Antennal detection of sex pheromone by female *Pandemis limitata* (Robinson) (Lepidoptera: Tortricidae) and its impact on their calling behaviour

NAOMI C. DELURY[1,2], GARY J.R. JUDD[1] and MARK G.T. GARDINER[1]

ABSTRACT

Previous observations lead us to believe that female *Pandemis limitata* (Robinson) (0 to 24 h old) are as attractive as their pheromone gland extract to males in clean air, but are more attractive in an environment permeated with their major pheromone component (*Z*)-11-tetradecenyl acetate. Therefore, in this study, we tested the hypothesis that females can detect and/or respond to their pheromone components. Using electroantennographic detection, we found female *P. limitata* able to perceive both of their known pheromone components, (*Z*)-11-tetradecenyl acetate and (*Z*)-9-tetradecenyl acetate. Female antennal response was found to be 46.3% weaker than that of males, under identical conditions, with male antennae producing significantly higher deflections to the higher pheromone doses tested and to the plant volatile, (*E*)-2-hexanal. Observations of females in clean air versus (*Z*)-11-tetradecenyl acetate-permeated air showed no significant differences with respect to onset time, frequency or duration of calling. Females moved significantly less often in a (*Z*)-11-tetradecenyl acetate-permeated portion of a flight tunnel than in the corresponding clean-air portion.

Key Words: (*Z*)-11-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate, female electroantennography, flight tunnel, mating disruption, threelined leafroller, sprayable pheromone, microencapsulated pheromone, movement

INTRODUCTION

Although pheromone-based mating disruption of Lepidopteran species is widely employed in some agricultural systems (Thomson *et al*. 2001), questions about the behaviour of female moths in the presence of their own sex pheromone often remain unanswered. In particular, while there is evidence that some female moths perceive (Michell *et al.* 1972, Birch 1977, Palaniswamy and Seabrook 1978, Light and Birch 1979, Barnes *et al*. 1992) and even modify their behaviour (Palaniswamy and Seabrook 1978, 1985, Palaniswamy *et al*. 1979, Sanders 1987, Weissling and Knight 1996, Evenden 1998) in response to their own sex pheromone, others apparently do not (El-Sayed and Suckling 2005) and information of this type is lacking for most species where commercial use of mating disruption is under study. During our own studies of pheromone communication disruption in the threelined leafroller, *Pandemis limitata* (Robinson) (Lepidoptera: Tortricidae), it appeared that males were more responsive to "calling" females (0 to 24 h old) in a pheromone-permeated atmosphere than they were to female gland extracts, although no difference in male response was detectable between these sources in clean air (N.C.D., unpublished data). One explanation for this observed difference is that female *P. limitata* can detect their pheromone environment and adjust their behaviour in a manner that

[1] Agriculture and Agri-Food Canada, Pacific Agri-Food Research Centre, 4200 Hwy 97, Summerland, BC, V0H 1Z0, Canada
[2] To whom correspondence should be addressed: Tel.: +1 250 494 7711; Fax: + 1 250 494 0755; E-mail: deluryn@agr.gc.ca

makes them more detectable to males than the female gland extract alone. Using electroantennograms (EAGs), we assessed the ability of female *P. limitata* to perceive their two known sex pheromone components, (*Z*)-11-tetradecenyl acetate [*Z*11-14:Ac] and (*Z*)-9-tetradecenyl acetate [*Z*9-14:Ac] (Roelofs *et al*. 1976) and asked the question, do females alter their calling behaviour in the presence of microencapsulated (MEC) *Z*11-14:Ac applied in laboratory flight-tunnel assays (Judd *et al*. 2005)?

