



Substrate Vibrations Elicit Defensive Behaviour in Leafminer Pupae

SVEN BACHER,*† JÉRÔME CASAS,† FELIX WÄCKERS,* SILVIA DORN*

Received 3 December 1996; revised 7 March 1997

Late instar larvae and pupae of the spotted tentiform leafminer *Phyllonorycter malella* (Ger.) (Lepidoptera: Gracillariidae) react with defensive behaviour when attacked by one of their parasitoids, the eulophid wasp *Sympiesis sericeicornis* Nees (Hymenoptera: Eulophidae). Vibrations produced during the insertion of the ovipositor into the mine are known to be important cues by which larvae detect the presence of their enemies. The aim of this study was to investigate which frequency components elicit defensive reactions in leafminer pupae using synthetic vibrations. Sine vibrations and bandlimited noise stimuli were offered to both free pupae and pupae concealed in their leafmines. Using laser vibrometry we measured the vibrations experienced by pupae inside their mines and assessed the influence of the mine. Pupae were shown to react to substrate vibrations, and do so over a broad range of frequencies. Behavioural reactions to noise stimuli were stronger than to pure sine stimuli. Mine tissue attenuated vibration amplitudes of the input signal from 5.1 to 22.6 dB. However, as response thresholds of concealed pupae were only twice as high as thresholds of free pupae (which is adequate to 3 dB) pupae inside their mine were more sensitive than expected. This discrepancy is discussed both in terms of the conditions of pupae and in terms of mine structure. The results indicate that broadbandness of vibrations produced by hunting parasitoids during ovipositor insertion into the mine may be a major criterion used by leafminers to perceive parasitoid presence and to escape ovipositor stings. © 1997 Elsevier Science Ltd. All rights reserved

Substrate vibrations Defensive behaviour Threshold curves Vibratory communication Sensory ecology
Leafminer

INTRODUCTION

Late instar larvae and pupae of the spotted tentiform leafminer *Phyllonorycter malella* (Ger.) (Lepidoptera: Gracillariidae) show characteristic defensive reactions when attacked by the parasitoid *Sympiesis sericeicornis* Nees (Hymenoptera: Eulophidae). Whereas pupae can only 'wiggle', larvae show a complex evasive behaviour (Meyhöfer *et al.*, 1997). In addition to wriggling, which is considered an evasive defence (Gross, 1993), larvae may also escape ovipositor stings by changing their location inside the mine. Wriggling can increase the handling time by the parasitoid. As a result, in about 10% of the cases the parasitoid abandons the mine without

parasitising the host (Casas, 1989). Therefore, it seems likely that defensive behaviour has an adaptive value for the leafminer.

For defensive behaviour to be effective, the leafminer must be able to detect the presence of a parasitoid at a stage that allows for an effective defence. We hypothesised that *P. malella* perceives parasitoid presence through vibrations produced when *S. sericeicornis* females forage on the mine. Indeed, such vibrations provide both detectable and reliable cues for the leafminer to identify its enemy (Bacher *et al.*, 1996). Through an analysis of combined ethograms Meyhöfer *et al.* (1997) recently demonstrated for *P. malella* larvae that vibrations are involved in the detection of parasitoid presence. However, the question of which components of the vibrational signal are actually involved in eliciting defensive behaviour remains to be answered.

The aim of this study was to determine which frequency components are involved in eliciting defensive behaviour. Pupae of *P. malella* were chosen for their monotypic behavioural reaction. Their behaviour in

*Institute of Plant Sciences, Applied Entomology, Swiss Federal Institute of Technology (ETH), 8092 Zürich, Switzerland.

†University of Tours, IRBI-UPRESA 6035, 37200 Tours, France.

‡Present address and to whom all correspondence should be addressed. Zoologisches Institut, Synökologie, Baltzerstr.3, CH-3012 Bern, Switzerland Tel:41-31-631 4539 Fax:41-31-631 4888 E-mail bacher@zos.unibe.ch.

response to direct as well as indirect stimulation was investigated using synthetic vibrations of different frequencies. In addition, the vibrations experienced by pupae inside their mines and the attenuation by the mine were measured by laser vibrometry.

