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Discrepancy in laboratory and field attraction of apple fruit moth *Argyresthia conjugella* to host plant volatiles

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Abstract – Apple fruit moth *Argyresthia conjugella* is a specialist seed predator of rowan *Sorbus aucuparia*. Large-scale synchronous fluctuation of seed production in rowan, named masting, drives apple fruit moth to seek alternative host plants such as apple, during years when rowan berries are not available for oviposition. The role of plant volatile compounds in attraction of gravid apple fruit moth females has been studied in a laboratory wind tunnel. Volatiles from rowan branches with green berries stimulate female moths to fly upwind and to land at the odour source. In contrast, females are not attracted to rowan branches without green berries, and they are not attracted to apple, showing that the chemical stimulus from rowan berries is required for attraction. Attraction to synthetic compounds identified from rowan, anethole and 2-phenyl ethanol, confirms the role of plant volatiles in host finding. These two compounds show, however, a discrepant behavioural effect in wind tunnel and field tests. Field traps baited with 2-phenyl ethanol capture female moths, but anethole does not produce significant captures. Wind tunnel tests produce opposite results: moths fly upwind towards the anethole lure, while 2-phenyl ethanol is not attractive at all. Wind tunnel attraction to 2-phenyl ethanol is achieved by adding odour from a rowan branch without berries, which is not attractive on its own. This finding demonstrates that interaction with the background odour contributes to the behavioural effect of plant volatile stimuli in the field.

Key Words – Host plant attraction, volatile organic compounds, background odour, anethole, 2-phenyl ethanol, rowan

Introduction

The principal mode of insect-plant communication is chemical. Deciphering the volatile signatures which guide insects to food sources and oviposition sites is a current urgent research challenge (Pichersky & Gershenzon, 2002; Bruce *et al*., 2005; Owen & Penuelas, 2005). These signals are sufficiently precise to let insects distinguish between host and non-host plants, and to choose plants in a suitable phenological or physiological state. Plant signals comprise substantial variation, on the other hand, since volatile emissions change continuously through phenological development, and in response to environmental and biotic challenges. And the message of any individual plant is blurred and diffused as it blends into the background odour released from surrounding vegetation.

Apple fruit moth *Argyresthia conjugella* (Lepidoptera, Argyresthiidae) is particularly suitable for studying the odour space that encodes recognition and attraction to different plant hosts. Apple fruit moth is, despite its common name, a specialist seed predator of rowan *Sorbus aucuparia*. Seed production in rowan shows large-scale yearly fluctuations, named masting, a reproductive strategy in shrubs and trees to minimize seed loss (Silvertown, 1980). Apple fruit moth females lay eggs on apple *Malus domestica* only during rowan intermasting years, when rowan berries are not available. Apple is, however, not suitable for larval development (Ahlberg, 1927; Kobro *et al*., 2003).

Co-occurrence of volatile compounds in rowan and apple, which are both rosaceous plants, may account for fatal attraction of *A*. *conjugella* females to apple for oviposition. A blend of 2-phenyl ethanol and anethole has been identified as an attractant for apple fruit moth, according to comparative chemical analysis and antennography of rowan and apple headspace. Traps baited with 2-phenyl ethanol and anethole captured a large number of females, but these field trapping tests do not answer the question whether the females were attracted over a distance, or whether they merely arrived from branches in close proximity to the traps (Bengtsson *et al*., 2006).

Wind tunnel bioassays have played an important role in the identification of sex pheromones, and are an essential tool also for the investigation of kairomones since they enable direct observation of the upwind attraction response under controlled stimulus and environmental conditions (Rojas, 1999; Pettersson *et al*., 2001; Tasin *et al*., 2006, 2007). However, unlike with sex pheromones, attraction to plant compounds in the laboratory does not always translate into attraction in the field and vice versa (Coracini *et al*. 2004; Mumm & Hilker, 2005; Yang *et al*., 2005). We here show the results of a first wind tunnel study of apple fruit moth *A. conjugella* attraction to their preferred host plant rowan and the substitute host apple. Attraction to single synthetic volatiles anethole and 2-phenyl ethanol is reversed in the laboratory and in the field. Stimulus interaction with background volatiles is proposed as an explanation for this discrepant behavioural effect.

Materials and methods

Insects

Rowan berries infested with last-instar larvae of apple fruit moth *A. conjugella* were collected in several localities in Southern Norway. Apple fruit moth *A. conjugella* is a specialized seed predator of rowan, undergoing obligatory diapause, and cannot be reared in the laboratory. Diapausing pupae were overwintered outdoors. In spring, pupae were kept at 4°C until they were transferred to Plexiglass cages for eclosion under a LD 18:6 h photocycle, 20-24°C and 55-70% rH. Newly eclosed insects were collected daily, males and females were kept in the same cages for mating. Insects were available for experimentation during 10 weeks.