MATERIALS AND METHODS

Laboratory cultures. Experiments were conducted with *P. limitata* from a laboratory colony maintained at the Pacific Agri-Food Research Centre, Summerland, British Columbia (BC) since 1992 that originated from wild populations collected in the Similkameen Valley of BC. *Pandemis limitata* were maintained on a modified pinto bean-based diet (Shorey and Hale 1965) at 25 °C under a 16:8 h L:D photoregime. Pupae were removed from diet, sexed and placed individually in 150 ml plastic cups provided with a wet cotton wick until adults eclosed. Male and female moths were isolated from each other in separate environmental chambers (25 °C, 65% r.h. with a 16:8 h L:D reversed photoregime).

Female and male perception of synthetic pheromone. Perception of synthetic pheromone by female and male *P. limitata* was measured using EAGs. Our EAG system consisted of an IDAC-02 computer-coupled data acquisition board, an INR-02 EAG-SSR system and AutoSpike software (Syntech, Hilversum, The Netherlands). Antennae excised from 0 to 24 h old females or males were mounted individually inside 10 µl glass capillaries containing silver-coated wire recording electrodes. Glass tubes were pulled at 300 °C on a Narishige (Model PN-30, Tokyo, Japan) micropipette puller. The distal antennal segment was removed and that end of the remaining antenna inserted into the indifferent electrode capillary. As a result, each antenna was suspended between two glass capillary electrodes filled with insect Ringer's solution (DeLury *et al*. 1999). Each antenna was challenged with six doses, in ten-fold increments from 0.1 ng to 10 µg, of *Z*11-14:Ac (97.5% purity, Sigma Chemical Company, St. Louis, MO), *Z*9-14:Ac (97% purity, Regine Gries, Simon Fraser University, BC), and a 94:6 ratio blend of both, respectively. The amount of *Z*11-14:Ac remained equal between the treatments of the main component and the blend; female antennae were also exposed to 100 µg doses from both *Z*11-14:Ac and the blend. Each antenna was exposed to each dose of each of the three compounds in order of increasing concentration. Treatments were puffed (200 ms; 10 ml per s) over each antenna, beginning with the lowest dose (0.1 ng) of each of the three compounds, which were presented in random order at each incremental dose. Baseline antennal responses were established using HPLC-grade hexane (two puffs) and a puff of the plant volatile, (*E*)-2-hexanal in paraffin oil, before and after each pheromone test concentration. All stimulus puffs were applied at 30 s intervals. All chemical stimuli were dispensed in 10 µl aliquots onto folded Whatman #5 filter paper (3 cm × 2 cm), placed in pipette tubes and connected to the puffer. The procedure was repeated using six female antennae and four male antennae.

Normalized percentage antennal deflections were calculated using the plant volatile response as the standard. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test to compare each treatment mean to the hexane control. All experimental error rates were set at $\alpha = 0.05$. Comparisons between female and male antennal responses at each dose were conducted on individual antennal deflections (mV) by first performing a two-way ANOVA, where the factors were sex and pheromone stimulus. A sex × pheromone stimulus interaction term was in-

cluded in the model. Following a significant ANOVA, linear contrasts using *t*-ratio statistics were used to make paired comparisons of mean antennal deflections for males and females at each dose. Experiment-wise error rates for these multiple-paired comparisons were fixed at 5% by adjusting α values for individual comparisons using the Bonferroni inequality. All statistical tests were performed with JMP 5.1 (2003).

