: (1) *R. mendax* flies deposit an egg under the fruit surface of ripe or ripening berries (Liburd et al. 1998) and (2) subsequently mark the fruit with an OMP, which inhibits other *R. mendax* females from ovipositing into the previously utilized resource for 6–12 days (Prokopy 1981a). Approximately 17 days following fly oviposition, the *R. mendax* maggot reaches the second larval instar when it is (3) maximally parasitized by female *D. alloeum* wasps (Stelinski et al. 2004), which (4) also deposit an OMP immediately following oviposition, informing other conspecific female parasitoids of the utilized resource (Stelinski et al. 2007). Given that both the fly and the wasp mark the external surface of the blueberry fruit to signal occupancy, we hypothesized that *R. mendax* flies detect and exploit the OMP of their predatory *D. alloeum* wasps. The use of the parasitoid's OMP to discriminate between utilized and available host fruit would represent a novel chemically mediated interaction between animals occupying different taxonomic orders and trophic levels for improved host selection.

Materials and methods

Insects

R. mendax-infested blueberry fruit were collected from a site in Fennville, MI, USA described in Stelinski et al. (2004) and from a site in Chatsworth, NJ, USA described in Stelinski et al. (2007). All fruit were collected during the summer of 2005. Parasitized and unparasitized *R. mendax* larvae were allowed to exit fruit naturally and pupate in moist vermiculite according to the procedure described in Liburd et al. (1998). Puparia were stored at 4°C for 140 days and thereafter transferred from cold storage to an environmental chamber at 24°C, 55–60% relative humidity (RH), under a light/dark (LD) 16:8 h photocycle. Flies began to emerge ca. 4–5 weeks following removal of puparia from 4°C. Approximately 20% of the fly puparia were parasitized by *D. alloeum* wasps, which began to emerge approximately 5–6 weeks following removal from cold storage. Both flies and wasps were maintained separately in aluminum screen cages (30×30×30 cm) at 24°C, 55–60% RH, under a LD 16:8 h photocycle. Flies were supplied with water and food (enzymatic yeast hydrolysate and sucrose; ICN Biomedicals Inc, Costa Mesa, CA, USA), and wasps were given 5% sucrose in water.

Field infestation of blueberry fruit

Blueberry fruit were infested with *R. mendax* larvae in the field or maintained uninfested according to the procedure described in Stelinski et al. (2006) at the Fennville, MI site in the summer of 2006. Briefly, blueberry fruit clusters consisting of 20–35 unripe fruit were enveloped in the field within 1.2-L nylon mesh bags on June 30th prior to the emergence of wild flies. This technique prevents wild females from ovipositing into blueberries and does not interfere with normal berry development and ripening (Stelinski et al. 2006). Following berry ripening, ten laboratory-reared and mated female flies were introduced into bags enveloping fruit clusters on three dates (July 20th, 30th, and August 5th) for 24 h intervals and then removed. At least 30 uninfested and bagged clusters were infested with flies on each date. Blueberry fruit infested by fly larvae were used for laboratory experiments approximately 3 weeks following infestation. At this time, flies reach the second larval instar becoming maximally acceptable to wasps for parasitization (Stelinski et al. 2004). Infested fruit clusters were maintained bagged and isolated from wild flies or wasps until use for laboratory assays.

Behavioral assays

General methods

A series of behavioral experiments were conducted to test the hypothesis that gravid female *R. mendax* flies avoid ovipositing into blueberry fruit that had been previously marked by the OMP of its larval parasitoid, *D. alloeum*. The role of previous experience with the wasp marking pheromone on the oviposition response of the fly was also investigated. The behavioral bioassay method for flies and wasps followed the protocols detailed by Prokopy (1981a) and Stelinski et al. (2007). Experiments were conducted at 24°C, 55–60% RH, and with a light intensity between 300–400 lx. Test fruit were infested by flies 17–21 days prior to assays as described above. Infested and uninfested picked fruit used in assays were tested 2–3 h after removal from the field. Fruit used for testing were removed from bushes by cutting off the entire fruit cluster at the stem. The stems were inserted into floral aquapicks, and entire clusters were placed into 1-L translucent plastic containers sealed with perforated lids.

