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Oriented responses of grapevine moth larvae *Lobesia botrana* to volatiles from host plants and an artificial diet on a locomotion compensator

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A B S T R A C T

Larvae of the grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae) are a major pest of vine, *Vitis vinifera*. As larvae have limited energy reserves and are in danger of desiccation and predation an efficient response to plant volatiles that would guide them to food and shelter could be expected. The responses of starved 2nd or 3rd instar larvae to volatile emissions from their artificial diet and to single host plant volatiles were recorded on a locomotion compensator. Test products were added to an air stream passing over the 30 cm diameter servosphere. The larvae showed non-directed walks of low rectitude in the air stream alone but changed to goal-oriented upwind displacement characterised by relatively straight tracks when the odour of the artificial diet and vapours of methyl salicylate, 1-hexanol, (*Z*)-3-hexen-1-ol, terpinen-4-ol, 1-octen-3-ol, (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*Z*)-3-hexenyl acetate were added to the air stream. This chemoanemotactic targeted displacement illustrates appetence for certain volatile cues from food by starved *Lobesia* larvae. Analysis of the larval behaviour indicates dose dependent responses to some of the host plant volatiles tested with a response to methyl salicylate already visible at 1 ng, the lowest source dose tested. These behavioural responses show that *Lobesia* larvae can efficiently locate mixtures of volatile products released by food sources as well as single volatile constituents of their host plants. Such goal-oriented responses with shorter travel time and reduced energy loss are probably of importance for larval survival as it decreases the time they are exposed to biotic and abiotic hazards.

Keywords: Grapevine moth, Servosphere, Olfaction, Chemoanemotaxis, Behaviour

1. Introduction

The European grapevine moth *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae) is one of the major pests in European vineyards. Damage to the vine (*Vitis vinifera*) is caused by larvae feeding on shoots and all phenological stages of grapes. Volatile compounds from the shoots, grape buds or fruits might reveal the location of potential food to larvae. However, *L. botrana* is found on more than 30 plant species from different plant families (Maher, 2002; Maher and Thiéry, 2006). Being polyphagous, *Lobesia* larvae would be exposed to a wider range of volatile plant compounds than larvae of specialised species. Nevertheless, sensitivity to and efficient localisation of food cues seems to be essential for the grapevine moth larvae that show a limited range of dispersal (Torres-Vila et al., 1997) with limited energy reserves. Oriented responses by the larvae to food would result in a shorter travel time and reduced energy loss, while shorter foraging time would result in a decreased risk of desiccation and predation.

Attraction to odours of different plants (*Tanacetum vulgare*, Gabel et al., 1992; *Rosmarinus officinalis*, Katerinopoulos et al., 2005; *Daphne gnidium*, Maher and Thiéry, 2006; *Vitits vinifera*, Tasin et al., 2005; Masante-Roca et al., 2007) has already been shown for adult female *L. botrana*. In wind tunnel studies, mated females showed upwind flight to different parts of the grapevine (Tasin et al., 2005; Masante-Roca et al., 2007), to an extract of the headspace volatiles of grapes (Tasin et al., 2006a,b) as well as to a synthetic blend of ten compounds present in grape headspace vapour that elicited significant antennogram responses from *L. botrana* (Tasin et al., 2005, 2006a). Similar upwind responses could be shown to a mixture of (*E*)-β-caryophyllene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*)-β-farnesene presented at the ratio found in grape headspace vapour (Tasin et al., 2006b, 2007).

In comparison to what has been achieved with adults, less attention has been paid to the sensory ecology of *L. botrana* larvae. Mondy et al. (1998) showed that the larvae were able to discriminate between different foods by olfaction: in a choice arena and a Y-tube olfactometer larvae showed a preference for

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the odour of artificial rearing media and grape berries that were infected with the fungus *Botrytis cinerea*. Attraction to volatiles from food resources has already been shown for larvae of other tortricid moths. Orientation to volatiles emitted by foliage of host trees (*Picea* sp., *Abies* sp.) was shown for larvae of the eastern spruce budworm *Choristoneura fumiferana* (Ascoli and Albert, 1985), and attraction to host plant volatiles was demonstrated for codling moth larvae, *Cydia pomonella* (Sutherland and Hutchins, 1972; Knight and Light, 2001). Codling moth larvae respond to the sesquiterpene α-farnesene that occurs in the waxy coat of several apple varieties (Sutherland and Hutchins, 1972) and, more recently, these larvae were shown to be attracted by ethyl and methyl esters of (*E*,*Z*)-2,4-decadienoic acid, emitted from pears (*Pyrus communis*) at threshold levels of 1 ng and 1 μg, respectively (Knight and Light, 2001). Neonate larvae of *Spodoptera frugiperda* were attracted to DMNT which was induced in cowpea (*Vigna unguiculata*) by conspecific herbivory, and showed even higher attraction when DMNT was supplemented with plant material (Carroll et al., 2008). In all of these studies on attraction to host plant odours by lepidopterous larvae the responses were mainly assessed by analysing the numbers of responding animals. In this study we have used a servosphere to record and analyse the tracks made by individual *L. botrana* larvae responding to artificial food odour and to host plant volatiles.

Odour-driven behaviour is an expression of the functional interplay between peripheral and central levels of the olfactory system. Olfactory systems of insects are of general interest because of the profound insights gained on their neuroanatomy and function and similarities with mammals (Vosshall and Stocker, 2007). However, recent studies on dipteran juvenile stages have pointed to a deficit of knowledge on larval-olfaction (Ramaekers et al., 2005; Xia et al., 2008). Parts of the larval olfactory system are integrated into the adult system and both share a basic architecture (Jefferis et al., 2004; Gerber and Stocker, 2007). Nevertheless, in addition to odour receptors expressed in larvae and adults, larvae of *Drosophila melanogaster* (Kreher et al., 2005), *Aedes aegypti* (Bohbot et al., 2007) and *Anopheles gambiae* (Xia et al., 2008) apparently express specific odour receptors that are significant for olfactory-driven behaviours.

Here we describe the oriented behavioural responses of juvenile *L. botrana* to food odours. The findings serve to complement previous studies on adult grapevine moth responses to host plant odours and thus contribute to our understanding of this insect's sensory ecology.

2. Methods and material

2.1. Preparation of L. botrana larvae

L. botrana (Denis and Schiffermüller) was reared on an artificial diet (see below) in 1.50 l polystyrene boxes (Semadeni, Switzerland) in a climate chamber (16:8 h L:D cycle, 85:65% RH and 25:18 °C temperature regime). 10–15-day-old larvae (second or third instar) were removed from the boxes at the end of the scotophase about 20 h before testing and kept individually starving in Eppendorf tubes plugged with a moistened piece of cotton. In the middle of the following scotophase the Eppendorf tubes with the larvae were transferred to a darkened laboratory (about 50% RH, 23 °C) and tested during the last 4 h of the scotophase. Under dim light a larva was gently transferred from the Eppendorf tube into the climatised main air stream (charcoal filtered air at 75% RH and 0.1 m/s; Taneja and Guerin, 1995) flowing over the apex of a locomotion compensator (see below). Each larva was tested only once.

