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Oviposition Response of the Moth *Lobesia botrana* to Sensory Cues from a Host Plant

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Abstract

The grapevine moth *Lobesia botrana* is a generalist insect herbivore and grapevine is one of its hosts. Previous studies have shown that insects use their olfactory abilities to locate hosts from a distance; whereas contact chemoreception mediates the stimulation of oviposition after landing. Little is known about the role of olfaction and its interactions with contact chemoreception and vision once the insect lands on the plant. Plant volatile compounds can be sensed by host-searching insects located some distance from the plant and insects sense both volatile and nonvolatile cues after landing on a plant. In the present study, we investigated the effects of these volatile and nonvolatile cues on the oviposition behavior of *L. botrana*. A behavioral bioassay with choice was developed in which insects were offered each sensory cue either alone or in combination with one or 2 other cues. Females were allowed to choose between a device with the stimulus and a blank device. Results were evaluated in terms of 2 parameters: quantity of eggs laid (egg counts) and preference for the stimulus (ODI: oviposition discrimination index). Our results suggest that olfaction significantly affects egg quantity and that there is significant synergism between olfaction and vision, in terms of their combined effect on egg quantity. In terms of preference (ODI), our results did not show a significant preference for any single cue; the highest ODI was measured for the full-cue stimulus (olfaction, vision, and contact). For ODI, a significant interaction was observed between olfaction and vision and a nearly significant interaction was observed between the olfactory and contact cues. The results are discussed in relation to the effects of plant sensory cues on the oviposition behavior of *L. botrana*.

Key words: contact chemoreception, dual-choice bioassay, grapevine moth, insect behavior, multimodal integration, olfaction, plant volatiles, vision

Introduction

Egg-laying females of phytophagous insects use sensory cues from host plants to search for and evaluate the suitability of individual plants for use as oviposition sites (Schoonhoven et al. 2005). Olfaction, contact chemoreception, and vision may all play roles in this process. In moths, olfactory cues released from host plants guide gravid females to potential oviposition substrates from a distance (Bruce et al. 2005). Once the insect has landed on the plant, volatiles, together with contact and/or visual stimuli, may play a role in the subsequent behavioral steps leading to oviposition (Renwick and Chew 1994).

Female grapevine moths, *Lobesia botrana*, are attracted by grapevine odors to lay eggs on flower buds and grapes at different phenological stages (Tasin et al. 2005; Masante-Roca et al. 2007). Although several studies have shown that insects' host-searching behavior is mediated by volatile signals acting at a distance (Hurtrel and Thiery 1999; Tasin et al. 2006, 2007), the means by which sensory cues from host plants affect the postlanding behavior of *Lobesia botrana* is still a controversial subject. A major role in this process has been attributed to contact chemosensory stimuli; gustatory receptors and their sensitivity to plant sugars have been well studied in this context (Maher and Thiery 2004b; Calas et al. 2006; Maher and Thiery 2006; Maher et al. 2006). In a recent study, volatiles from grapevine were found to increase egg deposition even in the absence of gustatory stimuli (Anfora et al. 2009). Little information is available in the literature on the behavioral effects of visual cues. A complete dissection of the behavioral role of each sense and their interactions has not yet been attempted in *L. botrana*. Therefore, the modalities through which the information from visual, olfactory, and contact-chemosensory plant cues are integrated into behavioral decisions require further study.

The present work investigates the oviposition preference of *L. botrana* as it relates to individual cues and combinations of those cues. We hypothesize that synergistic interactions among sensory cues may have significant behavioral implications for the oviposition behavior of this insect.