MATERIALS AND METHODS

Insects and plants

P. malella lives monophagously on apple trees. Pupae and apple leaves used in the experiments originated from our continuous laboratory rearing. Pupae were only used in one experiment. The age of the pupae was not determined.

General set-up

Vibrations were produced by means of an electromagnetic vibration exciter (B & K 4810) connected to a power amplifier (B & K 2706). The vibration exciter stood on an air-buffered, vibration attenuated table (Photon Control, Cambridge) to reduce background vibrations. In experiments in which stimuli were offered to free pupae (direct stimulation), the pupae were placed directly onto the moving platform of the vibration exciter. In experiments in which stimuli were offered to pupae concealed in their leafmines (indirect stimulation), leaves with a single mine were detached and their petioles placed in a water-filled vial through a hole in its lid. The petiole was fixed to the lid with plasticine at a distance of 0.5 cm from the leaf base. The vial was fixed on a small, vertically adjustable table such that the leaf was horizontal. The adjustable table holding the leaf was placed on the air buffered table next to the vibration exciter. The top of the vibration exciter was equipped with a sharpened screw. Using correction fluid, the tip of the screw was glued to the lower epidermis of the mine between the pupa and the leaf margin, approx. 1 cm from the pupa (Fig. 1). The behaviour of the pupae inside the mines was monitored through the lower epidermis of the leaf by means of a video camera (Panasonic WV-BL600) equipped with a macro zoom lens (Computar 18-108/2.5). The mine was illuminated from above by a glassfibre lamp (Fiberoptic-Heim AG, L100) to facilitate

observations. Experiments were conducted at an ambient temperature of $25 \pm 2^\circ\text{C}$.

We also measured the vibrations to which pupae were exposed during the indirect stimulation with a laser Doppler vibrometer (Dantec 41X62 Compact Laser Vibrometer, 2 mW He-Ne-Laser, tracker range 0.1–1.0 MHz). Leaves were prepared in the same way as described above. At the location of the pupa, a small window was cut into the upper side of the mine through which the pupa was removed. For better reflection of the laser beam and a better signal-to-noise ratio, a small piece of retroreflexive tape was glued to the lower inner mine surface at the former position of the pupa (Scotchlite, 3M; approx. 1 mm^2) with correction fluid. Vibrations perpendicular to the inner mine surface were measured at the location of the retroreflexive tape. Testing pupae concealed in their mines and measuring the response of a mine without its inhabitant is an attempt to estimate the influence of the mine on the behavioural response to stimuli. It is therefore imperative to keep the vibration testing set-up constant between these experiments. Other set-ups would be required to estimate mechanical parameters of leaves and mines (Pollard, 1977; McConnell, 1995).

Generating the vibrational signals

Sine wave signals were generated by a function generator (Stanford Research DS345) and fed directly into the power amplifier. Frequencies of 100, 200, 500, 800, 1000, 2000, 3000 and 4000 Hz were used in the experiments. This range comprised the most prominent frequencies produced by parasitoids during host location on mined apple leaves (Bacher *et al.*, 1996). Prior to the experiments, vibrations produced by the vibration exciter were measured with the laser Doppler vibrometer (for details see Bacher, 1996).

Noise signals were generated on a Macintosh Quadra 800 Computer using the software SoundScope (GW Instruments, 1993). A $\pm 1 \text{ V}$ Gaussian white noise signal (duration = 2 s, sampling frequency = 50 kHz, rise and fall times = 0.1 s) was filtered with digital bandpass filters (WLFDP, Zola Technologies, Inc.: filter length 63 points, stopband attenuation 80 dB, sampling frequency 50 kHz). Filters had the bandwidth of octaves and the border frequencies were chosen according to Bacher *et al.* (1996), i.e. band 1, 178–355 Hz; band 2, 355–708 Hz; band 3, 708–1410 Hz; band 4, 1410–2820 Hz; and band 5, 2820–5620 Hz. Due to technical limitations of the equipment, other frequency bands were not tested. The band limited noise signals were fed into the power amplifier via a D/A converter on a Mac Adios II/16 board (GW Instruments). The intensities were varied by multiplying the original stimuli by different factors (step size 0.5). A multiplication factor of 1.0 corresponds to an output root mean square (RMS) velocity of 2.23 mm s^{-1} at band 1, 3.66 mm s^{-1} at band 2, 3.74 mm s^{-1} at band 3, 0.89 mm s^{-1} at band 4 and 0.37 mm s^{-1} at band 5