Wind Tunnel Tests

The wind tunnel (Witzgall *et al*., 2001) has a flight section of 63 x 90 x 200 cm and was lit diffusely from above and from one side at 6 lux. Wind speed was 30 cm/s, and the temperature ranged from 19 to 22°C. Tests with plant material were done during seasonal flight period of *A. conjugella*. Newly cut branches with and without clusters of small green rowan berries and branches with green apples (cv. Aroma, Ø up to 5 cm) at the developmental stage suitable for apple fruit moth oviposition, were enclosed in 2-l glass jars. The apple cv. Aroma is most susceptible for attack by apple fruit moth (Bengtsson *et al*., 2006). Clean air from a tank passed over the plant material and left the jar at 30 cm/s, through a glass tube (4 mm ID x 20 mm). The glass jar was hidden behind a perforated metal grid (pore size 5 mm), and the outlet of the jar was fit into one enlarged pore of this metal grid, 30 cm from the ground, in the centre of the wind tunnel. The tip of the glass tube, protruding *c*. 2 cm into the tunnel, was covered by a glass cylinder (12.5 x 10 cm), which was mounted to the perforated metal grid, and which was covered with a metal mesh (2 x 2 mm mesh size). The rubber septa formulated with plant compounds (see below) were suspended in the centre of this cylinder. The odour source did thus not provide a visual cue for upwind orientation.

Synthetic plant compounds in hexane solution were formulated at 1 mg on red rubber septa (VWR International, Stockholm, Sweden). Treatments included 2-phenyl ethanol, anethole (93.8% and 96.2% chemical purity by GC, respectively; Shin-Etsu Chemical Co., Tokyo), and a 1:1-blend of both compounds. Synthetic pheromone (*Z*)-11-hexadecenyl acetate (*Z*11-16Ac) (Jaastad *et al*., 2002) was formulated on red rubber septa at 100 µg (99.7% isomeric purity, Pherobank, Wageningen, The Netherlands). A rubber septum impregnated with 100 µl hexane served as control treatment.

Wind tunnel tests were undertaken 3 to 6 h into the scotophase, which corresponds to the peak female and male activity period (Jaastad *et al*., 2005). Three- to four-day-old male and female moths were put singly into labeled glass tubes (2.5 x 15 cm) stoppered with gauze on both ends, 10 min before tests. Individual insects were introduced into the tunnel by positioning the glass tube onto a holder, 180 cm downwind from the source and 30 cm from the ground. They were given 3 min to respond and the following types of behaviour were recorded: take-off, flying upwind over 40 cm towards the source, and source contact after 180 cm of upwind-oriented flight in the centre of the wind tunnel. The time before take-off was also recorded. Insects were tested in batches of up to 25, the last moth was tested at the earliest 72 min after the first. After the wind tunnel session, all insects were sexed. Each odour source was tested with at least 40 females, on 3 to 6 different days, according to availability of insects eclosing from diapause. Six sources were also tested with least 40 males. Two treatments, in random order, were tested each day. Insects were used only once.

Field trapping tests

Synthetic 2-phenyl ethanol and anethole diluted in hexane were formulated on red rubber septa (VWR International). Treatments were 2-phenyl ethanol, anethole, and a 1:1 blend of both compounds. Treatments were tested in two concentrations 100 µg and 10 mg, adding to 200 µg and 20 mg in the blend, respectively. Tetra traps were hung at *c*. 2 m on rowan branches in forests (*n* = 10). Traps within one block were *c*. 5 m apart, and they were placed at random. All treatments were replicated once in each location (randomized complete block). Distance between blocks was at least 50 m. Traps were checked regularly during 2 weeks. The development of the flight period was followed according to the day-degree model for apple fruit moth (Kobro, 1988).

Statistical analysis

The number of moths recorded for each behavioural step in the wind tunnel was subjected to a 2x2 Fisher’s exact test. The results are presented as percentages to simplify comparison between treatments. In the field experiment, the number of female moths captured was subjected to an analysis of deviance for poisson-distributed data. Significance level of a post-hoc Tukey test was set to 0.05.

Results

Wind tunnel tests

In the wind tunnel, 38% of the test females flew upwind over 40 cm and 19% reached the source outlet of air, which had passed through a glass jar containing a freshly cut rowan branch with berries. The number of females landing was significantly different from blank (*P* = 0.0028). Few females started to fly towards air passing over a rowan branch without berries or an apple branch, the number of females responding was not different from blank (Fig. 1).