For each experiment, sexually mature (7–10 day-old) female wasps were used in pre-assay treatment manipulations. Sexually mature (14–20 day-old) female flies were also used during behavioral assays. Fruit were marked by wasp females prior to behavioral assays by first inserting a single female wasp into a 1-L plastic translucent cup containing a cluster of 10–15 blueberries that were infested with fly larvae. These female wasps typically oviposited into an infested berry within 2 min of introduction. Immediately following oviposition, wasps were gently transferred into a second container with a clean and unifested berry for marking by ovipositor dragging. Such fruit are henceforth defined as having received "wasp ovipositor marking." In a similar fashion, gravid female flies were allowed to oviposit into previously uninfested and unmarked berries and then transferred to clean and uninfested berries for marking by ovipositor dragging. Such fruit are defined as having received "fly ovipositor marking." Approximately 30–90 min after fruit were marked by either flies or wasps, behavioral experiments were commenced using two types of female flies. "Naïve" female flies had no previous experience with wasp-marked fruit, while "experienced" female flies had oviposited into wasp-marked and fly-infested fruit 18–24 h earlier.

Comparison of naïve and experienced flies

In the first experiment, naïve female flies (N=22) were presented sequentially and in random order with a berry that received: (1) wasp ovipositor marking, (2) fly ovipositor marking, or (3) no treatment (control). All assays were conducted in 1-L plastic chambers described above. Females were observed for 5 min, and the data recorded were acceptance (oviposition into fruit) or rejection (leaving fruit without oviposition) of fruit. Female flies typically accepted a berry for oviposition within 40 s of introduction into the plastic cup. The second experiment was identical to the first except that experienced female flies (N=20) were assayed. The third and fourth experiments were also conducted identically to the second experiment except that responses of experienced female flies were assayed either 3 (N=22) or 7 (N=20) days following fly and wasp marking of fruit.

Comparison of infested and uninfested fruit

In the fifth experiment, the oviposition response of experienced flies (N=20) was compared using fruit having received: (1) wasp ovipositor marking, (2) oviposition by female wasps without subsequent marking, or (3) no treatment (control). In this experiment, wasps were allowed to oviposit into a fly-infested berry in the second treatment but were not allowed to drag their ovipositor on the fruit by removing them immediately following oviposition. As before, the three treatments were presented sequentially and in random order. In the sixth experiment, the oviposition response of experienced female flies (N=20) was compared using two types of fruit having received wasp ovipositor marking versus an untreated control: (1) uninfested fruit and (2) fruit infested by a fly larva. The experimental procedures were otherwise identical to those described for experiment 2.

Removal and re-application of wasp OMP

In the seventh experiment, experienced female flies (N=18) were assayed, and two types of treatment fruit were compared versus an untreated control: (1) fruit receiving wasp ovipositor marking and (2) fruit receiving wasp ovipositor marking followed by a solvent wash to remove the deposited OMP. The fruit was washed following ovipositor dragging by gently rinsing the treated berry with a solution of 50% ethanol in distilled water immediately prior to the assay, which has been shown to effectively remove the wasp OMP (Stelinski et al. 2007). In the eighth experiment, the oviposition response of experienced female flies (N=15) was assayed for the following treatments versus an untreated control: (1) fruit sprayed with a solution of wasp OMP and (2) fruit sprayed with a solvent-positive control without pheromone. The solution of wasp OMP was made by rinsing 25 berries that had received wasp female ovipositor dragging in 10 ml of 50% ethanol in distilled water. The spray was applied using an atomizer with ca. 0.8 ml applied per berry. For the positive control, fruit were sprayed with the 50% ethanol solution alone. All sprayed fruit were air-dried for 1 h prior to testing. For both experiments, treatments were presented sequentially and in a random order.