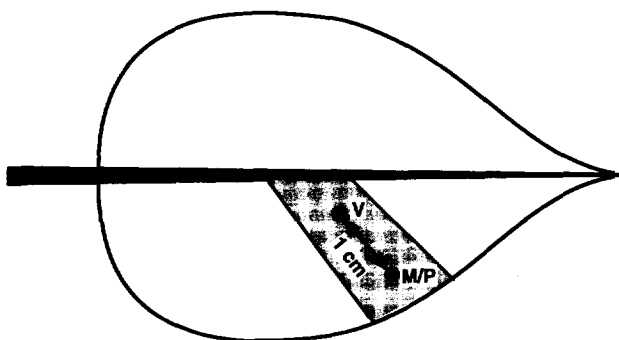


FIGURE 1. Relative location of vibration source (V) and measurement point/location of the pupa (M/P) on apple leaves during vibration measurements. Mine area indicated by grey pattern.

(settings of the power amplifier: 10 dB attenuation, gain control adjusted to the middle position).

Experimental procedure

Free pupae, sine stimuli. Pupae were removed from their leafmines and placed on top of the vibration exciter platform. Vibration signals were administered only when pupae were motionless, with no abdominal movements, for at least 3 min. Each individual was tested with all frequencies in random sequence. The intensity of the sine stimulus of a chosen frequency was increased on the function generator from zero in steps of 0.01 V_{pp} (Volt peak-to-peak) until the pupa showed wriggling behaviour. The duration of each intensity step was approx. 2 s, without a rest period between two intensities. The voltage at which individuals started 'wriggling' (reaction threshold) was recorded for each frequency. If the tested animal did not show a behavioural response until 1 V_{pp} (the intensity range tested is marked by the grey area in Fig. 2), the test was terminated and the result was classified as 'no reaction' for that frequency. Because it was impossible to evaluate the reason an individual did not respond (possible damage by handling, unresponsive physiological state/age, etc.) only experiments with 'responsive' individuals were considered for analysis. Individuals were defined as 'responsive' if they responded with wriggling to at least three of the frequencies tested. Valid results were obtained from 15 pupae; 3 pupae were discarded.

Concealed pupae, sine stimuli. The test procedure was the same as for free pupae. Unlike the previous experiment, intensities were increased until the signal was clipped by the power amplifier (the intensity range tested is marked by the grey area in Fig. 3). Animals that did not show a behavioural response even at the maximum intensity of a specific frequency were classified as 'no reaction' for that frequency. Valid results were obtained from 9 pupae; 1 pupa was discarded.

Concealed pupae, bandlimited noise stimuli. Frequency bands 1–5 were tested with each individual in random sequence. Intensities were increased from zero until a behavioural response was elicited. Each stimulus intensity was given three times with a pause of approximately 10 s between different intensities. Vibration signals were administered only when pupae were motionless, with no abdominal movements, for at least 3 min. Experiments were considered for analysis only if individuals showed a behavioural response to at least three of the frequency bands tested. Valid results were obtained from all 5 pupae tested.

Vibrations inside the mine. Sine waves of all frequencies used in the other experiments were tested at varying intensities. Three leaves were measured.

Data analysis

The behavioural thresholds (in V_{pp}) were converted to velocity. For sine wave data, displacement and acceleration were calculated from velocity. Peak-to-peak data

of experiments in which sine stimuli were used were transformed to RMS values by division by $\sqrt{2}$. For a direct comparison, the amplitudes of the bandlimited noise stimuli were also given as RMS values by numerical calculation of the standard deviation multiplied by a factor of 2 (GW Instruments, 1993). Thus, data are presented as 'effective' amplitudes in both directions of the mean. If not indicated differently, values are given as mean \pm SD. All tests in which the pupae showed no reaction were discarded for the calculation of means.

