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Inter-specific avoidance of egg-associated semiochemicals in four tortricids

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Abstract. Oviposition of four tortricid pests of vineyards (the European grapevine moth and the grape berry moth) and fruit orchards (the codling moth and the oriental fruit moth) is deterred by a blend of straight chain fatty acids and esters of fatty acids that have been identified in the eggs of one of them: the European grapevine moth (EGVM) *Lobesia botrana*. This is the first evidence of inter-specific recognition of an egg-like signal in moths. We demonstrate that oviposition site selection is influenced by population density, avoidance of deterrent being most important when females are isolated. Inter-specific egg recognition might be an important phenomenon, especially in species competing for a common food resource. We propose the term 'oviposition regulating synomone' for molecules and blends that affect the inter-specific spacing of eggs.
Key words. Lepidoptera; Tortricidae; synomone; pheromone; behavior; oviposition; *Lobesia botrana*; *Cydia pomonella*; *Cydia molesta*; *Eupoecilia ambiguella*; fatty acids; esters of fatty acids.

In a number of herbivorous insect species, females avoid ovipositing on sites already colonized by conspecific females[1,2]. Egg (or cluster) spacing behavior is an important reproductive strategy which reduces competition for food resources[3]. To date this phenomenon was considered to be cued by specific molecules produced by the females as is the case in *Rhagoletis cerasi*[4] or in pierids[2]. We propose that non-specific molecules commonly occurring on egg surfaces are detected by ovipositing females and alter the pattern of oviposition, and that the ability to modify behavior in response to cues from eggs of other species would be advantageous to phytophagous competitors.

Recognition and avoidance of eggs or egg masses has already been described in two tortricids: the oblique-banded leafroller *Choristoneura rosaceana*[5] and the European grapevine moth (EGVM) *Lobesia botrana*[6]. EGVM females avoid artificial and natural oviposition sites treated with methanol egg washes[6]. A synthetic oviposition regulating pheromone (ORP) made up of nine substances (C_{14} to C_{18} linear fatty acids and C_{16} to C_{18} methyl esters of fatty acids) has a similar effect on EGVM females. This effect is dose-dependent. The mixture is active at the dose of 2.5 egg equivalents per cm^2 (ref. 7). Five of the compounds used were identified in the eggs of the European corn borer, *Ostrinia nubilalis*, and deter its oviposition[8]. The relative simplicity of the molecules found in *L. botrana* and *O. nubilalis*, and the similarity of some constituents between these two species, led us to check the hypothesis that semiochemicals extracted from the eggs of EGVM could be recognized not only by its sympatric species the grape berry moth (GBM) *Eupoecilia ambiguella*, but also by two other species which are phylogenetically close: the codling moth (CM) *Cydia pomonella* and the oriental fruit moth (OFM) *Cydia molesta*. We tested our inter-specific hypothesis using four tortricid species that are severe pests of vineyards and fruit orchards. The EGVM and GBM occur sympatrically in European vineyards, while the CM and OFM are cosmopolitan species which attack apples and peaches. They all lay isolated eggs, and this egg-spacing behavior may be triggered by egg-associated semiochemicals, as already observed in *L. botrana*. Our results show that inter-specific avoidance of egg-like signals occurs in tortricids, and that the density of females affects the behavioral response. We report here the first instances of inter-specific egg spacing in moths, and the first observations of egg avoidance in tortricids that cause severe damage to vineyards and fruit orchards.

Materials and methods

Experiments were conducted on insects from laboratory colonies reared on a semi-artificial diet (photoperiod 16L:8D):EGVM – Station de Zoologie INRA, Pont de la Maye, France; CM and OFM – Station de Zoologie et Apidologie INRA, Montfavet, France, and GBM – Station fédérale de Recherches Agronomiques de Changins, Nyon, Switzerland. Experiments were conducted during one scotophase using 2-day-old mated females. Conditions during this scotophase were darkness; temperature 22 ± 1 °C; relative humidity $65 \pm 5\%$ except for GBM ($80 \pm 5\%$RH). The oviposition assays were conducted in the four species with similar oviposition arenas. Each arena was made of a Plexiglass tube (int. diam. = 4.7 cm, length = 13.5 cm) in which an artificial substrate (cardboard 13.5 cm × 7.5 cm) offered a choice between