2.2. Recording larval movements on a servosphere

A locomotion compensator (TrackSphere LC-300, SYNTECH, Hilversum, Netherlands) was used to record the movements of *L. botrana* larvae. The locomotion compensator, or servosphere (based on the system developed by Kramer, 1976) 0.3 m diameter sits on an air-cushioned ball of 6 cm diameter. Two servomotors placed orthogonally at the equator rotate the sphere to compensate for the movements of the larva. Larval movements at the apex of the sphere are processed by an infrared light-sensitive CCD camera positioned overhead and connected to a controller (TrackSphere Controller TRC-01, SYNTECH) that drives the servomotors. By compensating for the movements of the larva in this way the system maintains the insect at the apex of the sphere. Experiments were made under IR light transmitted by a ring of 12 diodes mounted around the camera. A grey sphere permitted a strong contrast of the larvae against background for the CCD device. Incremental larval displacements are supplied to a computer (TrackSphere Software, SYNTECH) by two pulse-generators (resolution of 0.1 mm, recording at 0.1 s intervals) mounted on the sphere's equator opposite the motors. Coordinates of tracks were calculated and analysed using the gLocTrack software (Institute of Biology, University of Neuchâtel, Switzerland; Otálora-Luna et al., 2004). Independent to this, an infrared-sensitive video camera (Panasonic WV-BP310) was used to observe and record larval behaviour on video tape.

2.3. Artificial food odour and host plant compounds

The artificial diet (based on the diet described by Rauscher et al., 1984) used as an odour stimulus as well as for the rearing of *L. botrana* was comprised of 800 g water, 25 g agar (Labolife, Pully, Switzerland), 30 g sucrose, 90 g wheat germ, 18 g brewer's yeast (Migros, Switzerland), 2.5 g cold-pressed sunflower oil (Coop, Switzerland), 25 g powder of alfalfa (*Medicaginis herba*, Dixa AG, St. Gallen, Switzerland), 40 g casein (Reactolab SA, Servion, Switzerland), 7.5 g Vanderzant vitamin mix, 12.5 g salt mixture W (MP Biomedicals, Solon, USA), 1.25 cholesterine, 2 g sorbic acid, 10 g ascorbic acid, 1.25 g chlortetracyclin hydrochloride, 2.5 g propionic acid, and 1 g linolenic acid (Fluka, Switzerland). About 20 g of this diet were placed in a 500-ml gas-wash bottle and its odour was tested for larval attraction as described below.

Ten host plant compounds were tested individually or in mixtures for their ability to induce oriented responses from *Lobesia* larvae. These compounds were chosen based on their occurrence in different phenological stages of *V. vinifera*, i.e. 1-hexanol (Shimizu and Watanabe, 1981), (*Z*)-3-hexen-1-ol, 1-octen-3-ol, (*Z*)-3-hexenyl acetate, methyl salicylate, (*E*)-β-caryophyllene, (*E*)-β-farnesene, DMNT, and nonanal (Tasin et al., 2005, 2006a, 2007). Terpinen-4-ol is found in grape juice (Schneider et al., 2001) and is also present in steam distillates of *T. vulgare* flowers that attract female *L. botrana* (Gabel et al., 1992). All compounds with the exception of 1-hexanol (not previously tested) are known to elicit antennogram responses from female *L. botrana* (Gabel et al., 1992; Tasin et al., 2005, 2006a). Methyl salicylate (>99%), 1-hexanol (99%), (*Z*)-3-hexen-1-ol (>98%), terpinen-4-ol (99%), (*E*)-β-caryophyllene (99%, sum of enantiomers) and nonanal (>95%) were obtained from Fluka, Switzerland, 1-octen-3-ol (>97%) was obtained from Merck, Germany, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (92%) was from Givaudan, Switzerland, (*E*)-β-farnesene (90%) was from Bedoukian (USA), and (*Z*)-3-hexenyl acetate (98%) was from Sigma-Aldrich (Germany). The compounds were dissolved in dichlormethane (SupraSolv® grade for gas chromatography; Merck) and doses between 1 ng and 10 μg of each were applied in a volume of 10 μl on filter paper disks (7 cm diameter) for tests. After evaporation of the solvent the filter paper disk was

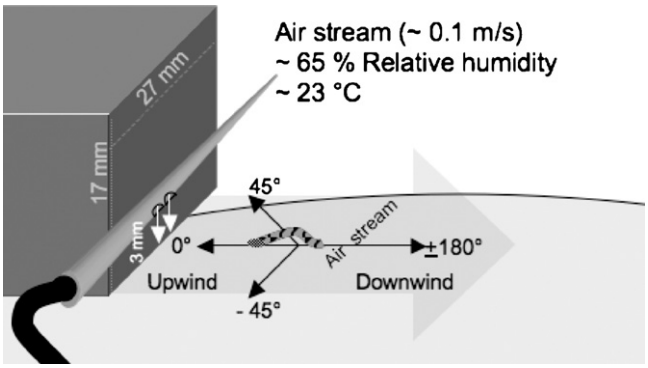

Fig. 1. The air stream containing the stimulus is injected downwards by a needle into a climatised air stream issuing from a rectangular tube of stainless steel. The larva exposed to a treatment at the apex of the sphere can respond with upwind displacement, defined as walking in a sector 45° either side of due upwind (0°) or by moving across or downwind.

placed in a 500-ml gas-wash bottle for testing as described below. Bottles were flushed with charcoal filtered air after each test and a new odour-impregnated filter paper was used for the next test. The same bottle was used for testing different doses of the same compound, starting with the lowest dose. Bottles (cleaned with acetone, Fluka-RBS detergent and demineralised water, dried at 140 °C) and filter papers were tested in control experiments with *Lobesia* larvae.

2.4. Stimulus delivery

Experiments started about 30 s after placing the larva on the servosphere. The responses of larvae were recorded in three successive 2-min periods: during pre-exposure (PreX) charcoal-filtered air passing through an empty 500-ml gas-wash bottle was added into the climatised main air stream; during the test period (*T*) charcoal-filtered air passing over the test stimulus (artificial diet or plant compounds applied on filter paper) placed in a gas-wash bottle was added to the main air stream; conditions during the post-exposure (PostX) period were identical to those of the PreX. These air flows of 35 ml/min were controlled by a software-operated stimulus delivery system (ST-05, SYNTECH). PreX, *T*, and PostX air streams were delivered through a stainless steel needle (2.0 mm × 80 mm, 2R2, Unimed, Lausanne, Switzerland), which was modified by blocking the tip and drilling two holes (0.85 mm diameter, 0.5 mm distant) on one side of the needle. This needle was placed horizontally 3 mm above the sphere surface with the two lateral holes pointing downwards and at right angles to the climatised main air stream that swept over the sphere (Fig. 1). The distance from the stainless steel needle to the sphere apex where the larva walked was about 9 mm. At this position, humidity and wind speed of the main air stream during PreX, *T* or PostX was 65% RH and 0.1 m/s.