Tests with synthetic compounds showed that anethole stimulated females to fly upwind and 15% of the test females landed at the source (significantly different from blank; P = 0.0119). In contrast, 2-phenyl ethanol did not attract any females to the source (Fig. 1).

The combination of odour from a rowan branch without berries, and synthetic 2-phenyl ethanol, released as a blend from the same glass jar, produced a synergistic effect on female attraction and landing at source (P = 0.0196). In comparison, landings at a blend of synthetic anethole plus rowan leaf odour (P = 0.116), or to a blend of anethole plus 2-phenyl ethanol were not different from blank (P = 0.2412) (Fig. 1).

Wind tunnel sessions on one day comprised batches of up to 25 females, which were tested within up to 120 min (3 min response time plus handling). There was no correlation between mean take off time and time before the moths were introduced into the tunnel (r = 0.06; P = 0.12).

Male moths were tested with six sources. Most males responded to the blend of anethole and 2-phenyl ethanol, but the but the number of males landing was not different from blank (F = xz, P = 0.2429). For comparison, 65% of males (n = 52) landed at a source of sex pheromone containing 100 µg (*Z*)-11-hexadecenyl acetate.

Field trapping tests

Field traps in rowan trees, baited with 2-phenyl ethanol or a 1:1 blend of 2-phenyl ethanol and anethole captured significantly more females than blank traps, which remained empty. Trap capture with anethole was not significant (Fig. 1). The poisson model showed highly significant variation between treatments (F = 47.2, df = 5, P < 0.001). Trap captures with 2-phenyl ethanol and anethole, at a 100-fold lower dose, were not significantly different from blank traps (data not shown).

The comparison of field and laboratory attraction of apple fruit moth to synthetic compounds 2-phenyl ethanol and anethole shows opposite results. As single compound, 2-phenyl ethanol was attractive in the field, while anethole was attractive in the wind tunnel. The combination of 2-phenyl ethanol and odour from a rowan branch without berries, both of which did not produce significant attraction by themselves, attracted a significant number of females in the wind tunnel (Fig. 1).

Discussion

Rowan volatiles attract mated apple fruit moth females

Attraction of apple fruit moth *A. conjugella* females to air passing over rowan branches with berries demonstrates the importance of odour cues for host finding in apple fruit moth. The number of females flying upwind over 180 cm to an odour source not providing visual cues for orientation, during a test period of only 3 min, is significant. A rate of 19% landings in response to odour from rowan branches with berries compares with the host plant attraction obtained with other lepidopteran species in wind tunnels (Landolt, 1989; Cossé *et al*., 1994; Phelan *et al*., 1991; Rojas, 1999; Olsson *et al*., 2005; Tasin *et al*., 2005, 2006, 2007).

Lack of attraction to apple branches, on the other hand, correlates well with the observation that apple is only an ersatz host of apple fruit moth (Ahlberg, 1927; Kobro *et al*., 2003). The wind tunnel test shows that females are not attracted to apple over a distance. Gravid females may become more responsive to apple volatiles with age, when deprived of suitable oviposition substrate during intermasting years (Mayhew, 1997).

Background odour effects female attraction to rowan volatiles

Attraction to synthetic rowan volatiles, 2-phenyl ethanol and anethole (Bengtsson *et al*., 2006), further corroborates that plant volatiles mediate attraction of mated apple fruit moth females. Interestingly, tests with these two compounds show opposite results in the laboratory wind tunnel and in the field.

Traps baited with 2-phenyl ethanol placed in rowan branches captured apple fruit moths, while 2-phenyl ethanol did not elicit a behavioural response in the wind tunnel. Contrasting field and laboratory results are most likely due to interaction of the test stimulus with the background odour: a combination of 2-phenyl ethanol and volatiles emanating from a rowan branch had a clear synergistic effect in charcoal-filtered wind tunnel air. On the other hand, lack of field attraction to anethole indicates that more active or more abundant rowan volatiles mask or interfere with anethole. This is corroborated by wind tunnel tests, showing that rowan volatiles did not enhance attraction to anethole.