Female behaviour in a pheromone-permeated environment. Female moths were placed in clean air or a pheromone-permeated environment and observed in side-by-side comparisons. To accomplish this, a sheet metal divider (73.3 cm × 37.4 cm) was installed vertically in the middle of the upwind portion of a pulling-style flight tunnel [75 cm wide × 73.3 cm high × 187 cm long flight section] covered with a replaceable 4 ml transparent polyester 'skin' (3M Canada Company, London, ON) (Judd *et al*. 2005), creating two identical isolated chambers (upwind area = 36.65 cm^2). The upwind cross-sectional surface was constructed of a set of replaceable horizontal sheet metal panes (73.3 cm × 3.7 cm with a 0.7 cm bend for stability) onto which pheromone was applied, with an untreated G200 Filtrete® with 0.5 oz Coverweb® (3M Canada Company, London, ON) that smoothed airflow, sealing the tunnel ca. 20 cm upwind of the panes. Smoke tests confirmed that air was drawn directly into each chamber at the upwind end and immediately moved downwind in a linear fashion through the tunnel. After observing females in both chambers in clean air, one chamber was randomly chosen to receive pheromone (10 mg ai · m^{-2}), which was applied to horizontal metal panes forming the upwind end of the tunnel. As a precaution, pheromone was not applied to a 1.5 cm strip immediately adjacent to the untreated chamber, creating a buffer to ensure no pheromone entered the clean-air portion of the tunnel. Untreated panes were shielded during all pheromone applications and the tunnel was allowed to ventilate for a minimum of 18 h between replicates. Pheromone treatment consisted of Phase I MEC *Z*11-14:Ac (3M Canada Company, London, ON) applied at a rate equivalent to 10 mg ai · m^{-2} to the cross-sectional area of the tunnel as described in detail by Judd *et al*. (2005).

Virgin female moths (0 to 24 h old) were chilled for 5 min at 2 °C and transferred individually into stainless steel mesh observation chambers (4.5 cm W × 3.5 cm H × 5 cm D). Chambers were stacked vertically to form a series of five individually-caged females. Treated and control portions of the tunnel each received one series of five individually-caged females ca. 2.5 h before scotophase, for a total of 15 females in each treatment. Females were observed for calling (raised wings with protruding abdomen) and movement (physically walking in the chamber) every 15 m until the initiation of scotophase, when they were observed continually for 3 h. Data gathered before scotophase was used solely for determination of onset of calling behaviour and was not included in any other analysis.

Frequencies, or the number of discrete observations, of calling and movement were analyzed separately using a two-factor ANOVA where treatment (*Z*11-14:Ac- and clean-air) and vertical position of female were the factors (JMP 5.1 2003). Onset and duration of calling were analyzed using a two-sided Wilcoxon Signed Ranks Test (JMP 5.1 2003), as the assumption of normality was not met for these data. The tunnel was assessed for positional bias between the two sides with respect to each behaviour before the application of pheromone and resulting data were analyzed as above.

RESULTS

Female and male detection of synthetic pheromone. EAGs confirmed that adult female *P. limitata* can perceive both components of their sex pheromone ($F_{21,224}$ = 19.40; $P < 0.0001$). However, antennal deflections significantly differed from those of the hexane control only for doses of 10 µg or greater for *Z*11-14:Ac and the 94:6

ratio of *Z*11-14:Ac: *Z*9-14:Ac (Fig. 1A). Response to *Z*9-14:Ac differed from the hexane control at each of the extreme doses tested, 0.1 ng and 10 µg, but did not differ from any of the doses in between (Fig. 1A). Antennae of male *P. limitata* were more sensitive to the pheromone components than female antennae (with the exception of 0.1 ng *Z*9-14:Ac), responding with deflections significantly higher than those to hexane at 1 µg for each individual compound and as low as 100 ng for the blend (Fig. 1B). Male response to the plant volatile was also significantly higher than their response to hexane (Fig. 1B). Females had significantly ($F_{1,19}$ = 125.85; $P < 0.0001$) lower antennal deflections (least squares mean ± SE: -6.13mV ± 0.30mV) when compared to males (least squares mean ± SE: -11.41mV ± 0.36mV). Comparisons of female and male antennal responses to individual doses showed that females had significantly ($F_{20,352}$ = 25.11; $P < 0.0001$) lower antennal deflections for 10 µg *Z*9-14:Ac, and for both 1 µg and 10 µg of *Z*11-14:Ac and the 94:6 ratio of *Z*11-14:Ac: *Z*9-14:Ac. Male antennal response to the plant volatile was also significantly higher than the female's ($F_{20,352}$ = 25.11; $P = 0.00134$).