2.5. Definitions, behavioural criteria and their quantification

The following criteria (for further explanation of definitions and criteria see below) were quantified from the larval tracks recorded by the servosphere: target vector, proportion of displacement in the upwind direction and the mean angular deviation of larval tracks from the upwind direction. These criteria are useful measures of oriented behavioural responses and were quantified for all larvae and stimuli tested. In addition, the following behaviours were quantified from the tracks for a more detailed investigation of the larval responses to the odour of the artificial diet: larval speed, the number of stops and their duration, time walking in the upwind direction, the distance covered by a larva and the resulting distance from the start point, the rectitude of a track and the mean angular deviation *s* of movement vector angles.

Movement vectors: Summing of five successive 0.1 s displacements and subsequent application of a running mean on two successive 0.5 s intervals was chosen to determine a single movement vector of a larva. The average speed of 9 straight-walking caterpillars of 4–6 mm body-length was about 1.5 mm/s such that vectors of 0.5 s corresponded to a displacement of about 15% of the caterpillar's body-length. These movement vectors matched with the motion sequence of the crawling larvae recorded on video and 0.5 s intervals were therefore used to analyse the number and distribution of movement vectors (Fig. 2). However, the limitations of transmission of very small larval movements when working at a resolution of 0.1 mm generated some artificial orientation angles at 0.00°, 45.00°, 90.00°, 135.00° and 180.00° (omitted in Fig. 2). Summing of eight successive 0.1 s displacements and subsequent application of a running mean on five successive 0.8 s intervals was used to eliminate artificially generated angles and to analyse the mean vector and target vector values. The latter settings were also used for calculating the duration of walking upwind and the proportion of upwind displacement. Summing of recording intervals and application of a running mean improved the recording of real movements by eliminating noise in the track records originating from the length of the caterpillars and their movement patterns that caused them to occasionally move out of focus.

Stops: By comparing the video-recorded and counted numbers of stops a velocity of 0.3 mm/s was determined as the minimum speed the animal had to achieve to be considered walking; slower displacements were discarded.

Mean vector angle (mean deviation from 0°): The mean vector angle was calculated from the angles of the single movement

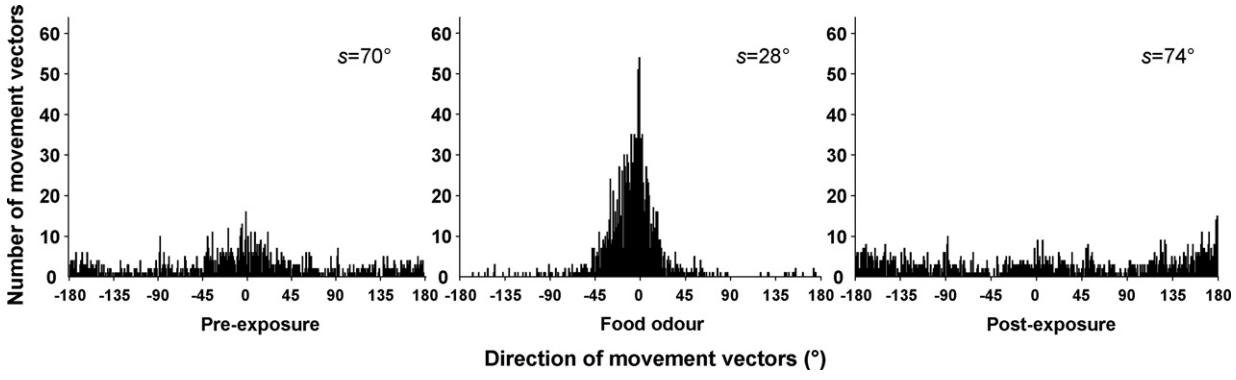

Fig. 2. Plots of movement vector angles described by 20 *L. botrana* larvae before, during and after exposure to food odour in an air stream on the servosphere (direction of airflow 0°). The mean angular deviation *s* is a measure of dispersion, equivalent to the standard deviation in linear statistics; Batschelet, 1981).

vectors made by a larva in relation to the stimulus source situated at 0° in the upwind direction.

Upwind displacement: A cone of 90° was defined as a sector in which the caterpillar met the applied odour plume in the upwind direction (0°). In what follows, walking in this sector is termed upwind displacement (Fig. 1). The range of this upwind cone was estimated from caterpillars showing left and right waggling with the head (as described below) at about 45° either side of 0°.

Resulting distance: Linear distance between the *xy* coordinates assigned to the position of the larva at the beginning and the end of an experimental period, equivalent to the beeline between start- and endpoint of a displacement.

Rectitude: Measure of straightness, calculated as the ratio of the resulting distance to the total distance walked during a PreX, *T*, or PostX period.

Target vector: The target vector is a measure of target-oriented behaviour. It combines straightness and directionality with respect to upwind of consecutive movement vectors, by multiplying *r* (mean vector length as a measure of the movement vectors' angular concentration; Batschelet, 1981) by the cosine of the mean vector angle. Whereas *r* indicates the degree of straightness, the cosine of the mean vector indicates the degree of orientation in the target direction. When both factors reach their theoretical maximal value for a straight walk upwind, the target vector achieves a maximal value of 1.

Most of the larvae started to move upon transferring them into the air stream on the servosphere, however, some of the larvae showed sluggish or no movement, so were not included for analyses. The behaviour to food odour was analysed for 20 larvae. For all other treatments the behaviour of 10 larvae was analysed (compounds at different concentrations or combinations of compounds). Altogether, the dataset presented here represents the analysis of 6-min walks by 400 larvae.

Calculations of movement vectors mean vector angles, rectitude, distance travelled, duration of upwind displacement and target vectors were performed by the gLocTrack software. Statistical analyses of values calculated for the PreX, *T* and PostX periods were performed using Wilcoxon's matched pairs test for datasets obtained from tests with the same stimulus at one concentration. Analyses of datasets obtained from tests with different stimuli or different concentrations of the same stimulus were performed using the Mann–Whitney *U*-test.