Background odour has been shown to affect attraction of several other insects to plant volatiles, although the difference is not as striking as shown here with apple fruit moth. Pear ester is used to monitor codling moth *Cydia pomonella* populations in orchards, but does not attract codling moths in the wind tunnel (Light *et al*., 2001; Knight & Light, 2005a,b; Yang *et al*., 2005). Likewise, (*E*)-β-farnesene attracts codling moth males in apple orchards, but not in charcoal-filtered wind tunnel air. The main volatile compound of apple headspace, (*E*,*E*)-α-farnesene, has a synergist effect on attraction to (*E*)-β-farnesene in the wind tunnel, but not in the field (Coracini *et al*., 2004). An olfactometer bioassay of the parasitoid *Chrysonotomyia ruforum* in response to Scots pine with host eggs of the sawfly *Diprion pini* provides another example (Mumm & Hilker, 2005): (*E*)-β-farnesene was attractive only when embedded in pine odour.

Discrepancies in insect behaviour in the field and laboratory may result from visual and olfactory stimulus interaction (Schoonhoven *et al*., 2005). Visual cues, however, cannot explain the mismatch of apple fruit moth laboratory and field attraction to anethole and 2-phenyl ethanol. Furthermore, the amount of compound used in field and laboratory cannot account for these differences. Field tests with different amounts on rubber septa show that 10 mg of 2-phenyl ethanol on rubber septa is suitable for field attraction. A tenfold lower dose was used in the wind tunnel, which produces attraction similar to a rowan branch.

A contrasting behavioural effect of single plant volatiles in the laboratoy and field re-emphasizes that it is crucial to study plant-insect communication in ecologically realistic settings. Plant volatiles are, for one, not perceived as single compounds, since plants release hundreds of compounds. The use of point sources in wind tunnel and field trapping tests may produce another artefact. It is conceivable that rowan trees produce large and diffuse odour clouds of varying composition: leaves and fruit clusters release different volatile blends (Bengtsson *et al*., 2006), and these plumes would, through turbulences created by leaves and branches, intermingle and fuse with plumes from adjacent plant organs and from surrounding plants. The temporal structure of sex pheromone plumes is a principal factor for male moth upwind orientation to pheromone-releasing females. Males respond to fluctuating and intermittent plumes, while continuous, uniform pheromone clouds are not sufficient to elicit orientation flights (Kennedy *et al*., 1981; Baker *et al*., 1985; Murlis *et al*., 1992).

Towards the identification of apple fruit moth kairomone

Anethole, which is a major component of anise and fennel aroma, has been reported from only four plant genera, including apple (Knudsen *et al*., 1993, 2006; Bengtsson *et al*., 2001). It has been shown to attract scarabid beetles (Tóth *et al*., 2004) and bibionid flies (Cherry, 1998). In comparison, 2-phenyl ethanol is widespread throughout the plant kingdom. It occurs in 34 of 174 genera listed by Knudsen *et al*. (1993, 2006), and is frequently found in insect-pollinated plants (Andersson *et al*., 2002). Accordingly, 2-phenyl ethanol is known to attract a wide range of species from different taxa, including Lepidoptera (Haynes *et al*., 1991; Honda *et al*., 1998; Imai *et al*., 1998; Zilkowski *et al*., 1999).

A synergistic effect of 2-phenyl ethanol and rowan leaf volatiles demonstrates that rowan headspace contains yet unidentified behaviourally active compounds. The role of anethole as host signal for apple fruit moth is still unclear, since it does not show a synergistic interaction with leaf volatiles or 2-phenyl ethanol.

Females become attracted to rowan branches with fruit clusters both in the laboratory and in natural habitats, suggesting that a more complete signal facilitates perception against a noisy background. Such a more complete kairomone blend is expected to produce an even stronger behavioural effect in an apple orchard, where the females migrate in search of oviposition sites during rowan intermasting years. The identification of further attractant volatiles and the behavioural mechanisms of host orientation in apple fruit moth is subject of an ongoing study.