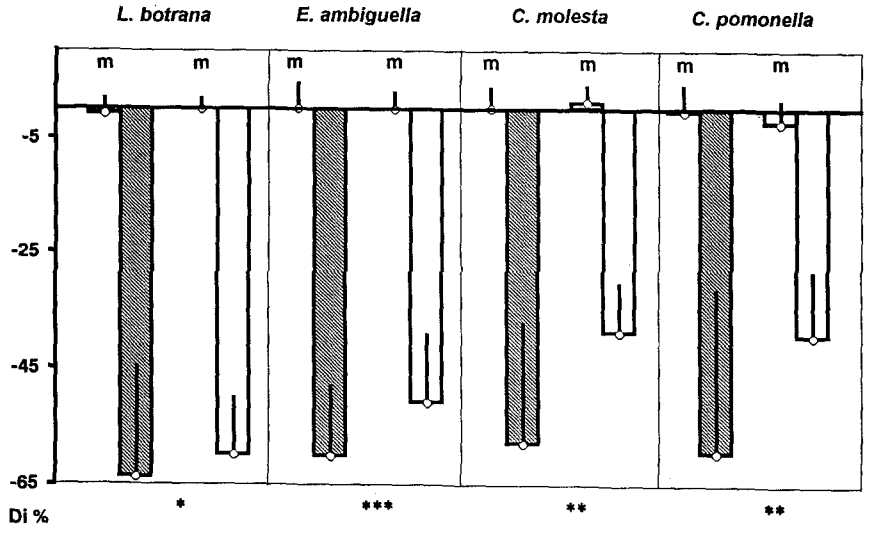
treated areas and non-treated areas[6]. The treated areas were 23 circles (diam. = 1.2 cm) with centers 2.1 cm apart, with a total area of 26 cm^2. Regular sized and equidistant dots of 5 µl of ORP solution were applied by means of a Plexiglass mask. The non-treated area was the area between the circles (76 cm^2). There were at least 5 replicates of grouped females (5 per arena) and 22 replicates of isolated females per treatment (1 replicate = 1 female). After experiments, all females were isolated for one night in small glass tubes in order to determine whether they laid eggs after exposure to semiochemicals (yes or no type response). We used two parameters: 1) the mean number of eggs per female laid during the exposure on treated sites (T) (corrected according to the area ratio) and the non-treated area (NT) and 2) the deterrence index, (Di) which was calculated as follows: Di% = (T-NT)/(T + NT) × 100; a value of −100% indicates total avoidance of treated areas and 0% no preference. We used non-parametric statistics, the Mann Whitney U test, to compare numbers of eggs and deterrence indexes. We performed 3 different treatments: 1) blank: where the cardboard is left blank, 2) control: application of pure methanol and 3) application of EGVM synthetic pheromone.

The synthetic pheromone used for these experiments was a blend of the nine constituents of the EGVM ORP which were mixed in pure methanol according to the concentration ratios found in the egg extract[7]: myristic acid (0.26), palmitic acid (1.37), stearic acid (0.18), methyl palmitate (0.59), methyl palmitoleate (0.08), methyl inoleate (0.23), methyl linolenate (0.20), methyl oleate (0.61) and methyl stearate (0,29) (amounts in ng per egg equivalent). The concentration was adjusted to a dose corresponding to 4 EGVM egg equivalents × $µl^{-1}$. (about 17 eggs × cm^{-2}).

Results and discussion

In the four species, we obtained eggs evenly distributed on blank cardboard by both grouped and isolated females. Pure methanol alone had no significant effect on the oviposition behavior of the four species, i.e. it had no effect on the egg distribution, the deterrence index being close to 0% in each species at both densities (fig.).

The EGVM ORP applied in dual choice bioassays strongly modified the oviposition behavior of isolated females in all four species. Females significantly avoided areas treated with the EGVM ORP (figure). Four CM females, 3 EGVM females, 2 OFM females and 1 GBM female completely avoided treated areas. Furthermore, 20% (GBM and OFM) to 33% (CM) of the females in each species did not lay any eggs when ORP was applied. This was never the case in the blank or with pure methanol. For example, 18 ovipositing females of CM laid a total of 395 eggs on the areas treated with solvent but only 43 eggs on areas treated with ORP. Females which did not lay eggs when exposed to ORP laid eggs during the night following exposure. This is interpreted as suppression of egg-laying in some individuals during the exposure to the synthetic mixture. We also observed



Selection of an oviposition site in four tortricid species by isolated and grouped females in response to *Lobesia botrana* synthetic oviposition regulating pheromone (ORP): *L. botrana* (EGVM), *Eupoecilia ambiguella* (GPM), *Cydia molesta* (OFM) *and Cydia pomonella* (CM). The choice is expressed as a deterrence index (see Methods). Thick bars represent deterrence indexes, and thin black bars the S.D. around this parameter. Striped bars correspond to isolated females and white bars to females grouped in fives. Letter m corresponds to methanol treatment. The ORP is a blend of nine molecules, adjusted to the dose of 20 egg equivalents per dot (see ref. 6). Non-parametric statistics (Mann Whitney U test): each Di value obtained with ORP is different from its control at $p < 0.001$; stars indicate differences of Di between isolated and grouped females, *at $p < 0.05$, **at $p < 0.01$, ***at $p < 0.001$.