3. Results

3.1. Walking behaviour of Lobesia larvae in an air stream and their responses to the addition and removal of an artificial diet odour

3.1.1. Walking behaviour of larvae in an air stream (pre-exposure)

Of 23 larvae that were transferred into the air stream on the servosphere, three showed sluggish or no movement whereas 20 showed persistent walking behaviour and stayed motionless for less than 90 s of the 360 s test period. These 20 larvae walked on the servosphere at an average speed of 1.12 mm/s (±0.39) covering a mean distance of 71.19 mm (±48.11) within 2 min. Video recordings revealed that walking by *Lobesia* larvae is achieved by first setting forward the anal prolegs followed by pushing the abdominal pro- and thoracic-legs forward, thus generating a wave running over the body from back to front of the caterpillar. Walking was regularly interrupted by stops, numbering a mean of 36 (±10) in the 2-min period each with a mean duration of 1.68 s (±0.64). During most of these stops larvae lifted the head and thorax to wave in the air. Changes in course direction were regularly observed following this lifting behaviour by left- or right-directed touch down of the thorax. Changes in direction were also performed while walking where the larva regularly made left- and right "waggling" movements with the head held down. The average resulting distance between the start and endpoint of the unbroken sections of tracks in the air stream alone was 22.88 mm (±20.33). The tortuous character of these walks is reflected by a mean rectitude value of 0.36 (±0.22). No preference was evident for the upwind direction during the PreX period. The median target vector, a measure of the efficiency of upwind displacement, was 0.21 (minimum −0.53, maximum 0.67) and larvae spent less than one third (minimum 0.00, maximum 0.60) of their way oriented upwind (Tables 1 and 2). The median deviation of the tracks was 48.08° (minimum 2.05°, maximum 134.35°) from 0° (Table 3) and the mean angular deviation *s* of movement vector angles was 70° (Fig. 2).

3.1.2. Walking behaviour during delivery of artificial diet odour

The larvae travelled at a mean speed of 1.33 mm/s (±0.46) in the presence of food odour, making a mean number of 39.6 (±9.5) stops of 1.19 s (±0.35) duration during the 2 min exposure to the odour of the artificial diet. The mean displacement of 102.18 mm (±56.47) as well as the resulting distance of 79.65 mm (±46.63) were significantly higher in the presence of food odour than in the air stream alone ($p < 0.01$ and $p < 0.0001$, respectively). Rectitude reached a mean value of 0.77 (±0.16) for the twenty larvae tested, indicating that track straightness reached a significantly higher level during delivery of the food stimulus than during the PreX ($p < 0.0001$) or PostX periods ($p < 0.001$). Both head lifting and left and right waggling with the head held down while walking could be observed as in the initial PreX period. In particular, waggling was observed when larvae walked close to 45° either side of upwind, indicating perception of the stimulus air stream limits. This left-right sampling was followed by a larva entering or leaving the cone 45° either side of due upwind.

The median target vector was significantly higher at a value of 0.89 (minimum 0.73, maximum 0.99) during exposure to the food odour than in the PreX and PostX periods (Table 1). The preference for walking upwind during delivery of food odour in the air stream is also illustrated by the mean vector angles of tracks walked by *Lobesia* larvae (Fig. 3) and the low median deviation of these tracks of 9.64° (minimum 0.25°, maximum 19.80°) from 0° (Table 3). The courses of the recorded tracks also demonstrate a strong propensity by the larvae to walk in the direction of the food stimulus at 0° (a typical track is shown in Fig. 4). This preference for the direction of the food odour source is also visible in the frequency distribution plot of movement vector angles (Fig. 2) which is unimodal around 0° with a deviation *s* of 28°. There is a minor deviation of the movement vector angles from 0° due to a slight offset in the airflow direction, detected after this experiment was completed. Furthermore, orientation in the direction to the food odour is indicated by the increased time larvae spent walking in the cone 45° either side of due upwind (Fig. 5) and the proportion of displacement (median 0.64, minimum 0.29, maximum 0.90) made within this portion of the Cartesian plane, both significantly higher than in the PreX or PostX periods (Table 2).

3.1.3. Reactions of the larvae to removal of food odour (post-exposure)

Following removal of food odour from the air stream the larvae undertook a different pattern of displacement. Larvae on average covered a distance of 82.39 mm (±62.20) at a speed of 1.17 mm/s (±0.43) and made 33.7 stops (±8.7) lasting a mean of 1.70 s (±0.74). Head-lifting behaviour and waggling could regularly be seen as in the preceding test periods. As observed during the PreX period, larval tracks were much less directional in the absence of food odour (Fig. 4), characterised by a low rectitude (on average 0.27 ±0.15) such that the average resulting distance in the PostX periods was 20.39 mm (±17.16), lower than in the PreX period.

The diverging mean vector angles (Fig. 3), the wide distribution of movement vector angles (*s* of 74°, Fig. 2) and the significantly lower

Table 1
Target vectors calculated for tracks of *L. botrana* larvae walking on a locomotion compensator in an air stream alone (Pre-exposure), in response to the odour of an artificial diet, single host plant compounds and their mixtures added to the air stream (Treatment), and after removal of the treatments (Post-exposure).

