2. *Material & Methods*

2.1 *Insects.*

Pupae were obtained from an insecticide-susceptible laboratory colony (Pree *et al.*, 1998). The larvae were reared on 3–4 cm diameter green apples that had not been sprayed with insecticide (Pree, 1985). Male and female pupae were held separately for emergence at 23°C, 60% RH, and at a 16L:8D photoperiod in a Plexiglas cages (33 x 33 x 33 cm, H x V x L). Adults of both sexes were maintained isolated from each other in separate rooms.

2.2 *Phermone and citral.*

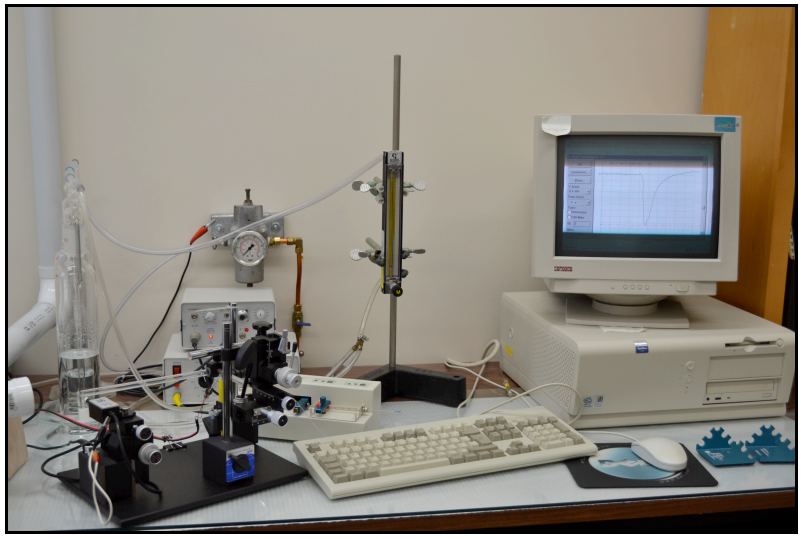
The main component of *G. molesta* sex pheromone, (*Z*)-8-dodecen-1-yl acetate ((*Z*)-8-12:OAc) (Roelofs *et al.*, 1969) was obtained from the Pherobank, Plant Research International, Wageningen, The Netherlands. It was 99.0% chemically pure and contained 0.2% (*E*)-8-dodecen-1-yl acetate ((*E*)-8-12:OAc). Citral (3,7-dimethyl-2,6-octadienal) was obtained from Sigma-Aldrich Canada, Oakville, Ontario. It had ≥ 96% chemical purity.

2.3 *Pheromone dose-EAG response.*

The effect of pheromone stimulus dose on electroantennogram (EAG) response of ten antennae of 2-3 days old male *G. molesta* was determined using the Syntech (Hilversum, The Netherlands) EAG system (Fig. 25) described by Trimble and Marshall (2007). Airflow of 2 L/min was delivered to the antennal preparation through a 30 cm-long glass air delivery tube with a single 2 mm-diameter hole 10 cm from the outlet. Test stimuli were applied to a 1 x 5 cm piece of Whatman® No. 1 filter paper (Whatman International Ltd, U.K.) in 50 µL of 99.9% pure ethanol (Commercial Alcohols Inc., Brampton, Ontario). The filter paper rectangles were placed in a ≈ 3 mL-capacity Pasteur pipette after the solvent had evaporated for 30 min in a fume hood. A test stimulus was delivered during 0.5 s in a 5 mL “puff” of air while the tip of Pasteur

pipette was inserted through the hole in the air delivery tube. An antenna was first stimulated with the control (i.e. 50 µL ethanol) and then with ten increasing doses of (*Z*)-8-12:OAc at 1 min intervals over the range 1.0 x 10^{-11} – 1.0 x 10^{-2} g of pheromone. The stimulus source was renewed after 4 h of use.

Figure 25: Electrophysiology (EAG) equipment.



2.4 *Effect of citral on EAG response to pheromone.*

The EAG response of ten male OFM antennae to mixtures of (*Z*)-8-12:OAc and citral was measured using the same EAG system and methods used to determine the effect of (*Z*)-8-12:OAc dose on EAG response. An antenna was first stimulated with the control (i.e. 50 µL ethanol). After a 1-min recovery period it was stimulated with 10 µg (*Z*)-8-12:OAc, and at 1-minute intervals thereafter with 10 µg (*Z*)-8-12:OAc combined with 1 (0.1 µg), 10 (1.0 µg), 100 (10 µg), 1000 (1000 µg) and 10,000% (1,000 µg) citral. The effect of repeated stimulation with pheromone alone on EAG response was measured in ten antennae. An antenna was first stimulated

with the control and after a 1-min recovery period with 10 µg (*Z*)-8-12:OAc six times at 1-minute intervals.