The attenuation of the mine was calculated as sound intensity level (SIL) and given in decibel (dB): $SIL = 10 * \log_{10}$ (vibration amplitude at the measurement point/amplitude of the vibration exciter).

RESULTS

Free pupae

Pupae reacted to all frequencies in the range tested (Fig. 2). Displacement thresholds were highest at 100 Hz (on average $45 \pm 25 \mu\text{m}$) but decreased steadily over more than two orders of magnitude with increasing frequency to an average of $0.16 \pm 0.07 \mu\text{m}$ at 4 kHz. When converted to velocity, thresholds showed a similar tendency to decrease with increasing frequency, but decreased only half an order of magnitude, from an average of $28 \pm 16 \text{ mm s}^{-1}$ at 100 Hz to an average of $5.5 \pm 1.9 \text{ mm s}^{-1}$ at 1 kHz. Between 2 and 4 kHz the threshold remained nearly constant between 3.1 and 4.2 mm s^{-1} . In contrast, the acceleration threshold curve showed a positive relationship with frequency. The lowest behavioural threshold occurred at 100 Hz ($17.7 \pm 9.8 \text{ m s}^{-2}$) and the highest at 4 kHz ($104 \pm 48 \text{ m s}^{-2}$). Thus, the results did not reveal an unambiguous frequency window to which pupae of *P. malella* are especially sensitive.

Up to 1 kHz all individuals tested showed a behavioural response to intensities below the arbitrarily chosen highest test amplitude of 1 V_{pp} (Fig. 2). Above 1 kHz the percentage of animals reacting decreased with increasing frequency to only 57% of the tested individuals responding at 4 kHz. This indicates that the actual threshold values may be underestimated at high frequencies.

Concealed pupae

The mean threshold values of concealed pupae exposed to sine stimuli were always higher than those of free pupae (Fig. 3). At frequencies up to 1 kHz the threshold values were about twice as high as thresholds of free pupae (between 1.5 times at 200 Hz and 2.4 times at 800 Hz). At higher frequencies, however, no reliable threshold values could be established due to the low percentage of responding animals. The low number of animals responding indicates that far higher amplitudes may be necessary to elicit wriggling behaviour in concealed pupae at high frequencies.

Reaction thresholds of concealed pupae to bandlimited noise stimuli ranged between $8.6 \pm 1.7 \text{ mm s}^{-1}$ at band