Compounds	Source dose (ng)	Median of target vectors (25th/75th percentiles)		
		Pre-exposure	Treatment	Post-exposure
Food odour	$\sim 20000 \times 10^6$	0.21* (0.08/0.37)	0.89 (0.81/0.93)	−0.2*,† (−0.38/−0.02)
Methyl salicylate	1	−0.1* (−0.17/0.14)	0.5** (0.01/0.70)	−0.06* (−0.26/0.09)
	100	−0.11* (−0.32/0.25)	0.52** (0.43/0.56)	−0.33* (−0.45/−0.22)
	10000	−0.1* (−0.49/0.12)	0.68** (0.46/0.81)	−0.22* (−0.38/0.01)
1-Hexanol	1	0.01 (−0.11/0.45)	−0.1 (−0.21/0.10)	−0.04 (−0.63/0.05)
	100	−0.08* (−0.41/0.30)	0.68** (0.62/0.82)	−0.01* (−0.40/0.48)
	10000	0.06* (−0.20/0.44)	0.75** (0.46/0.83)	0.16* (−0.07/0.51)
(*Z*)-3-Hexen-1-ol	1	0.12 (−0.20/0.49)	0.04 (−0.27/0.07)	−0.13 (−0.50/0.06)
	100	0.3* (0.16/0.38)	−0.06** (−0.26/0.02)	−0.23 (−0.45/−0.02)
	10000	−0.01* (−0.28/0.26)	0.44** (0.30/0.68)	0.09* (−0.39/0.26)
1-Octen-3-ol	1	−0.05* (−0.21/0.17)	0.43** (0.23/0.75)	0.31 (−0.08/0.77)
	100	−0.05 (−0.19/0.22)	0.38 (−0.14/0.51)	0.00 (−0.10/0.12)
	10000	0.06 (−0.18/0.43)	0.38 (0.10/0.63)	0.07 (−0.42/0.52)
Terpinen-4-ol	1	0.23 (−0.16/0.29)	0.23 (−0.40/0.56)	−0.54* (−0.69/−0.04)
	100	−0.02 (−0.10/0.26)	0.36 (0.19/0.51)	−0.29*,† (−0.51/−0.03)
	10000	0.03* (−0.30/0.24)	0.46** (0.31/0.67)	0.22* (−0.20/0.57)
DMNT	1	0.24 (−0.08/0.71)	0.34 (0.01/0.64)	0.01* (−0.08/0.17)
	100	0.22 (0.03/0.32)	0.46 (0.16/0.61)	−0.11*,† (−0.35/−0.06)
	10000	0.04* (−0.26/0.26)	0.47** (0.43/0.72)	−0.14* (−0.30/0.11)
(*E*)-β-Caryophyllene	1	0.35 (0.11/0.47)	0.19 (−0.24/0.48)	−0.29 (−0.48/0.34)
	100	−0.29 (−0.33/0.09)	−0.04 (−0.14/0.24)	−0.27* (−0.34/−0.20)
	10000	−0.22 (−0.59/0.08)	0.06 (−0.08/0.25)	0.08 (−0.13/0.46)
(*E*)-β-Farnesene	1	0.06 (0.04/0.23)	0.12 (−0.09/0.25)	−0.13 (−0.37/0.03)
	100	−0.07 (−0.33/0.17)	−0.19 (−0.45/−0.04)	−0.07 (−0.27/0.36)
	10000	0.17 (−0.14/0.41)	0.57 (−0.24/0.73)	−0.07* (−0.53/0.24)
(*Z*)-3-Hexenyl acetate	1	−0.05 (−0.30/0.11)	−0.05 (−0.19/0.03)	−0.21 (−0.43/0.06)
	100	0.01* (−0.30/0.28)	0.43** (0.27/0.55)	0.02* (−0.20/0.27)
	10000	−0.15* (−0.30/−0.01)	0.28** (0.07/0.49)	0.00 (−0.29/0.13)
Nonanal	1	0.33 (0.04/0.48)	−0.04 (−0.15/0.38)	0.17 (−0.17/0.44)
	100	0.22 (−0.02/0.38)	−0.01 (−0.36/0.27)	−0.05 (−0.49/0.05)
	10000	0.17 (−0.14/0.43)	0.32 (−0.11/0.66)	0.14 (−0.24/0.61)
1-Hexanol + DMNT	100 + 100	−0.24 (−0.32/0.10)	−0.13 (−0.51/0.14)	−0.05 (−0.57/0.42)
1-Hexanol + Methyl salicylate	100 + 100	−0.05 (−0.16/0.20)	0.41 (−0.03/0.64)	0.02 (−0.25/0.11)
1-Hexanol + Terpinen-4-ol	100 + 100	−0.25 (−0.33/0.26)	0.13 (−0.44/0.53)	−0.1 (−0.29/0.02)
1-Hexanol + Terpinen-4-ol	50 + 50	0.05 (−0.23/0.07)	0.51 (0.39/0.81)	−0.01* (−0.37/0.15)
1-Hexanol + (*Z*)-3-Hexenyl acetate	50 + 50	0.36* (0.24/0.65)	0.03** (−0.22/0.14)	0.04 (−0.20/0.65)
1-Hexanol + Nonanal	50 + 50	0.38 (0.06/0.49)	−0.10 (−0.43/0.32)	−0.21† (−0.39/−0.08)
1-Hexanol + (*E*)-β-Caryophyllene	50 + 50	0.24 (−0.11/0.57)	0.18 (−0.05/0.46)	−0.23 (−0.62/0.06)
(*E*)-β-Caryo-phyllene + DMNT + (*E*)-β-Farnesene	100 + 78 + 9	0.01 (−0.33/0.27)	−0.16 (−0.57/0.33)	0.33 (−0.13/0.54)

Values in the table are the medians for 10 replicates for the host plant chemicals and for 20 replicates of the artificial diet odour.
* A significant difference between the target vector values for the pre- or post-exposure period from the corresponding test period (Wilcoxon's matched pairs test, $p < 0.05$).
† Significant difference between pre- and post-exposure periods (Wilcoxon's matched pairs test, $p < 0.05$).
** Target vectors values for host plant chemicals showing significant differences to the pre-exposure period were compared to the target vector value for food odour and were significantly different in all cases (Mann–Whitney *U*-test, $p < 0.05$).

target vector values (median −0.20, minimum −0.79, maximum 0.84, Table 1) all indicate that the courses taken by the larvae were even more non-directional after exposure to the food stimulus than before. Furthermore, the deviation from the upwind direction of the tracks (median 153.65°, minimum 15.82°, maximum 179.68°) was significantly higher PostX to the food odour (Table 3). The time spent in the cone 45° either side of upwind was significantly shorter following removal of the food odour from the air stream than before exposing the larvae to same (Fig. 5), with a consequent lower proportion of upwind displacement following odour removal (median 0.11, minimum 0.00, maximum 0.40, Table 2).

3.2. Walking behaviour during delivery of plant volatiles

1-Hexanol and methyl salicylate, the compounds inducing the highest target vector values of 0.75 and 0.68, respectively, showed dose dependent effects on larval attraction (Fig. 6) with significant effects compared to the PreX and PostX periods (Table 1). Methyl salicylate caused significant attraction at 1 ng, the lowest source dose tested. At source doses of 100 ng and 10 μg larvae responded to 1-hexanol and methyl salicylate with increased upwind displacement and decreased deviation from the upwind direction at 0° (Tables 2 and 3). Target vector was also dose dependent for terpinen-4-ol and DMNT (Fig. 6). A drop in target vector values was significant at all doses of these two products during the PostX period. An increase from PreX to test period was recorded for the 10 μg source dose of terpinen-4-ol and DMNT (Table 1), and significant effects were also found for upwind displacement and mean deviation from the upwind direction at this dose (Tables 2 and 3). (*Z*)-3-Hexenyl acetate induced a significant increase in target vector over the PreX value at 100 ng and 10 μg and (*Z*)-3-hexen-1-ol at the 10 μg source

Table 2
Upwind displacement calculated for tracks of *L. botrana* larvae walking on a locomotion compensator in an air stream alone (Pre-exposure), in response to the odour of the artificial diet and to single host plant compounds added to the air stream (Treatment), and after removal of the treatments (Post-exposure). Only treatments causing significant shifts in target vector values over the initial air stream exposure values (Pre-exposure in Table 1) are presented.

Compounds	Source dose (ng)	Median of the proportions of upwind displacement (25th/75th percentiles)		
		Pre-exposure	Treatment	Post-exposure
Food odour	$\sim 20 \times 10^9$	0.30* (0.17/0.37)	0.64 (0.57/0.75)	0.11*,† (0.08/0.27)
Methyl salicylate	1	0.15 (0.06/0.23)	0.34 (0.18/0.49)	0.14 (0.04/0.31)
	100	0.15* (0.04/0.24)	0.49 (0.29/0.52)	0.03* (0.00/0.10)
	10000	0.04* (0.00/0.13)	0.49 (0.41/0.55)	0.08* (0.05/0.16)
1-Hexanol	100	0.08* (0.04/0.28)	0.48 (0.34/0.61)	0.13* (0.08/0.35)
	10000	0.22 (0.10/0.33)	0.51 (0.32/0.58)	0.26* (0.15/0.42)
(*Z*)-3-Hexen-1-ol	10000	0.20* (0.03/0.25)	0.37 (0.24/0.43)	0.17* (0.05/0.40)
Terpinen-4-ol	10000	0.11* (0.00/0.27)	0.36 (0.30/0.40)	0.30 (0.01/0.35)
1-Octen-3-ol	1	0.10 (0.10/0.18)	0.32 (0.19/0.58)	0.20 (0.10/0.67)
DMNT	10000	0.14* (0.10/0.25)	0.53 (0.32/0.64)	0.16* (0.09/0.19)
(*Z*)-3-Hexenyl acetate	100	0.12* (0.10/0.26)	0.35 (0.28/0.39)	0.17* (0.07/0.25)
	10000	0.07 (0.01/0.14)	0.22 (0.12/0.38)	0.15 (0.01/0.23)