Female behaviour in a pheromone-permeated environment. Observations of females in clean air and *Z*11-14:Ac-permeated environments showed that there were no significant differences in onset of calling ($P > 0.934$), frequency of calling ($F_{1,24}$=0.6859; $P \geq 0.4157$), or duration of calling ($P > 0.890$) (Fig. 2) among females. However, females moved significantly less in the *Z*11-14:Ac environment ($F_{1,11}$=5.0999; $P \leq 0.0452$) than in clean air (Fig. 2). There was no observed positional bias in the tunnel for either chamber with respect to any of the observed behaviours [($P \geq 0.152$), ($F_{1,14}$=3.5383; $P \geq 0.0809$), ($P \geq 0.193$) and ($F_{1,10}$=1.1703; $P \geq 0.3047$) respectively]. No vertical positional effects were observed for frequency of calling or movement [($F_{1,24}$=0.9835; $P \geq 0.4352$) and ($F_{1,11}$=0.8725; $P \geq 0.5107$) respectively].

DISCUSSION

We have found that adult female *P. limitata* (0 to 24 h old) are able to perceive both of their pheromone components; however, significant responses to the main component alone or in a blend were only detected for doses of 10 µg or greater, in contrast to males, which responded to 1 µg of the individual compounds and to 100 ng of the blend. Interestingly, female antennae gave a significant response to only the lowest (0.1 ng) and the highest (10 µg) doses of the minor component *Z*9-14:Ac. It is possible that as treatments were presented randomly in increasing concentrations, 0.1 ng *Z*9-14:Ac would have contacted a relatively 'fresh' antenna compared to subsequent doses, and as *Z*9-14:Ac is the minor pheromone component, occurring at approximately 6 to 9% in the female gland (Roelofs *et al*. 1976), the antennae may be able to perceive it at a lower level than *Z*11-14:Ac; however, a similar phenomenon was not observed for the blend or for the males.

The ability of female *P. limitata* antennae to perceive their own pheromone components, albeit at high doses, is not surprising as this has been shown for other tortricids. Palaniswamy and Seabrook (1978) noted that female *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae) displayed threshold response levels corresponding to pheromone concentrations above which various behavioural patterns became evident, such as increased walking, extension of the ovipositor and antennal grooming. Ross *et al*. (1979) examined response of female *C. fumiferana* antennae to differing doses of pheromone and with increasing age, finding that females have a higher threshold for pheromone response than males and that female antennae are at their peak responsiveness in 3 to 6 d old insects. Higher response thresholds may be explained by the fact that antennae of female *C. fumiferana* have one-third to one-half the number of sensilla trichodea as