Materials and methods

Insects

The *L. botrana* moths used in this study came from the laboratory culture maintained in San Michele all'Adige (Italy). Wild larvae collected from host plants have been introduced into the rearing annually, in order to avoid inbreeding effects. These wild larvae were collected in the field and kept on host plants as they were transported to the laboratory. Larvae were reared in plastic boxes (35 × 20 × 6 cm) that were kept in a climate chamber (22.5 °C, 65% relative humidity, 16:7 light:dark photoperiod) until the insects' pupation. They were fed on a semi-artificial diet. Insects were provided unlimited access to food (water 74%, agar 1.5%, sucrose 3%, alfalfa 2.5%, yeast 1.8%, minerals 1.2%, cholesterol 0.1%, wheat germ 8.9%, casein 4%, sorbic acid 0.2%, ascorbic acid 1%, vitamin mix 0.8%, tetracycline 0.1%, propionic acid 0.2%, linoleic acid 0.1%, maize seed oil 0.2%, nipagine 0.4%). Adults emerged in the plastic boxes (30 × 30 × 30 cm; Megaview Science Co., Taiwan) and were not exposed to the food. A 24-h-old female was placed inside a plastic container (20 mm ID, 50 mm long) along with a male of the same age for one night. In order to be sure that all females were mated and able to lay eggs, only couples in which the female laid 1–10 eggs were used in oviposition experiments. No food was provided to the adult insects during the oviposition experiments. Females had never been exposed to plants prior to the experiments and each female insect was used only once.

Behavioral assay

Experiments were arranged in an illuminated room with gray walls and the environmental conditions were the same as those described for the rearing. Choice experiments were conducted in cylindrical metal cages (250 mm ID, 500 mm long, 1.5 mm mesh; Figure 1) similar to those described by Tasin et al. (2009). Because *L. botrana* is a crepuscular moth, cages were not exposed to direct light. Moths were allowed to choose between 2 possible oviposition sites in each cage; the sites in each cage were 30 cm apart from each other. This distance was chosen in order to provide the test insect with a sufficiently large discriminatory environment, which might facilitate a response relatively close to what might occur under natural conditions. After the oviposition stimuli were put into position, a mated female was released in the center of each cage. In our experiment, a period of 72 h was needed for the moth to discriminate among the stimuli. Within a shorter period, females may not choose among the stimuli and the number of eggs laid may be reduced (Anfora et al. 2009). After 72 h, we removed the moth and counted the eggs that had been deposited at each site. Ripe clusters of cv. Cabernet Sauvignon, similar in color and number of berries, were used in the bioassay. Clusters were detached from the plant 1 h before the beginning of the bioassay. We chose to use mature grapes (stage 38; Eichhorn and Lorenz 1977) for this study, based on the relevant literature (Maher et al. 2006).

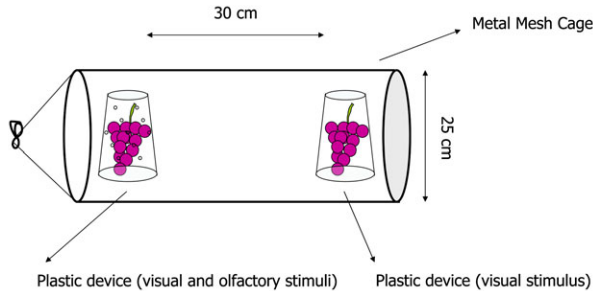

Figure 1 Schematic drawing of the behavioral choice test with 2 oviposition devices. As an example, a test between Olfaction + Vision (left) and Vision (right) is presented.

In order to provide the test insects with several combinations of sensory cues, polyethylene conical cups (matte transparent; base diameter 88 mm, top diameter 61 mm, height 130 mm), which each either contained a bunch of grapes or were empty, served as oviposition devices (for details, see Anfora et al. 2009). This kind of oviposition substrate offers a smooth surface that serves as a basic thigmotactic stimulus (Maher and Thiery 2004a; Sambaraju and Phillips 2008). The base opening of the cup was covered with a plastic petri dish and placed on the bottom of the cage. We wore gloves when we handled the cups, in order to avoid any contamination from human hands. Each pair of gloves was used only once. For the treatments in which olfactory cues were examined, 30 holes (1.5 ± 0.2 mm ID) were pierced in the sidewall of the cup to allow volatile compounds from the grapes to exit the device (Table 1).