Values in the table are the medians for 10 replicates for the host plant chemicals and for 20 replicates of the artificial diet odour.
* A significant difference between the proportions of upwind displacement for the pre- or post-exposure period from the corresponding test period (Wilcoxon's matched pairs test, $p < 0.05$).
† Significant differences between pre- and post-exposure periods (Wilcoxon's matched pairs test, $p < 0.05$).

dose. These effects were also seen in larval upwind displacement and mean low deviations from 0° (Tables 2 and 3). Higher target vectors over the PreX and PostX periods were also recorded for 1-octen-3-ol, though this was only significant between PreX and test period at the 1 ng source dose (Table 1). The significant effect on the target vector recorded for the PreX in comparison to 100 ng of (*Z*)-3-hexen-1-ol was caused by high upwind orientation during the PreX (Table 1). No significant differences in larval behaviour were recorded between PreX and the tested source doses of (*E*)-β-caryophyllene, (*E*)-β-farnesene and nonanal. The highest target vector was recorded for the responses of the larvae to the odour of the artificial diet, significantly higher than any other recorded value (Table 1).

Binary mixtures of 1-hexanol that itself induced the highest target vector responses with a compound from the other chemical classes tested (acyclic C_{11} homoterpene DMNT, terpene alcohol terpinen-4-ol, sesquiterpene (*E*)-β-caryophyllene, aliphatic ester (*Z*)-3-hexenyl acetate, aliphatic aldehyde nonanal and aromatic methyl salicylate) were tested at a ratio 1:1. No significant increase in target vector was recorded for any of these mixtures (Table 1). For the mixture of 1-hexanol plus terpinen-4-ol tested at two source doses (50 ng + 50 ng and 100 ng +100 ng), a significant decrease in target vector was recorded after removal of the mixture at the lower dose (Table 1) and the target vector value recorded during exposure to vapour from the lower source dose was significantly higher ($p < 0.05$) than that recorded at the higher source dose. No behavioural effects were recorded for the ternary mixture of (*E*)-β-caryophyllene + DMNT + (*E*)-β-farnesene (Table 1) which is attractive to female *L. botrana* (Tasin et al., 2006b, 2007).

Table 3
Deviations from 0° calculated for tracks of *L. botrana* larvae walking on a locomotion compensator in an air stream alone (Pre-exposure), in response to the odour of the artificial diet and to single host plant compounds added to the air stream (Treatment), and after removal of the treatments (Post-exposure). Only treatments causing significant shifts in target vector values over the initial air stream exposure values (Pre-exposure in Table 1) are presented.

Compounds	Source dose (ng)	Median of the mean deviations from 0° (25th/75th percentiles)		
		Pre-exposure	Treatment	Post-exposure
Food odour	$\sim 20 \times 10^9$	48.08* (16.25/66.86)	9.64 (3.43/14.19)	153.65*,† (115.80/167.82)
Methyl salicylate	1	108.38 (77.81/134.80)	33.38 (23.71/97.83)	96.95 (52.27/119.27)
	100	104.52* (52.58/153.42)	29.56 (25.75/48.00)	133.81* (119.60/141.51)
	10000	98.24* (55.80/145.39)	12.81 (7.79/25.58)	129.79* (83.95/153.80)
1-Hexanol	100	97.61* (64.33/156.73)	27.24 (14.85/32.38)	96.73* (48.72/162.30)
	10000	63.07* (40.09/131.68)	20.80 (6.82/32.63)	74.97* (32.32/124.90)
(*Z*)-3-Hexen-1-ol	10000	92.81* (63.47/144.65)	34.56 (29.21/50.80)	71.85 (38.83/152.30)
Terpinen-4-ol	10000	76.64* (48.17/139.83)	20.97 (8.47/35.38)	63.16* (37.32/102.80)
1-Octen-3-ol	1	98.65* (41.89/131.40)	22.05 (15.09/58.69)	61.63 (8.41/99.75)
DMNT	10000	87.26* (48.55/138.06)	27.37 (14.88/40.01)	127.26* (62.11/144.89)
(*Z*)-3-Hexenyl acetate	100	92.62* (57.46/130.84)	22.79 (9.44/34.37)	89.23* (59.12/143.27)
	10000	107.96* (89.93/130.60)	52.16 (16.57/77.72)	89.89 (70.70/121.25)

Values in the table are the medians for 10 replicates for the host plant chemicals and for 20 replicates of the artificial diet odour.
* A significant difference between the mean deviations from 0° for the pre- or post-exposure period from the corresponding test period (Wilcoxon's matched pairs test, $p < 0.05$).
† Significant difference between pre- and post-exposure periods (Wilcoxon's matched pairs test, $p < 0.05$).

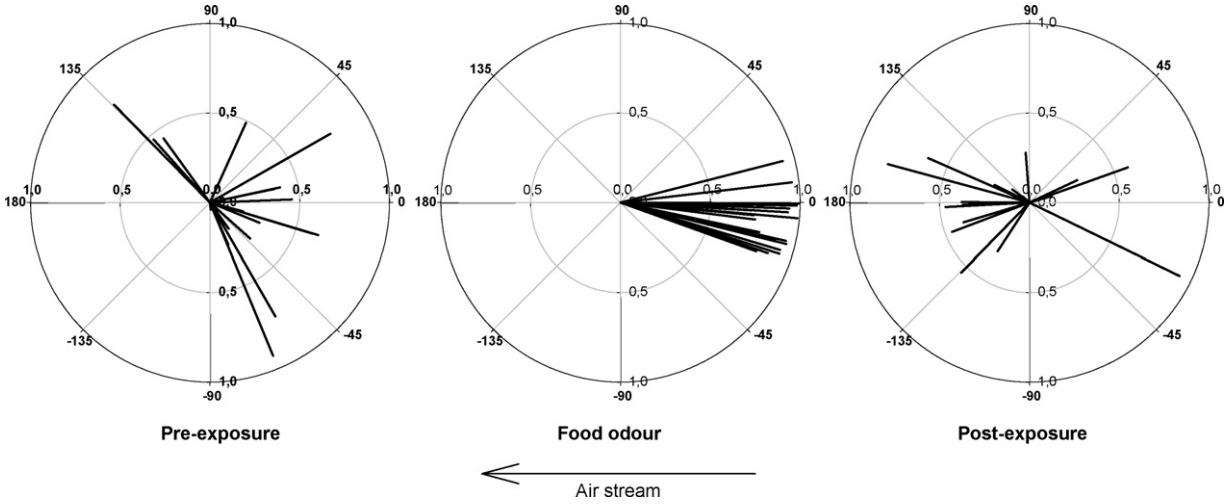

Fig. 3. Polar plot of the mean vector angles of the tracks of 20 *L. botrana* larvae before, during and after exposure to food odour in an air stream on a servosphere. The mean vector angles were calculated from the angles of the single movement vectors (Fig. 1) of a larval track. The nearer the mean vector angle weighting to the perimeter the straighter the walk in that direction (a value of 1 equals a straight walk). The air stream direction is indicated by the arrow.