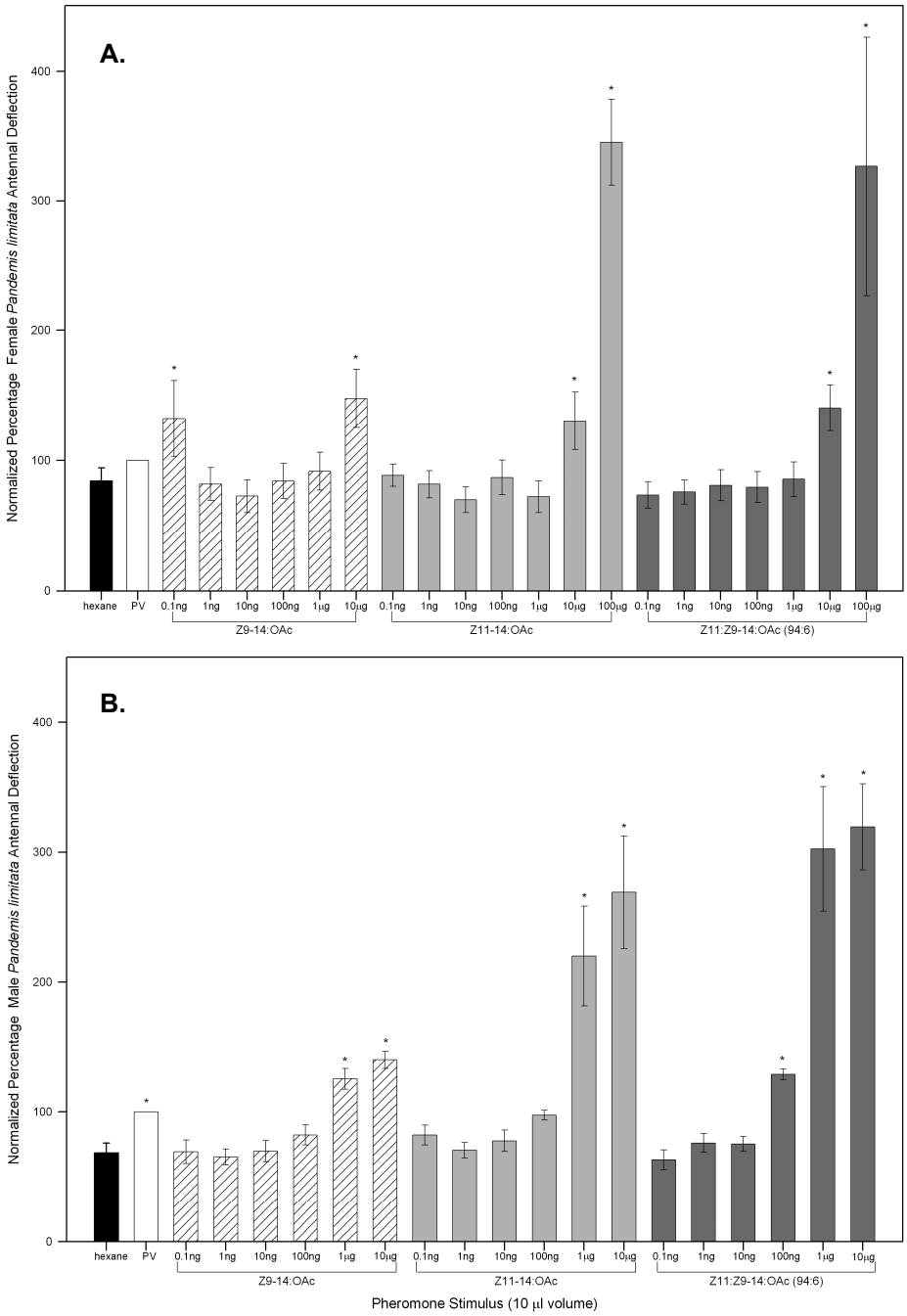

Figure 1. Mean (± SE) normalized electroantennogram responses of antennae from 0 to 24 h old A) female (n = 6) or B) male (n = 4) *Pandemis limitata* to synthetic (*Z*)-11-tetradecenyl acetate (Z11-14:OAc) or (*Z*)-9-tetradecenyl acetate (Z9-14:OAc), or both in a ratio of 96:4. (*E*)-2-hexanal was puffed over each antenna before and after each pheromone source for the purpose of normalization of each antenna over time and HPLC-grade hexane was puffed over each antenna at the beginning and end to establish a base response. All stimulus puffs were spaced by 30 s intervals. Normalized percentage deflections were calculated using the plant volatile (PV) response as the standard. Asterisks indicate a significant difference ($P \leq 0.05$) from the hexane base response as determined by an ANOVA followed by Dunnett's test, treatment versus hexane ($\alpha = 0.05$).