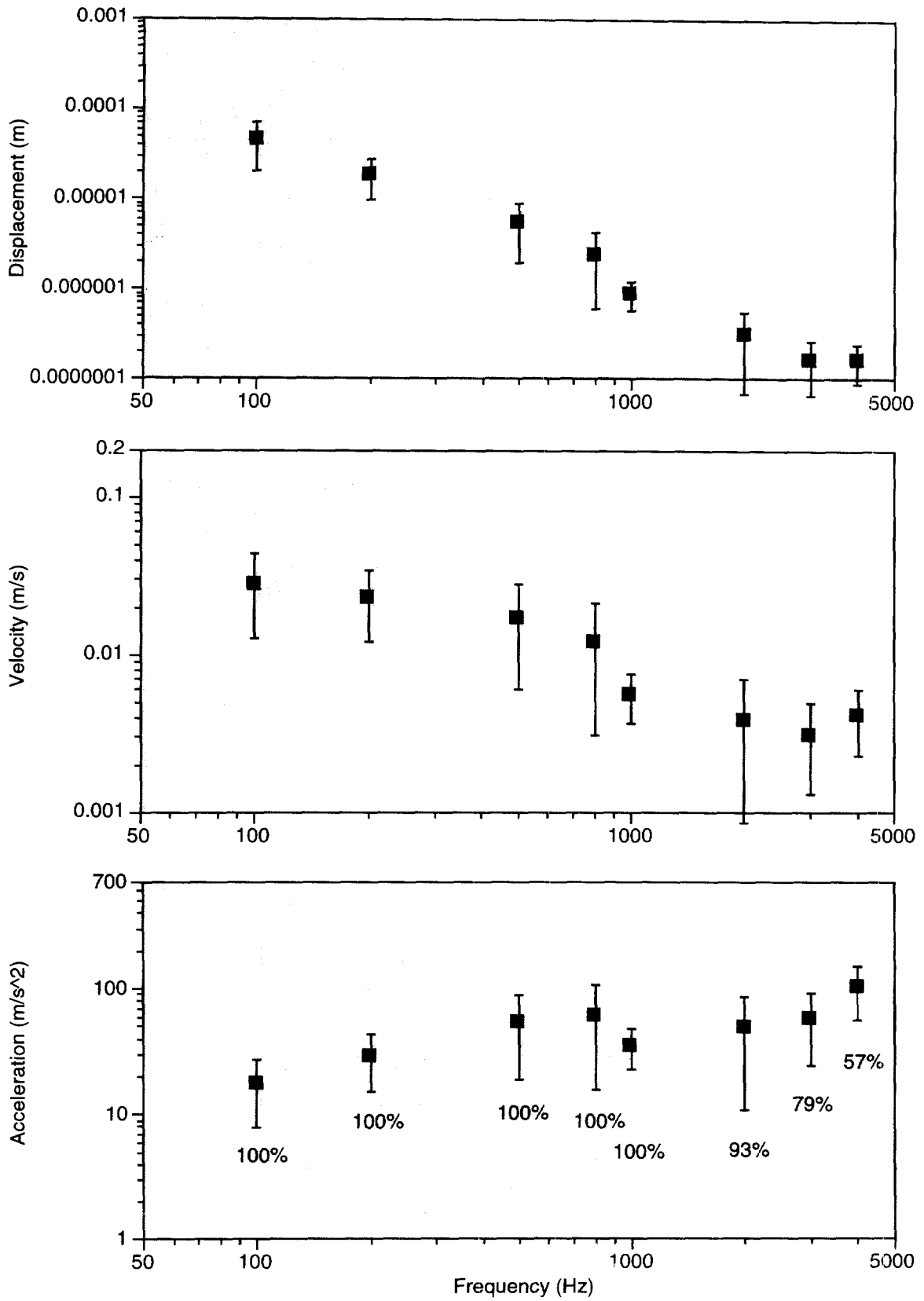


FIGURE 2. Reaction thresholds of free pupae to sine stimuli (mean \pm SD). The data are displayed as RMS displacement, velocity and acceleration amplitudes. The grey area indicates the tested range up to the arbitrarily chosen highest intensity (1 Vpp). In the lowest graph the percentages of responding animals are given at each frequency. $N = 15$.