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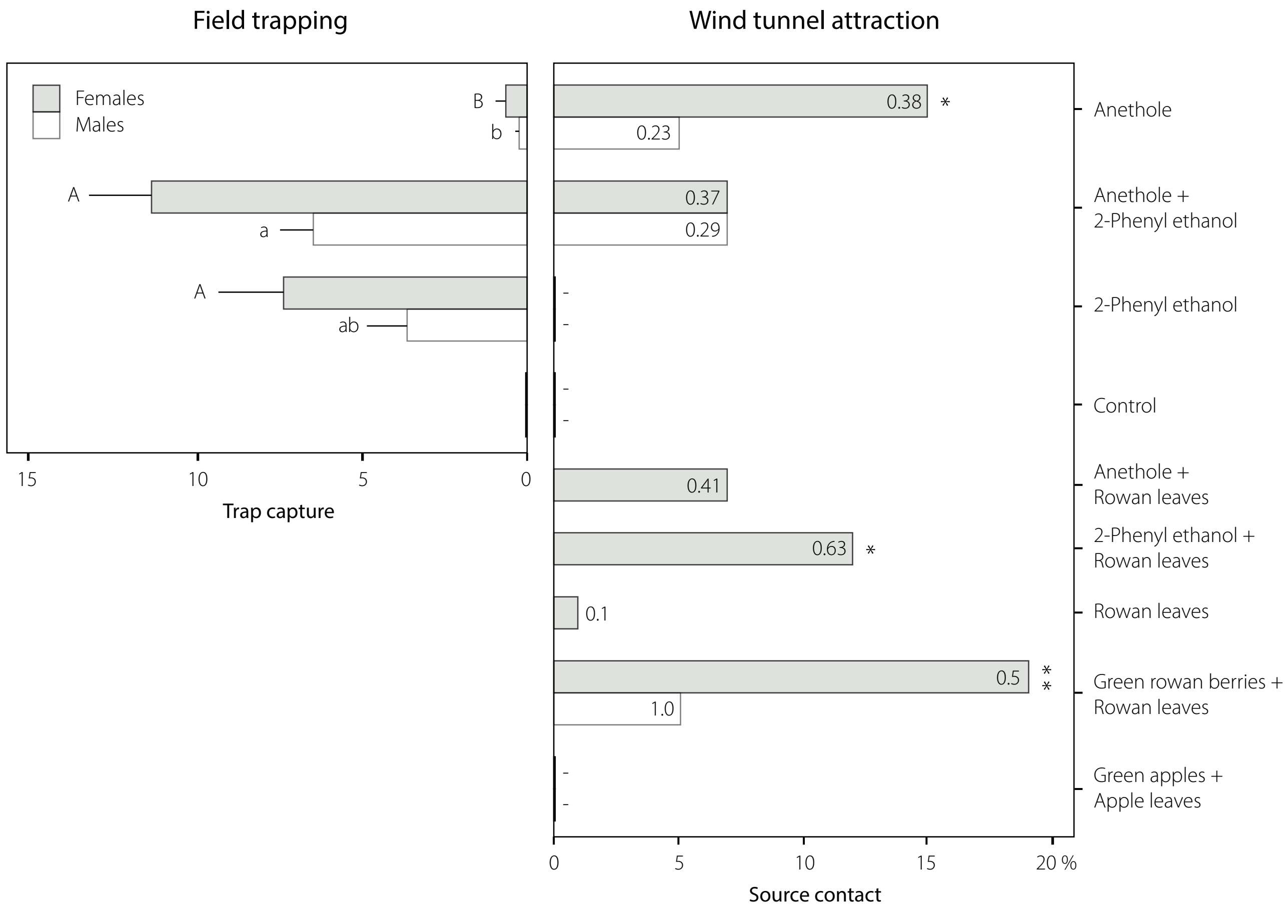
Legend

Fig. 1. Field and wind tunnel attraction of apple fruit moth *Argyresthia conjugella* to synthetic rowan volatiles anethole and 2-phenyl ethanol, and natural volatiles from rowan and apple twigs. Anethole and 2-phenyl ethanol were formulated on rubber septa, at 1 mg for wind tunnel, and at 10 mg for field tests. Field: Mean captures of female and male moths in traps hung to rowan branches (n = 10). Treatments with different letters are significantly different (Tukey test; $P < 0.05$). Wind tunnel: A rowan branch (*Sorbus aucuparia*) with or without berries, a branch with green apples (*Malus domestica* cv. Aroma), and rubber septa containing 1 mg of synthetic compound, were held in a 2-l glass jar. An airstream passed through the glass jar and through a glass tube outlet into the wind tunnel. Individual moths (n = 40 to 80) were scored for upwind orientation flight over 40 cm and source contact, after 180 cm upwind flight. Numbers in bars show the ratio between source contacts and upwind orientation flights over at least 40 cm. Three treatments were tested with females only. For each bioassay, asterisks show significant differences between each treatment and control (Fisher's exact test; * $P < 0.05$; ** $P < 0.01$).


Field trapping
Wind tunnel attraction
Females
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Trap capture
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0.37
0.29
-
-
-
-
0.41
0.63 *
0.1
0.5 * *
1.0
-
-
0
5
10
15
20 %
Source contact
Anethole
Anethole +
2-Phenyl ethanol
2-Phenyl ethanol
Control
Anethole +
Rowan leaves
2-Phenyl ethanol +
Rowan leaves
Rowan leaves
Green rowan berries +
Rowan leaves
Green apples +
Apple leaves