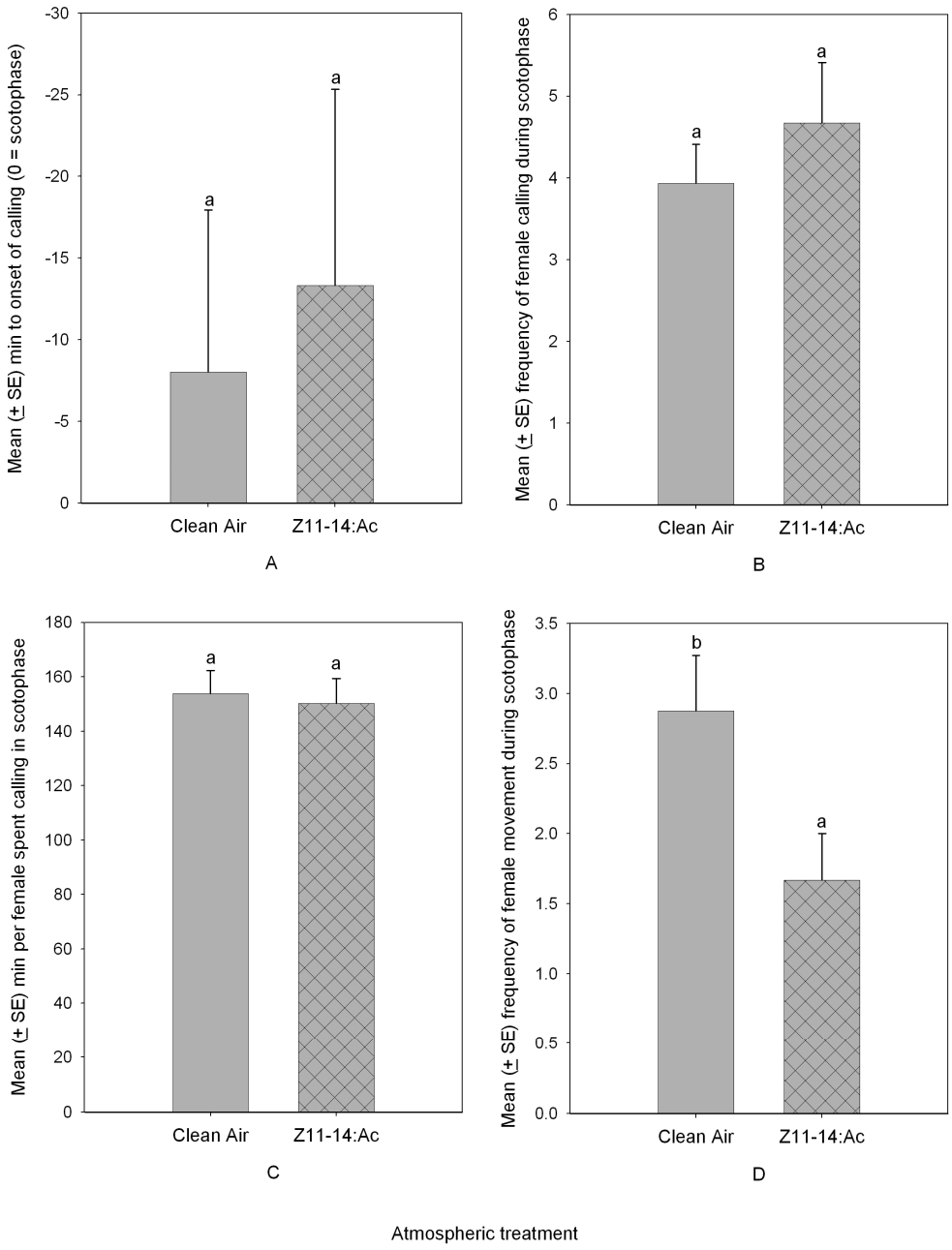

Figure 2. Mean (+ SE) duration (A and C) or frequency (B and D) of 0 to 24 h old female *P. limitata* in either side of the divided permeation tunnel, exposed to either clean air or (*Z*)-11-tetradecenyl acetate (Z11-14:Ac) released from 3M microcapsules applied to the upwind portion of one side of the tunnel. Individually-caged females were placed in the tunnel (n = 15), immediately before pheromone application ca. 2 h before scotophase. Females were observed every 15 m from application until initiation of scotophase for onset of calling, and continuously once scotophase began for frequency and duration of calling and frequency of movement. For each behaviour observed, bars with the same letter are not significantly different ($P > 0.05$).

male antennae (Albert and Seabrook 1973). In *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), Light and Birch (1979) found that antennal response in females was 25% that of males. Our results show that antennal responses in female *P. limitata* over a wide range of pheromone doses is 46.7% less than that of males, with significant differences observed at high doses for all compounds tested. A difference was even observed for the plant volatile.