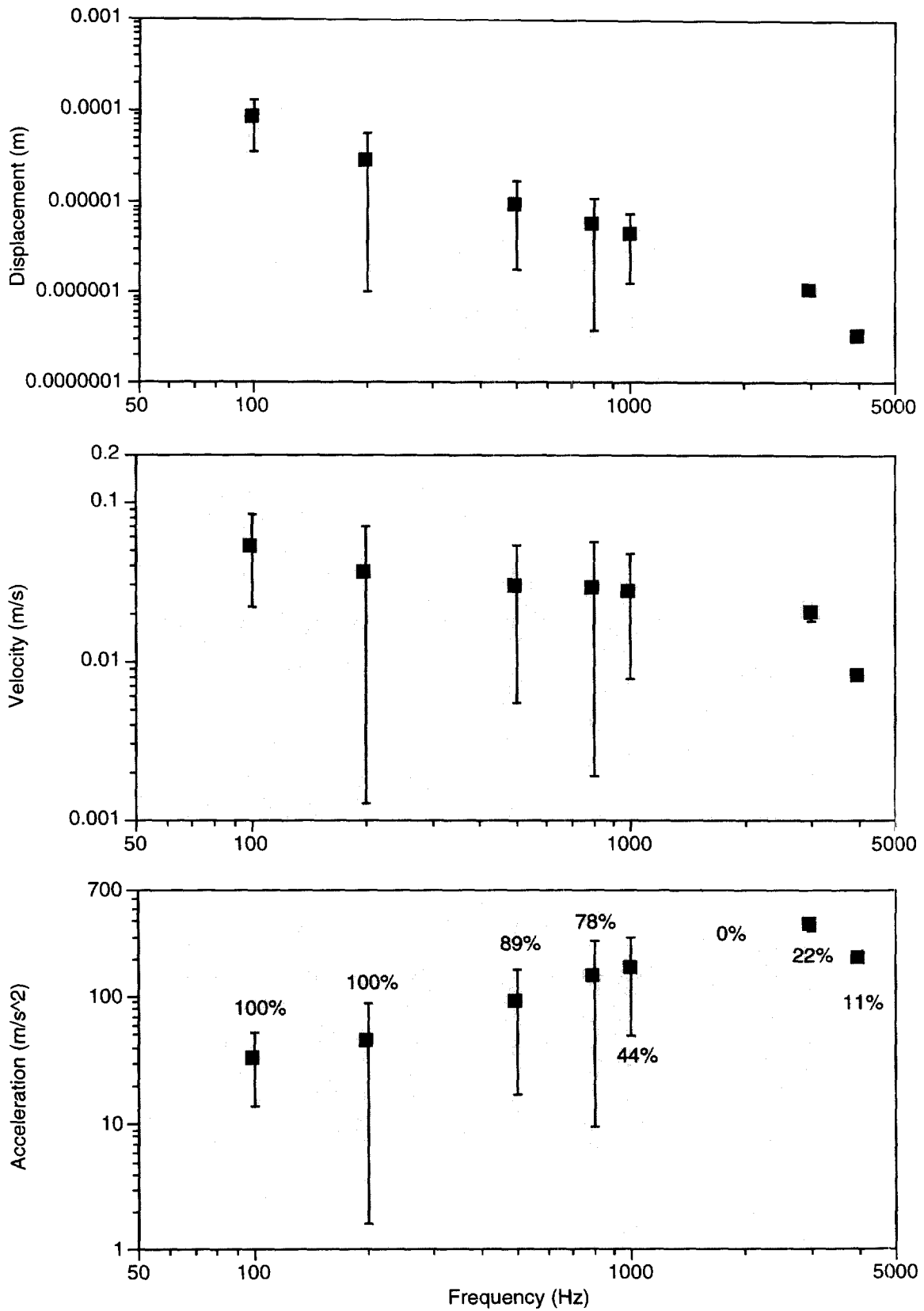


FIGURE 3. Reaction thresholds of pupae concealed in their leafmines to sine stimuli (mean \pm SD). The data are displayed as RMS displacement, velocity and acceleration amplitudes. The grey pattern indicates the tested intensity range (until the amplifier clipped the signal). In the lowest graph the percentages of responding animals are given at each frequency. $N = 9$.