Statistical analyses

Logistic regression analyses were used to test the significance of differences between the numbers of female flies attempting oviposition in experimentally manipulated versus control berries in laboratory experiments based on the null hypothesis that fly acceptance of an oviposition substrate is random (R Development Core Team 2004). In all cases, the significance level was $\alpha<0.05$.

Results

Comparison of naïve and experienced flies

Preliminary logistic regression analyses revealed no significant differences between behaviors assayed for wasps collected from Michigan and New Jersey, USA, and thus, these data were combined for further analyses. Oviposition by naïve female flies that had not previously oviposited into wasp-marked fruit was significantly (χ^2=16.4, *df*=1, *P*=0.0004) reduced on fruit previously marked by conspecific flies but not on fruit previously marked by wasps (χ=0.21, *df*=1, *P*=0.52; Fig. 1a). However, for experienced female flies that had previously oviposited into wasp-marked fruit, oviposition was significantly (χ^2=11.3, *df*=1, *P*=0.004) reduced on fruit previously marked by a conspecific female as well as on fruit previously marked by a parasitoid female (χ^2=13.1, *df*=1, *P*=0.002; Fig. 1b). For experienced flies, oviposition was significantly reduced by both the fly (χ^2=8.4, *df*=1, *P*=0.003) and wasp (χ^2=9.8, *df*=1, *P*=0.002) OMP for up to 7 days after ovipositor dragging (Fig. 1c, d).

Comparison of infested and uninfested fruit

Oviposition by experienced female flies was significantly (χ^2=6.8, *df*=1, *P*=0.006) reduced on wasp-marked fruit which did not receive oviposition but not so on fruit which received wasp oviposition without subsequent marking (χ^2=0.42, *df*=1, *P*=0.21; Fig. 1e). Ovipositor dragging by parasitoid females significantly reduced subsequent oviposition by experienced flies on both fly-infested (χ^2=13.4, *df*=1, *P*=0.005) and uninfested (χ^2=6.9, *df*=1, *P*=0.03) fruit (Fig. 1f).

Removal and re-application of wasp OMP

Oviposition by experienced *R. mendax* female flies into wasp-marked fruit that had been subsequently rinsed with an ethanol solution was not significantly (χ^2=0.09, *df*=1, *P*=0.87) reduced compared with the control; however, oviposition was reduced on marked fruit that were not rinsed (Fig. 2a). Oviposition by experienced female flies into fruit treated with a solution of wasp OMP was significantly (χ^2=5.4, *df*=1, *P*=0.03) reduced compared with untreated as well as solvent-treated fruit (Fig. 2b).

Discussion

Our results provide convincing behavioral evidence that gravid female *R. mendax* flies recognize and avoid ovipositing into fruit that was previously marked by the oviposition-marking pheromone deposited by its wasp parasitoid, *D. alloeum*. Although interspecific recognition of host-marking pheromones is uncommon (see review by Nufio and Papaj 2001), it has been demonstrated to occur among closely related parasitoid species (Vet et al. 1984; van Baaren et al. 1994) and closely related *Rhagoletis* fly species (Prokopy et al. 1987). There are also rare examples of interspecific recognition of host-marking pheromones among relatively unrelated species such as among species within different families of Hymenoptera (Bolter and Laing 1983) as well as among species within different genera of Lepidoptera (Thiéry and Gabel 1993). Also, interspecific recognition of OMPs has been suggested to occur in another *Rhagoletis* species. Hoffmeister and Roitberg (1997) reported that *R. basiola* females took longer to probe and oviposit on rose hips that had been searched by the egg parasitoid, *H. rosae*, but oviposition would occur after this longer latency period.