Use of an oviposition device as a substitute for grapes and evaluation of position biases

The quantities of eggs deposited by female grapevine moths on grapes and on the plastic cups described above were

Table 1 Description of the experiments in this study

Type of experiment	Investigated effect	Stimulus	Control	Number of insects tested
Bioassay setup	Substitution	Grape	Grape	12
		Grape in a pierced cup	Grape in a pierced cup	12
	Position bias	Grape in a pierced cup at the eastern side	Grape in a pierced cup at the western side	12
	Deterrence	Grape in a pierced cup that is replaced daily	Grape in a pierced cup that is not replaced during the course of the experiment	15
Individual cue	Contact	Cup sprayed with surface extract	Cup sprayed with methanol	30
	Vision	Grape in an unpierced cup	Blank unpierced cup	41
	Olfaction	Pierced cup containing a grape hidden under white gauze	Blank cup containing white gauze	33
Combinations of cues	Olfaction + Contact	Grape hidden in white gauze inside a pierced cup sprayed with surface extract	Cup containing gauze sprayed with methanol	28
	Vision + Contact	Grape in an unpierced cup sprayed with surface extract	Unpierced cup sprayed with methanol	30
	Olfaction + Vision	Grape in a pierced cup	Blank cup	30
	Olfaction + Vision + Contact	Grape in a pierced cup sprayed with surface extract	Cup sprayed with methanol	30

compared using 2 no-choice tests. In the first test, moths were exposed to 2 grapes that were not inside the device. In the second test, the site for oviposition was the same as in the first test but was inside 2 pierced cups. Moths were confronted with the same olfactory and visual cues, but a different contact stimulus. In these experiments, we also evaluated the presence of any positional (east–west) bias in our setup. An unpaired 2-tailed *t*-test was used to evaluate the capacity of the device for acting as a substitute for a bunch of grapes.

Presence of deterrents

We tested the hypothesis that a female would lay more eggs during a 3-day-long experiment if the oviposition device were changed every day. This is in accordance with several studies that have presented evidence for the release of oviposition deterrents (epideictic pheromone) by *L. botrana* eggs (Thiery et al. 1992; Calas et al. 2006). To verify that oviposition deterrents are released by *L. botrana* eggs, the performance of a female (i.e., the number of eggs laid) was evaluated during a 3-day experiment. In the first test, a female was placed in a cage with 2 pierced cups holding similar grapes and each cup was offered to her over a 3-day period. The same setup was used in the second test, except that one of the 2 cups was replaced with a new cup each day. Females were expected to lay more eggs in the second treatment. An unpaired 2-tailed *t*-test was used to evaluate the presence of deterrents.

Oviposition experiments to test synergism

For a setup in which insects could see, but not smell cues, we placed a bunch of grapes inside an unpierced cup. In this case, only visual cues were available to the test insect. To test the effect of visual cues, an experiment involving an oviposition choice between a cup containing a bunch of grapes and an empty cup was arranged. A setup in which moths could use their olfactory abilities but not their vision was constructed using a pierced cup containing a bunch of grapes that was covered with white gauze. To test the effect of olfaction, oviposition on this cup was compared with that observed on an empty pierced cup containing only white gauze.

A setup in which olfactory and visual cues were both available to the insects was constructed by placing a bunch of grapes inside a pierced cup. To test the effect of the combination of olfactory and visual cues on oviposition, a choice bioassay was performed in which ovipositioning activity on a pierced cup containing a bunch of grapes was compared with that observed on an empty pierced cup.

A device in which only "contact compounds" were offered to the insects was constructed by spraying a plastic cup with a berry-surface extract. One hundred and twenty mature grape clusters cv. Cabernet Sauvignon were harvested 1 h before dusk and then individually immersed in 3 L of methanol (Fluka) for 5 min, partially following the protocols found in the literature for grape-surface extractions (Maher and Thiery 2006; Maher et al. 2006). Methanol was chosen because it evaporates rather quickly from the oviposition device, protects plant compounds from enzymatic degradation, and has been used previously to effectively extract known oviposition stimulants (Sato et al. 2004; Varandas et al. 2004). In order to minimize the extraction of compounds from the interior of the grape and from the pedicels, we used only fresh grapes and did not detach any berries from the cluster. Immediately after extraction, the methanolic extract was filtered (45 μm) and then concentrated by rotary vacuum evaporation (40 °C, 240 mbar) to 55.5 g equivalent of fresh weight per milliliter. The extract was refrigerated at –80 °C until use.