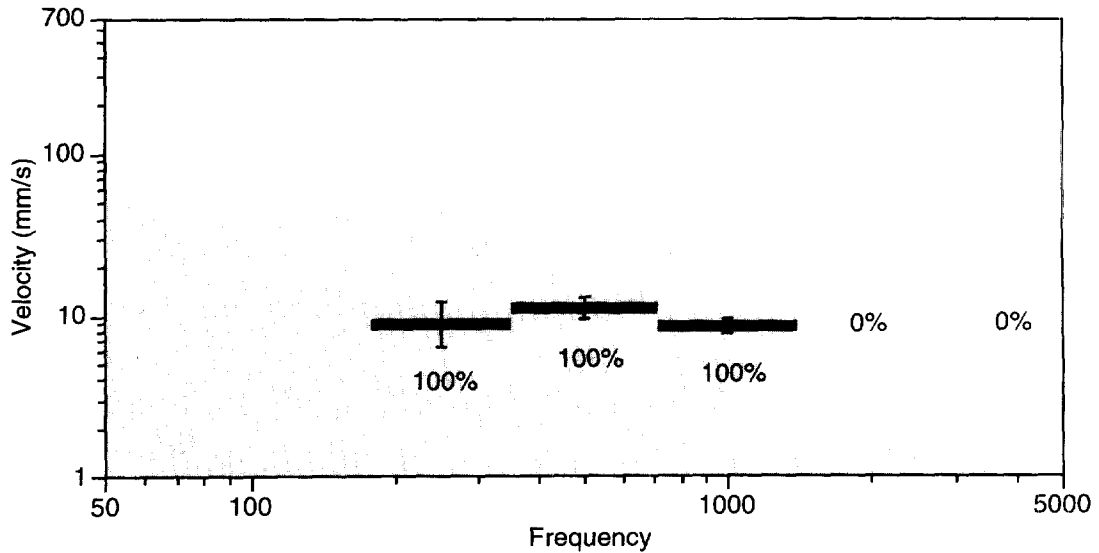


FIGURE 4. Reaction thresholds of pupae concealed in their leafmines to bandlimited noise stimuli (mean \pm SD). The data are displayed as RMS velocity amplitudes. The horizontal bars indicate the width of the frequency bands. The grey pattern indicates the tested intensity range (until the amplifier clipped the signal). The percentages of responding animals are given at each frequency. $N = 5$.

3 and $11.4 \pm 3.3 \text{ mm s}^{-1}$ at band 2 (Fig. 4). These thresholds were 2.6 to 3.8 times below the thresholds for sine stimuli in the same frequency range (200–1000 Hz). No behavioural reaction was elicited in response to stimuli of frequency bands 4 and 5 up to the highest intensities tested. However, these intensities were quite low compared to intensities at lower frequencies.

Vibrations inside the mine

Vibrations were attenuated linearly with increasing stimulus intensity. Mine tissue attenuated vibration amplitudes of the input signal between 5.1 and 22.6 dB at a distance of 1 cm from the source (Fig. 5). Different frequencies were transmitted with varying attenuation in

the mine. Moreover, frequency specific attenuation varied considerably between leaves.

DISCUSSION

Threshold curves and behavioural frequency sensitivity

The results of this study demonstrate that pupae of *P. malella* react with defensive behaviour to vibratory stimuli over a broad frequency range. Irrespective of the scale used, the results show no sharp tuning to any frequency. The importance of broadbandedness as signal parameter is further indicated by the lower thresholds to noise stimuli compared to sine stimuli. The same result was found e.g. in the spider, *Cupiennius salei*, where a marked

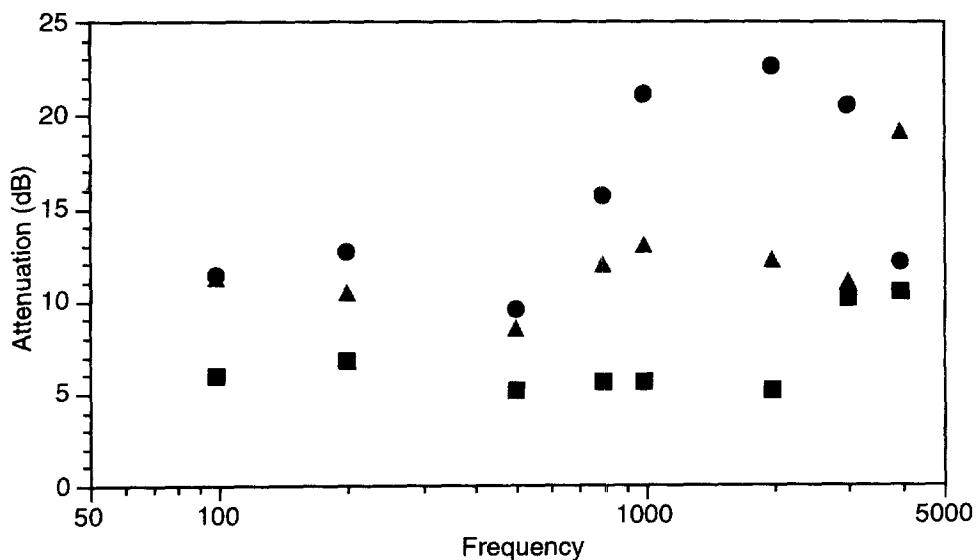


FIGURE 5. Attenuation of vibration amplitudes travelling 1 cm through mine tissue at different frequencies. Different symbols represent measurements from different leaves. $N = 3$.

decrease of threshold with noise stimuli is found in both behavioural and physiological experiments (Hergenröder and Barth, 1983; Speck-Hergenröder and Barth, 1987). The question whether a specific frequency range is the most important to elicit defensive behaviour in pupae cannot be answered on the basis of the available data because it is not yet known whether the relevant receptors and the associated neural processes are responding to the displacement, velocity or acceleration component of a vibratory signal.