The chemical and behavioral ecology of host selection has focused on kairomones used by herbivores for detection and recognition of plants or infochemicals exploited by natural enemies for prey finding (Dicke 2000). Cues or signals originating from the presence of predators or parasitoids that modify utilization or colonization of a plant resource by an herbivore have received less attention. Infochemicals from all trophic levels could potentially influence herbivore host selection and population regulation. For example, spider mites are less likely to colonize and lay eggs on leaf discs that were exposed to phytoseiid mite predators compared with unexposed controls (Grostal and Dicke 1999). In addition, parasitization of *Plutella xylostella* larvae is reduced in the presence of larval *Pieris rapae*, and oviposition by female *P. xylostella* is greater on plants colonized with *P. rapae*, suggesting that infochemicals modify host selection in a multitrophic context (Shiojiri et al. 2002). Even within the same (third) trophic level, parasitoids of *Drosophila* that compete for a given host avoid one another via volatile infochemicals (Janssen et al. 1995). In the tephritid fruit flies, closely related *Rhagoletis* (Prokopy et al. 1987) and *Anastrepha* species (Aluja and Díaz-Fleischer 2006) reject fruits marked with their own or a heterospecific OMP.

In the present system, there is an evolutionary advantage for both the fly, *R. mendax*, and the wasp, *D. alloeum*, to

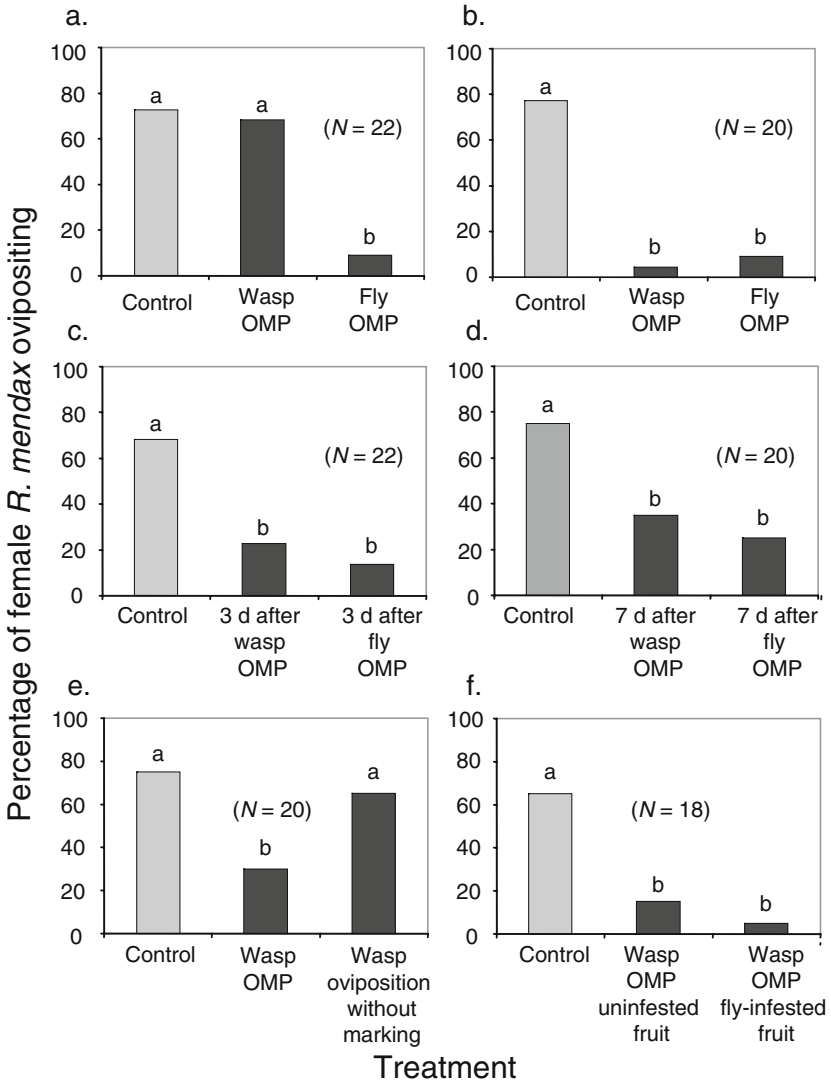

Fig. 1 Percentage of naïve *R. mendax* females ovipositing into unmarked blueberries (control) versus identical berries having received wasp or fly oviposition marking 30–90 min prior (**a**); percentage of experienced *R. mendax* females ovipositing into unmarked (control) berries versus identical berries having received wasp or fly oviposition marking 30–90 min (**b**), 3 days (**c**), or 7 days (**d**) prior; percentage of experienced *R. mendax* females ovipositing into unmarked (control) berries, berries having received wasp ovipositor marking without previous oviposition, or berries receiving oviposition without subsequent marking (**e**); percentage of experienced *R. mendax* females ovipositing into unmarked (control) berries versus identical berries having received wasp oviposition marking that were either uninfested or infested with an *R. mendax* larva (**f**). Significant differences in the numbers of wasps ovipositing are indicated by *different letters above bars* according to analyses by logistic regression ($P<0.05$)