The behavioral effect of this surface extract was measured in a bioassay in which moths were allowed to choose between a cup sprayed with an amount of extract corresponding to that extracted from a bunch of grapes and a cup sprayed with methanol only. A bunch of grapes was placed in a cup and the cup was then sprayed with the methanolic extract. This setup provided the insect with visual and contact cues and was compared with a blank cup sprayed with the solvent in a choice experiment. Olfaction and contact were coupled by spraying a pierced cup containing a bunch of grapes and then wrapping it in a piece of white gauze. The same gauze was placed into the blank cup, whose outer surface had been treated with methanol. Olfactory, visual, and contact cues were combined by placing a bunch of grapes inside a pierced cup that had been sprayed with the methanolic extract. This was done with the aim of reconstructing the original stimuli present on and around the host plant. A pierced cup sprayed with methanol only served as a control.

Parameters of oviposition preference

Preferences for a given stimulus were scored using an oviposition discrimination index (ODI) (Maher and Thiery 2006). This index was calculated using the following formula: ODI = [(no. eggs on substrate A – no. eggs on substrate B)/total no. eggs] × 100. The value of ODI varies from –100, when all eggs are laid on A, to +100, when all eggs are laid on B. A paired 2-tailed t-test was used to test for the presence of a significant preference for the stimulus.

Statistical evaluation of synergistic effects of the cues on egg counts

The synergism between cues was tested using a generalized linear model (GLM) followed by a factorial parametric analysis of variance (ANOVA), as suggested by Slinker (1998). Because ODI represents a percentage, we could not use this index as a dependent variable in the analysis. Instead, we used the actual number of eggs laid, which is a quantitative parameter more suitable for measurements of synergism. This parameter had a negative binomial distribution. An analysis of variance with this distribution was carried out using R software (R Development Core Team 2004).

Results

Use of an oviposition device as a substitute for grapes and evaluation of position biases

The introduction of an artificial oviposition device with the aim of separating out the different sensory cues broadcast by the host plant did not influence the mean number of eggs (±standard error) deposited by a female moth (6.8 ± 1.1 [grape inside the cup] vs. 7.0 ± 1.0 [grape]; unpaired t-test: $t_{12} = 2.07$; $P = 0.82$). In addition, no bias related to the position of the stimuli (eastern or western side of the cage) was detected in our setup (6.7 ± 1.8 vs. 6.8 ± 1.4; paired t-test: $t_{12} = 2.20$; $P = 0.95$). Females laid eggs exclusively on the oviposition device and not in any other part of the cage.

Effect of deterrents on the number of eggs laid

There was no difference between the mean number of eggs laid in the cages in which neither of the 2 cups was replaced (7.3 ± 2.1) and the cages in which one of the cups was replaced daily (10.1 ± 3.6) (unpaired t-test: $t_{15} = 2.05$; $P = 0.52$). In our experiment, the presence of deterrents from previously laid eggs was not significant. Accordingly, we did not replace the cup in later tests.

Effects of plant cues on oviposition

Oviposition discrimination index

Positive ODI values were observed for a number of stimuli. Among them, the 3-cue treatment had the highest ODI (28) value, followed by the Olfaction + Vision (21.5), Olfaction + Contact (18.2), Vision + Contact (6.1), and Olfaction alone (1.5) treatments. Negative ODI values were observed for the treatments in which only visual cues (–5.8) and only contact cues (–17.0; Figure 2) were presented. Significant ODI values were observed for the full set of all 3 cues and the Olfaction + Vision treatment (paired t-test: $t_{30} = 2.63$ and $P = 0.01$ for the full set of cues; $t_{30} = 2.14$ and $P = 0.04$ for Olfaction + Vision). None of the other factors were significant.