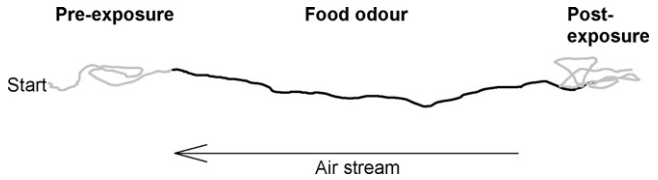

Fig. 4. A typical track described by a *L. botrana* larva on the locomotion compensator in response to the air stream alone (pre- and post-exposure periods, grey) and the odour of the artificial diet (black) used for rearing *L. botrana*. The larva showed significant upwind displacement when exposed to the food odour but moved at random in the absence of food odour in the air stream.

4. Discussion

4.1. Behaviour of L. botrana larvae in an air stream

Walking by *L. botrana* larvae is achieved in a manner similar to that described for caterpillars by Weber (1933) by pushing forward in a wave running from the back to the front of the body. The step length used here to account for a single movement was estimated at about 15% of the larval body length. This is in agreement with a step length of about 18% of body length employed in a detailed investigation of locomotion in the eastern tent caterpillar, *Malacosoma americanum*, by Stulce (2002).

When walking in the air stream larval tracks showed a mainly winding character with some straight sections. These non directed walks of low rectitude with a high degree of variance in mean directions are indicative of searching behaviour. Larvae regularly undertook displacement interrupted by short stops to reorientate rather than to repose. Throughout most of these stops larvae did not stay motionless but showed characteristic lifting and sideward waving of the head and thorax, often followed by a change in walking direction. Side-to-side waving as well as back and forth waving usually with a subsequent change in walking direction has already been described for 1st instar *C. pomonella* larvae (Jackson, 1982). Similar searching behaviour was named "head-waving" for cater-

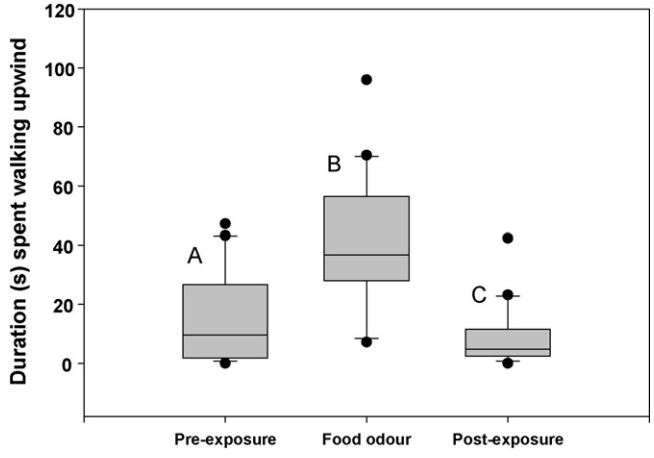

Fig. 5. Box plots illustrating the spent time walking by 20 *L. botrana* larvae in a cone extending 45° either side of upwind (0°) during exposure to food odour compared to the pre- and post-exposure periods. Exposure to food odour significantly increased the time spent walking upwind but this decreased significantly after removal of food odour. The line within a box marks the median, the boundaries of a box indicate the 25th and 75th percentiles, error bars indicate the 10th and 90th percentiles, and the points represent data beyond these limits. Treatments labelled with dissimilar letters are significantly different ($p < 0.05$, Wilcoxon's matched pairs test).

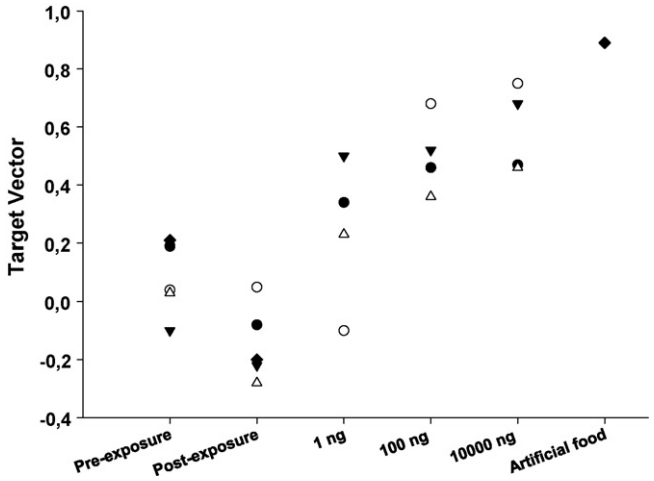

Fig. 6. Target vectors (medians) calculated for tracks of *L. botrana* larvae walking on a servosphere in an air stream alone (medians for data recorded pre- and post-exposure to treatments), and in the air stream bearing vapours from three doses of the host plant compounds 1-hexanol (○), methyl salicylate (▼), 4,8-dimethyl-1,3,7-nonatriene (●), terpinen-4-ol (△) or the odour of the artificial diet (◆). Target vector that provides an index of a caterpillar's attraction to different treatments is comprised of measures of track straightness and the mean track angle relative to the direction to the odour stimulus and approaches a maximum of 1 for a straight walk upwind to the odour source.

pillars of the cabbage butterfly *Pieris rapae*, the diamond-back moth *Plutella maculipennis* and the alfalfa looper *Plusia californica* (Jones, 1977), and described as "rearing" for *Drosophila* larvae (Green et al., 1983). That this behaviour serves as a mechanism to scan for useful cues seems evident: through lifting the head and thorax sense organs are exposed to the environment to facilitate the capture of chemical, visual and/or mechanical information, permitting the larvae to come into contact with for example foliage. Periodic scanning for resources during stops is known as saltatory search (O'Brien et al., 1986) and has been described for different insect species (Miller, 1979; Otálora-Luna et al., 2004). Periodic left and right head casting movements have also been described for larvae of coccinellidae and syrphidae (Banks, 1957; Chandler, 1969). For *Lobesia* larvae, smaller left-right movements with the head held down were regularly observed during walking in the air stream alone as well as during exposure to odours. Possibly, this head waggling also serves to increase the range within which to encounter potential stimuli or to provide a comparison between the perceived physical or chemical stimulus with information perceived just prior. The strong motivation to walk in the air stream by 20 of the 23 *Lobesia* larvae studied here indicates good pre-conditioning of the larvae (starved and tested during the scotophase) and test conditions (darkness and high humidity).