recognize and respond to their species-specific OMPs, which likely function as signals (sensu Nufio and Papaj 2001) among conspecifics. Resources for both the fly and the wasp are short-lived and only support the potential development of one offspring per fruit. Therefore, marking and recognizing an occupied host is highly adaptive, reducing misallocation of eggs and time spent examining an occupied host. Recognition of wasp-marked fruit by *R. mendax* female flies is an interesting strategy that could further optimize the fly's reproductive potential. The OMP of the blueberry maggot remains active for only 6–12 days (Prokopy 1981a), but maggot development within the berry lasts ca. 3–4 weeks (Lathrop and Nickels 1932). Detection and recognition of the wasp's OMP potentially extends the fly's ability to avoid a previously utilized host fruit, but this interaction does not appear to benefit the wasp. Both species' OMP reinforce rejection of a previously exploited and unsuitable resource reducing competition among fly progeny as modeled by Roitberg and Mangel (1988), irrespective of whether the fly or wasp OMP induces the avoidance response. Thus, within the fly–wasp interaction, the wasp OMP is likely a cue (sensu Nufio and Papaj 2001) exploited by the fly for a nonmutual benefit. Given that parasitization of *R. mendax* by *D. alloeum* can reach 50% in unsprayed blueberry plantings (Stelinski et al. 2004), the selection pressure for the development of interspecific avoidance of *D. alloeum*-marked fruit by *R. mendax* may have been similar to that acting on the development of intraspecific avoidance of OMPs among *R. mendax* conspecifics.

The effect of the wasp's pheromone on blueberry maggot host acceptance required previous experience with wasp-marked fruit by the fly in the form of an ovipositional bout. It has been shown that experience is required for *R. pomonella* flies to recognize and avoid their conspecific OMP; however, this experience occurs while the gravid female fly examines the marked fruit and does not require associated oviposition (Roitberg and Prokopy 1981).

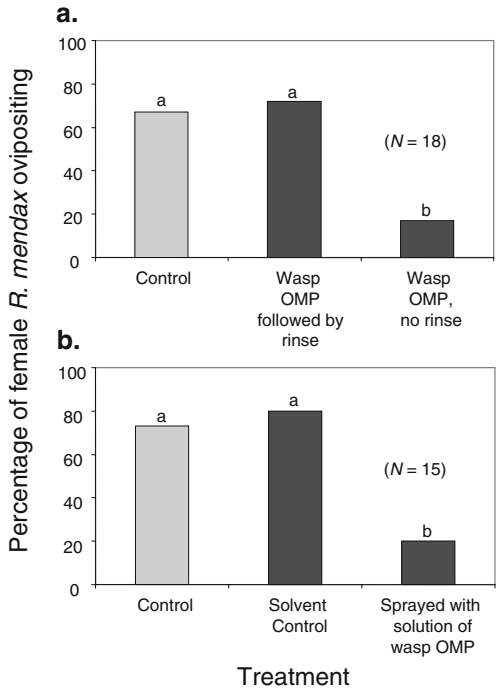

Fig. 2 Percentage of experienced *R. mendax* females ovipositing into unmarked (control) berries, berries receiving wasp ovipositor marling, or berries receiving wasp ovipositor marking followed by rinsing with ethanol solution (**a**); percentage of experienced *R. mendax* females ovipositing into unmarked (control) berries, berries sprayed with an 50% aqueous solution of ethanol, or with an aqueous ethanol solution of wasp marking pheromone (**b**). Significant differences in the numbers of wasps ovipositing are indicated by *different letters above bars* according to analyses by logistic regression ($P<0.05$)