Evaluation of synergism between cues to affect egg counts

A synergistic interaction was observed between the olfactory and visual cues (GLM followed by ANOVA; deviance = 4.54;

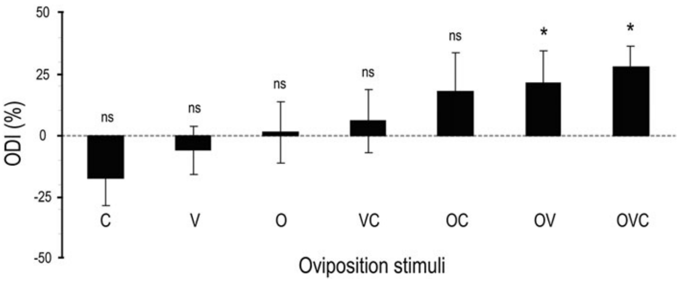

Figure 2 ODI of *Lobesia botrana* in laboratory choice bioassays for individual stimuli from grapes and combinations of these stimuli (c = contact, v = vision, o = olfaction). An asterisk above a bar indicates a significant difference ($P < 0.05$) between the number of eggs laid on a treated device and the number of eggs laid on the blank according to a paired 2-tailed t-test (ns = no significant difference). Error bars represent the standard errors of the means.

$P = 0.033$; degrees of freedom [df] = 1,248). No significant interactions were observed between the olfactory and contact cues or between the visual and contact cues. There was also no significant 3-way interaction (Table 2). Only the olfactory cue emerged as significant (3-way ANOVA; deviance = 13.96; $P = 0.001$; df = 1,248).

Discussion

In the present study, we investigated the effects of different plant cues on the oviposition behavior of *L. botrana*. A behavioral bioassay with choice was developed and used to offer each test insect a single type of sensory cue or a combination of 2 or 3 different types of sensory cues. A significant interaction was observed for the combination of olfactory and visual cues.

Effect of plant cues on oviposition

Visual cues alone did not affect the preferences of the egg-laying females. The visual image of the grapes was not sufficient to stimulate oviposition under our experimental conditions (Figure 2 and Table 2). Visual stimuli are thought to play a minor role in the ovipositioning behaviors of nocturnal moths, in contrast to the situation for insects that are active during the day, such as butterflies (Talsma et al. 2008).

In terms of ODI (Figure 2), we observed a positive but not significant effect when the volatile cue was presented alone. Despite the fact that, in a previous study, volatiles from actual unripe grapes stimulated oviposition, synthetic volatiles identified from these grapes have been shown to stimulate oviposition only at a specific dose, meaning that females lay fewer eggs when concentrations of these substances are too high or too low (Tasin et al. 2008; Anfora et al. 2009). In the field, these same compounds have been shown to both attract gravid females and stimulate oviposition on the vegetation near the lure (Anfora et al. 2009). Ripe berries (as were used in this study) are known to release both a smaller amount and a different profile of plant volatiles than unripe bunches (Tasin et al. 2005). In particular, the release of compounds such as terpenoids and methyl salicylate, which were identified as oviposition stimulants by Anfora et al. (2009), strongly decreases during ripening. It is thus possible that our grapes released only suboptimal quantities of these compounds, which were not sufficient to trigger any significant preference in terms of ODI. However, the relatively low emission of plant compounds did not compromise the positive effect on egg counts (Table 2), suggesting a role of volatiles as egg-laying stimulants. In previous studies, volatiles from host plants have been shown to not only affect oviposition but also attract *L. botrana* to host plants from a distance (Masante-Roca et al. 2007; Tasin et al. 2010).

The contact stimulant extracted from the berries had a non-preferential effect on moth choice (Figure 2). Similar results have also been reported in another moth (Rojas et al. 2003). This may be explained by either the co-occurrence of inhibitory and stimulatory compounds in the extract, as reported by Honda (1995) and/or by the solvent extraction itself, which may alter the relative proportions of the different non-volatile compounds from the phylloplane (Lombarkia and Derridj 2002, 2008). Our extract may also contain compounds from the interior of the plant, to which the moths are not exposed in nature. In another study of *L. botrana*, surface extracts from branches of *Daphne gnidium*, an alternative host plant for this species, were found to have a deterrent effect on oviposition (Maher and Thiery 2006). Maher et al. (2006) reported stimulatory effects of certain raw extracts from grapes on *L. botrana* oviposition. However, only extracts from ripe berries elicited oviposition; extracts from other phenological stages suitable for egg laying did not. It thus appears that the particular methods used to extract materials from the surface of the plant may greatly affect the amounts of behaviorally active compounds present in the extracts. What is perceived as a stimulant by an egg-laying insect and what is extracted by a solvent from the surface of a plant are not necessarily the same (Muller and Riederer 2005; Stadler and Reifenrath 2009). A further factor that makes it difficult to compare our study with those already published is the type of bioassay employed. In Maher et al. (2006), for example, the volume of the arena available for the test insect was much smaller than the area used in our bioassay. The use of a larger discriminatory environment may lead to a more natural response than might be observed in smaller arenas. In general, our results on the effect of contact compounds from grapes differ from those published by other authors (Maher and Thiery 2004a, 2006; Maher et al. 2006; Moreau et al. 2008). It thus appears that investigations based on surface extracts obtained through improved