4.2. Behavioural response to the odour of the artificial diet and to host plant compounds

In this study the 20 second or third instar larvae tested all strongly reacted to the rearing medium odour. In a study by Mondy et al. (1998) only 43% and 7%, respectively, of second and third *Lobesia* larval instars reacted to the rearing medium. On the servosphere, all *Lobesia* larvae reacted by walking upwind to the odour source. This chemoanemotactic response indicates appetence for food by the larvae. The target vector value of 0.89 during delivery of food odour into the air stream illustrates how perception of this stimulus leads to targeted displacement. The relatively straight tracks, approaching the minimal linear distance, minimises energy use by the larvae. This efficiency in goal oriented displacement combined with a higher, though not significant, mean speed in response to the odour of the artificial diet is probably of importance for larval survival as it also results in a decrease in the time they are exposed to both biotic and abiotic hazards. Previous experience during development of the larvae on the artificial diet might have influenced their strong attraction to the food odour. Induction of a feeding preference and attraction to the rearing medium has already been described for other lepidopteran larvae (Jermy et al., 1968; Saxena and Schoonhoven, 1978; Carlsson et al., 1999). Associating the odour of a previously experienced diet, although artificial, with a food resource might be reasonable for the polyphagous *Lobesia* larvae. In fact, the odour of the artificial diet contains a variety of plant volatiles as well as CO_2 (data not shown), which also emanate *Lobesia* host plants.

Previous studies have shown that lepidopteran larvae respond to single host plant compounds: *C. pomonella* to (*E*,*E*)-α-farnesene and to ethyl- and methyl esters of (*E*,*Z*)-2,4-decadienoic acid (Sutherland and Hutchins, 1972; Knight and Light, 2001), *Depressaria pastinacella* to octyl acetate (Carroll and Berenbaum, 2002), *S. frugiperda* to linalool and DMNT (Carroll et al., 2006, 2008), but no such studies had been made with *Lobesia* larvae. Though the strength of attraction to plant volatiles was lower than for the odour of the artificial diet, seven out of the ten individually tested host plant compounds caused significant larval attraction. However, no increase in attraction compared to the single compounds was recorded for the binary mixtures of host plant compounds tested or for a ternary blend of host plant volatiles that attracts female *L. botrana* (Tasin et al., 2006b, 2007).

Lobesia larvae were attracted by the green leaf volatiles 1-hexanol, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate. These ubiquitous plant compounds are important in food-location by phytophagous insects as green leaf volatiles are known to induce behavioural and electrophysiological responses in moths and other insects (Visser et al., 1979; Guerin et al., 1983; Hansson et al., 1999; Reddy and Guerrero, 2000, 2004; Bruce et al., 2005). 1-Hexanol caused a dose dependent increase in attraction and was the compound that induced the highest target vector response values from *Lobesia* larvae. This attraction indicates that 1-hexanol plays a fundamental role in the olfactory responses of these larvae, as has also been found for *Drosophila melanogaster* larvae (Cobb and Domain, 2000; Oppliger et al., 2000). 1-Hexanol was also tested here in 1:1 (v/v) binary mixtures with host plant compounds of other chemical classes. Though the target vector values for the mixtures with methyl salicylate or terpinen-4-ol indicated larval attraction, neither these values nor those for any of the other binary blends differed significantly from the PreX, i.e. less attractive than to 1-hexanol alone. Interestingly, the target vector value of the lower dosed 50 ng + 50 ng mixture of 1-hexanol + terpinen-4-ol indicated stronger attraction than the higher dosed 100 ng + 100 ng mixture. Absence of a significant response might be due to inappropriate amounts or ratio of the products in the air. This might also account for the lack of larval response to the ternary blend of grapevine volatiles β-caryophyllene, DMNT and (*E*)-β farnesene that is attractive to female *L. botrana* in a wind tunnel (Tasin et al., 2006b, 2007).

Methyl salicylate attracted the apple fruit moth *Argyresthia conjugella* in field-trapping tests (Bengtsson et al., 2006) and was also shown to induce upwind flight in *Lobesia* females in wind tunnel experiments when mixed with other host plant volatiles (Tasin et al., 2007). Here, *Lobesia* larvae showed significant attraction and a high efficiency in locating methyl salicylate even at a 1 ng source dose. Increasing target vector values by larvae at 100 ng and 10 μg methyl salicylate source doses indicate a dose-dependent pattern of response to this product. A similar pattern was recorded in the responses of *Lobesia* larvae to terpinen-4-ol and DMNT although the test period target vector values were different from the PreX period values only at the highest doses tested. Though a significant effect was only found at 1 ng, high target vector values for all three doses of 1-octen-3-ol indicate activity of this compound on the behaviour of *Lobesia* larvae. No significant increase in the target vector values were recorded for β-caryophyllene, (*E*)-β-farnesene or nonanal. Most of the single compounds tested were also identified in the artificial food (data not shown) such that previous experience might have influenced the behavioural responses to the individually tested compounds. But as β-caryophyllene, which occurs in the artificial food, caused no attraction when tested, it would appear that the behavioural experiments described here show how *Lobesia* larvae can discriminate for host plant compounds independent of previous experience.

The ten host plant compounds tested here were selected because of their ability to induce electroantennogram responses (except for 1-hexanol for which biological activity to our knowledge has not been previously documented for *Lobesia*) from female *L. botrana* (Tasin et al., 2005, 2006a; Gabel, 1992). The attraction to seven of these individually tested host plant compounds by *Lobesia* larvae underlines sensitivity to identical compounds in both juveniles and adults. Attraction of larvae to single compounds applied at source doses as low as 1 ng indicates the low threshold sufficient to induce this behavioural response (considering the dilution of the products in the air stream). This is comparable to the effects of behaviourally active doses of plant volatiles and pheromones on adult *Lobesia* (Tasin et al., 2006a; El-Sayed et al., 1999). The lack of response to the mixtures of products applied at

ratios that are most likely different to that found in natural blends points to the discriminative capacity of the larval olfactory system and the importance of precise ratios of products in host odour blends as has been shown for adults (Tasin et al., 2006b).

4.3. Behaviour of Lobesia larvae after removal of the test stimuli from the air stream

Removal of the food odour from the air stream induced local search behaviour by *Lobesia* larvae as indicated by tracks with movement vectors with highly deviating angles and the lower overall displacement. This change was also significant in comparison to the PreX period, indicating a different behaviour by the larvae in the post-treatment exposure period in the air stream. Similar effects on behaviour were also observed after removal of single host plant volatiles from the air stream even at a source dose of 1 ng. A primary function of these non-directed movements is to regain contact with the stimulus. *P. rapae* caterpillars exhibited local search after loss of contact with the host-plant with decreased directionality in tracks that covered only a small area (Jones, 1977).

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