Associative learning has been demonstrated for the closely related *R. pomonella* (Bush 1966; Smith and Bush 2000) given that previous experience with host fruit increases subsequent fruit acceptance for oviposition (Prokopy et al. 1982). Female *R. pomonella* are able to detect the presence of conspecific larvae within host fruit and reject that fruit for oviposition (Averill and Prokopy 1987). Thus, female blueberry maggot flies may associate fruit infested by conspecific larvae with the presence of the wasp OMP during oviposition. After learning, encounters with subsequent wasp-marked fruit may trigger rejection, reducing the time investment on an unsuitable host. Detecting an unsuitable resource soon after landing on fruit and prior to oviposition likely increases the reproductive efficiency of the fly compared with solely relying on detection of a conspecific larva hidden within the fruit resource during the lengthy oviposition process.

Ruther et al. (2002) recently proposed specific categories of kairomones including "foraging kairomones": "used by the benefiting organism in the context of the location of food for the organism itself or its offspring" and "enemy avoidance kairomones": "used by the benefiting organism to reduce the negative impact of a natural enemy." In the host–prey system described here, it is more likely that the wasp OMP functions as a foraging kairomone for the fly than an enemy avoidance kairomone. A blueberry fruit is rendered unsuitable for subsequent use by another fly whether it contains an unparasitized or parasitized fly larva, since a single blueberry can support the development of only one larva. Additionally, there is a long temporal displacement (17–21 days; Stelinski et al. 2004) between initial infestation of the fruit resource by the fly and utilization of fly-infested fruit by the wasp. In the absence of parasitoids, the fly uses host color, shape, size, plant odors, and its own OMP to select an ovipositional substrate. If the fly OMP is no longer present due to degradation but the wasp OMP is detected, determination of occupancy involves incorporation of new information in the form of a learned response. If a fly oviposits into an occupied host that has been wasp-marked and parasitized, we suggest that it associates the external pheromone marker deposited by the wasp with fruit occupation by a conspecific larval fly. This behavioral association results in rejection of subsequently encountered wasp-marked fruit following detection of a chemical marker on the fruit surface. Detection of the wasp's OMP by the fly therefore may increase foraging efficiency and maximize reproductive potential by reducing handling time on an unsuitable host. The likely benefit of detecting the wasp's OMP by the fly is a reduction in misallocation of eggs. Thus, it is more likely that the wasp's OMP functions as a foraging kairomone exploited by the fly than an enemy-avoidance kairomone since its primary function appears to be increasing efficiency of locating a food resource for the fly's offspring. Field studies will need to be conducted to test this hypothesis. While examining their hosts, herbivores process a bouquet of chemicals including information regarding host presence, quality, as well as presence of competitors and natural enemies. The results of the current study emphasize that host-plant selection by herbivores has to be considered not only in a bitrophic context but that other levels have to be included as well. Furthermore, new definitions for chemically mediated interactions may need to be formulated as our understanding of multi-trophic interactions increases.

The vast majority of OMPs described to date mediate interactions among female conspecifics (Nufio and Papaj 2001). The unique example presented herein has a broader function: exploitation of an infochemical deposited by one species by another belonging to a different taxonomic order and trophic level in a context where both herbivore and parasite examine and mark the same oviposition substrate. *D. alloeum* also parasitizes populations of the apple maggot fly, *R. pomonella* (Walsh) (Glas and Vet 1983; Stelinski and Liburd 2005). The apple maggot fly species occurs in two host races preferentially infesting either hawthorn or apple fruit (Feder et al. 1994). Therefore, the *Rhagotetis* fly–*D.*

alloeum parasitoid interaction occurs on at least two other oviposition site resources (hawthorn and apple fruit) and may occur in many other host–parasitoid associations. Given that wasp parasitoids comprise up to 20% of all insects (Price 1980), it is possible that many other examples of interspecific pheromone recognition remain to be discovered.

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