Simultaneous observations of females in clean and pheromone-permeated air, from ca. 2 h before initiation of scotophase until 3 h into scotophase, showed no difference in onset of female calling time (Fig. 2A). However, a larger sample size may reveal that females in environments permeated with *Z*11-14:Ac do initiate calling earlier as there was a large variance among females. No differences were detected with respect to duration or frequency of calling once scotophase began (Fig. 2B, C). We did not have the ability to measure female output of pheromone directly in a background of *Z*11-14:Ac, therefore we must rely on observations of behaviour to give us an indication of female response. We did not observe changes in frequency or duration of calling, as one might expect if females were altering concentrations or outputs of their effluvia to compete with the level of compounds perceived in the background (N.C.D., unpublished data). Of course, these changes, as well as a change in onset of calling, may not be observable in the first 3 h of scotophase, only becoming apparent after the female has been in the environment continuously for more than 24 h (Palaniswamy and Seabrook 1985). In fact, studies on female *C. fumiferana* have found that female EAG responses to their sex pheromone increase with age at least until three days after emergence (Palaniswamy and Seabrook 1978), indicating that females may become more sensitive to their pheromone over time. As we only looked at response in 0 to 24 h old *P. limitata* females for a 5 h period, we would not have observed such a phenomenon if one existed.

Our observations showed females moved less often in air permeated with Z11-14:Ac than females in the clean air (Fig. 2D). It is possible that the tendency to remain still in the pheromone background may translate, in time, to increased calling, as observed in *C. fumiferana* (Palaniswamy and Seabrook 1985). Sanders (1987) found that although pheromone in the background clearly increased flight activity of both virgin and mated females, virgin females remained inactive for 48 h after emergence, even in the presence of the pheromone. As we observed increased movement of females in clean air, this does not appear to be happening in *P. limitata*, although observations over time would determine if the level of activity increases with age.

Similar to our results in *P. limitata*, El-Sayed and Suckling (2005) found that permanent exposure of female *Eupoecillia ambiguella* (Hübner) (Lepidoptera: Tortricidae), *Lobesia botrana* (Denis and Schiffermüller) (Lepidoptera: Tortricidae), or *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) to their main pheromone compounds did not alter the timing, duration or frequency of calling. In addition, Weissling and Knight (1996) found that the temporal pattern of calling in *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) was unaffected by the major component of their pheromone; however, the likelihood of calling was increased. Nevertheless, other species have been found to initiate calling earlier in pheromone environments, such as female *C. fumiferana* (Palaniswamy and Seabrook 1985). At the other extreme, Evenden (1998) observed a delay in calling for female *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) in field plots treated with their complete pheromone blend, followed by a potential reduction in time spent calling compared to their counterparts in clean air. It is important to remember that our experiments, as well as those of El-Sayed and Suckling (2005), did not use the complete pheromone blend in the background, which could have a greater impact on female response.

While experiments described here do

not encompass all of the potential factors that may impact females in a pheromone environment, such as background pheromone dose, completeness of pheromone blend, age of females or changes in mating status, our results are relevant to previous work on disruption of pheromone communication in *P. limitata* (Judd *et al*. 2005; N.C.D., unpublished data). The presence of the major pheromone component (*Z*11-14:Ac, applied at 10 mg ai · m^{-2}) does not appear to cause female *P. limitata* to change their calling behaviour, except with respect to frequency of movement, during the first 3 h of the first scotophase. As such, alternative explanations, such as mode of delivery of the gland extract, as well as the use of nonchemical cues to attract males like sound or vision (Castrovillo and Cardé 1980), or even chemical cues not associated with the sex pheromone gland, need to be explored to determine what cues become important to males as they search for female moths in pheromone-permeated environments (N.C.D., unpublished data).

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