The behavioural thresholds of *P. malella* pupae are relatively high compared to other arthropods. There are few studies on behavioural thresholds in arthropods to vibrations transmitted through solid substrate. The displacement thresholds releasing prey capture behaviour in the wandering spider *Cupiennius salei* are in the same range as those for *P. malella* (Hergenröder and Barth, 1983). Other insects, however, are much more responsive to vibrations than *P. malella* pupae. The damp-wood termite *Zootermopsis nevadensis*, for example, shows an alarm reaction to vibration intensities 60 times below the threshold of leafminer pupae (Kirchner *et al.*, 1994), and the honeybee *Apis mellifera* is nearly 15 times more responsive than *P. malella* to vibrations of the comb (Michelsen *et al.*, 1986).

The influence of the mine

Behavioural thresholds of concealed pupae to sine vibrations up to 1 kHz were only twice as high as thresholds of free pupae. This twofold attenuation is adequate to 3 dB and, therefore, is much lower than attenuations measured with empty mines. A first explanation is that the attenuation of the mine as measured is not the one experienced by pupae. Indeed, the location of a pupa in the mine as well as its coupling to mine tissues through silk threads spun before pupation all determine the intensity of vibrations as received by the pupa. Major differences in intensity can be expected over a short distance. A detailed understanding of the mine construction and the spatial organisation of the host's life is now being studied by the second author. A second possible reason for the observed attenuation is that a pupa outside its mine may exhibit major differences in its behavioural reactions.

Ecological relevance

The ectoparasitoid *S. sericeicornis* produces the most prominent vibrations when inserting its ovipositor into the mine (Bacher *et al.*, 1996). Such probing behaviour is the only behaviour that triggers a marked reaction in larvae without being touched (Meyhöfer *et al.*, 1997). For example, larvae almost always stop feeding after the first insertion of the ovipositor and, if they do, never resume feeding before the parasitoid abandons the leaf.

Velocities of the leaf surface during probing amount to up to 8.9 mm s⁻¹, with an average of 3.0 mm s⁻¹ (Bacher *et al.*, 1996; for a direct comparison with thresholds of this study the values were multiplied by a factor

of 2 to obtain amplitudes in both directions of the mean). These vibrations are in the same intensity range as the bandlimited noise vibrations to which *P. malella* pupae do react. Moreover, the frequency composition of natural stimuli is similar to the composition of noise stimuli (Bacher *et al.*, 1996). A major difference between our experiments and the probing behaviour is in the duration of the stimuli. Probing lasted on average 7.5 ± 5.2 s SD, but spells of broadband vibrations within a single probing event lasted only 1–2 ms (Bacher *et al.*, 1996).

The results obtained in this study are consistent with previous results obtained from leafminer larvae and parasitoids (Bacher *et al.*, 1996; Meyhöfer *et al.*, 1997). They demonstrate that pupae, too, do perceive and react to vibrations. Furthermore, they strongly suggest that broadbandness of vibrations produced by the probing action of hunting parasitoids is likely to be an important aspect of the signal triggering escape reactions in their leafmining hosts.

Leafminers are the group of phytophagous insects which harbour the most diverse parasitoid fauna (Hawkins, 1994). For example, *P. blancardella*, a sister species, is attacked by more than 20 different parasitoid species in Switzerland (Casas and Baumgärtner, 1990). The patterns of foraging behaviour of the different parasitoid species show a high degree of variability (J.C., personal observation) and the defensive behaviour of leafminers cannot be based against a single enemy. However, the insertion of the ovipositor into the mine by different parasitoid species may well produce the same type of vibratory signal, and, together with the response of leafminers to a broad range of frequencies, may allow the host to escape attacks of different enemies.

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Acknowledgements—We are grateful to C. Fornallaz for his technical help, D. Stenzel for assisting in the experiments, and T. Breithaupt (Konstanz, D), H. Gu (ETH), W. Kirchner (Konstanz, D), J. Tautz (Würzburg, D), M. Wolfe (ETH) and two anonymous referees for helpful comments on earlier versions of this manuscript.