Table 2 Three-way ANOVA of egg counts for the different treatments in the oviposition bioassay with choice (8 combinations of cues)

Factor	df	Sum of squares	Mean of squares	*F*	*P*
Olfaction (7.0 vs. 2.9)	1	13.9	13.9	13.9	0.001
Vision (3.9 vs. 4.2)	1	1.3	1.3	1.3	0.257
Contact (1.3 vs. 2.6)	1	<0.01	<0.01	<0.01	0.909
Olfaction + Vision (6.5 vs. 1.3)	1	4.5	4.5	4.5	0.033
Olfaction + Contact (11.1 vs. 4.0)	1	0.5	0.5	0.5	0.494
Contact + Vision (2.9 vs. 2.3)	1	0.3	0.3	0.3	0.570
Olfaction + Vision + Contact (7.1 vs. 0.9)	1	<0.01	<0.01	<0.01	0.953
Corrected total	248	224.12			

Egg-laying females could choose between a device with a sensory stimulus (listed in the first column) and a blank device. Numbers in parentheses represent the average egg counts on the device with the stimulus and on the blank device.

protocols are needed before we can draw final conclusions concerning the behavioral effects of this type of stimulus. Substitution of solvents with a noninvasive tool (ex. arabic gum) may be useful as we work toward this target.

Synergism between volatile and nonvolatile cues

The combination of visual and contact cues did not elicit any synergistic effect. However, the effect of contact cues alone was compensated for by the effect of vision (Figure 2). This phenomenon was also seen in the combination of the 2 chemical senses (olfaction and contact chemoreception; Figure 2; $P = 0.06$). Although only a subsignificant synergism was observed for this combination, we detected an increase in the number of eggs laid in this treatment, as compared with the treatments in which each of these 2 cues was tested individually (Table 2). These results seem to imply that the co-occurrence of olfactory and contact cues reverses the negative effect of contact compounds alone, suggesting the presence of stimulants in our surface extract. The stimulatory effect of these compounds seems to be triggered by the presence of plant volatiles. In some insects, volatiles have been found to synergize stimulation by nonvolatiles; the perception of volatile compounds via both olfactory and gustatory circuits may partially explain this behavior (Roessingh et al. 2007).

The addition of olfactory cues to the visual cues significantly augmented both the ODI and the number of eggs laid (Figure 2 and Table 2). Although in the study of Masante-Roca et al. (2007), long-range orientation to grapes was not affected by vision, in our experiment, vision appeared to play a role in host–plant recognition only when in association with olfactory cues. The existence of an interaction between olfaction and vision during host location from a distance has been documented previously (Raguso and Willis 2002; Gilbert and Kuenen 2008; Holopainen 2008). Our data suggest that there is also a positive synergistic interaction between these 2 senses after insects land at the source of the stimuli (i.e., during oviposition) (Figure 2 and Table 2).

The addition of contact cues to olfactory and visual cues had a positive effect on ODI. This was as expected, as the full-cue stimulus is supposed to mimic the grape itself, providing more complete information to the insect to use to recognize the plant as a suitable host. Although the behavioral effects of combinations of plant cues on insect oviposition have been documented in a number of studies (Harris and Miller 1988; Harris and Foster 1995; Kanno and Harris 2000; Schoonhoven et al. 2005), to the best of our knowledge, this study represents the first attempt to reconstruct a natural stimulus for *L. botrana* by isolating and then re-combining 3 different plant cues in a unique device for behavioral assays. The capability of this device to mimic actual grapes is corroborated by the fact that no difference was found between the number of eggs laid on this device and the number laid on actual grapes.

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