Effect of solvent and EGVM egg-like signal on the number of eggs (means ± S.D.) laid by isolated females in the four species during the night of exposure.

Treatments	*L. botrana*	*E. ambiguella*	*C. molesta*	*C. pomonella*
Blank	42 ± 11.2	29.7 ± 8.8	43.1 ± 21.4	32.5 ± 15.1
Solvent	43.4 ± 15.6	31.3 ± 10.7	45.3 ± 14.7	28 ± 1.3
EGVM ORP	27.1 ± 23.1	18.10 ± 12.8	31.3 ± 27.7	16.2 ± 16.6

In each species the number of eggs was not affected by the solvent, but was reduced by the ORP treatment at $p < 0.001$(Mann Whitney U test). Equivalent parameters obtained with grouped females in the blank were 39.8 (EGVM), 19.0 (GBM), 41.6 (OFM) and 25.1 (CM)(no S.D. because of grouping).

that EGVM ORP caused a significant reduction in the total number of eggs per female (between 25% in GBM and 50% in CM) (table). Inter-individual differences in behavioral responses could be due to variability in sensitivity or to incompleteness in the chemical blend used, this may account for the variability in the avoidance observed in the two *Cydia* species (S.D. of deterrence index; see fig.)

We checked the effect of female density on the behavioral response. In isolated females, the mean numbers of eggs when no treatment was applied were: 43.1 ± 21.4 (OFM), 42.0 ± 11.2 (EGVM), 32.5 ± 15.1 (CM) and 29.7 ± 8.8 (GBM). Grouping females in fives slightly reduced the number of eggs per female in EGVM (39.8) and OFM (41.6). It caused a higher reduction in CM (25.1 eggs) and GBM (19.0 eggs) (table). The result we obtained with CM is in contradiction with other data which report that CM females in the presence of males laid more eggs during 4 days when they were in groups of 5 than when isolated or in groups of more than ten[9]. The deterrence provoked by the synthetic EGVM egg-associated semiochemicals is reduced, in the four species, when females are grouped in fives. In the EGVM and the GBM, the deterrence indexes (Di) are −60% and −64% (EGVM), and −51% and −60% (GBM) in grouped and isolated females respectively (fig.). In similar experiments, the value of Di obtained in groups of 10 EGVM females is −55%[6]. In the two *Cydia* species, this index is close to −39%, while in isolated females it is close to the values obtained in the EGVM and the GBM (fig.). The ability of *Cydia* species to select oviposition sites free of egg-like chemicals also depends on the number of females per oviposition arena. This clearly indicates that female density influences the oviposition site selection in the four species. A similar phenomenon occurs with the Alfalfa blotch leafminer: at high density more than 50% of the leaflets undergo multiple attacks (more than one individual per leaflet) but only 5% at low density[10].

We conclude from our results that the oviposition behavior of the four tortricid species is affected by molecules identified in EGVM eggs, and that inter-specific egg-like signal recognition occurs in different species belonging to the same family. The present results support previous reports that semiochemicals isolated from the European corn borer *O. nubilalis* eggs deterred oviposition behavior in other moth species (EGVM and the noctuid *Sesamia nonagrioïdes*)[11], and give further evidence that inter-specific egg (or egg mass) avoidance exists in moths as it has been observed to be the case in butterflies[12]. We postulate that, besides specific molecules, simple and generally-occurring molecules (C_{14} to C_{18} saturated and unsaturated straight chain fatty acids and derivative esters) are recognized by different female species and could provoke egg spacing. Such molecules could also be used by species from other trophic levels (parasitoid insects or predators) to locate eggs on a plant. From our results, the use of the term 'pheromone' to refer to such generally occurring molecules appears to be questionable. Avoidance of previously exploited sites by laying females can reduce larval competition for food[3,10]. An egg-dispersing signal that acts inter-specifically is therefore probably advantageous for both producer and receiver, and would be considered as a synomone (sensu Nordlund and Lewis[13]). We therefore propose the term oviposition regulating synomone (ORS) for molecules which cue inter-specific egg spacing.

Our results suggest that population density may influence the behavioral response. This is important in the perspective of plant protection, since the population level would affect the response of females to such chemicals. Therefore, the development of choice-enhancing semiochemicals which regulate the oviposition behavior of herbivorous insects has obviously to be considered as part of a broader strategy of integrated population management. One could expect a rather good activity of such behavior-modifying chemicals at low population levels. Interestingly, first field applications conducted in the 'Côtes du Rhone' vineyards (France) on natural EGVM populations confirmed the biological activity of the chemicals used, and reduced the oviposition[14]. The application of our findings to the control of these four serious pests of vineyards and orchards is one of our objectives.

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