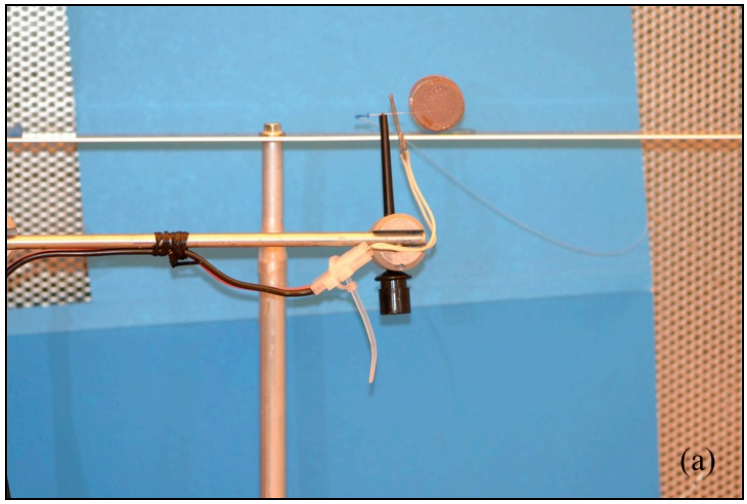
2.5 *Effect of citral sexual response of males to females and synthetic pheromone lure.*

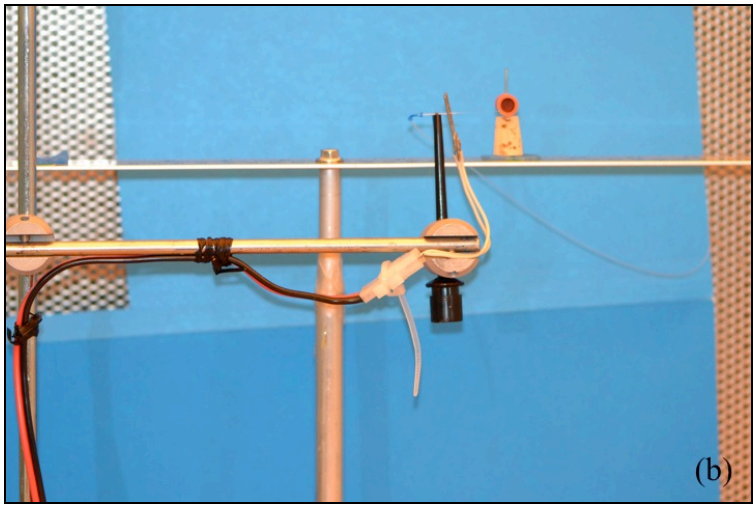
The effect of citral on the response of an OFM male to a virgin, calling OFM female or to a Trécé Pherocon OFM synthetic pheromone lure (Trécé Inc., Salina, CA) (Fig. 26) was observed in an acrylic plastic flight tunnel (55.5 x 87 x 160 cm, H x W x L) (El-Sayed *et al.*, 2001) using an air velocity of 30 cm/s, temperature of 22–24° C and 50–70% RH. Light intensity was 75 on the floor of the tunnel and 150 lx at the release point of the males. The pheromone sprayer (Fig. 27) developed by El-Sayed *et al.* (1999) and modified by Trimble and Marshall (2007) to permit the atomization of pheromone in ethanol solutions at rates as low as 0.125 µL/min was used to dispense citral. The sprayer components are a microdialysis pump, a 50 µL gas-tight syringe connected to an atomization nozzle with fluorinated ethylene propylene (FEP) tubing, and a function generator that excites a piezo-electric bending motor attached to the nozzle. Experiments were conducted during the three hours before the onset of the scotophase when OFM males and females exhibit the greatest sexual activity (Baker and Cardé, 1979). One hour before the test period, a 1–2 day-old virgin female was placed into a glass tubing "cage" (2 x 2 cm, L x D) that was closed at each end using 0.8 x 0.8 mm-mesh copper screen. Two to 3-day-old males were placed individually in glass release tubes (15 x 2.5 cm, L x D) and the ends of the tubes were closed using cotton wool. These tubes were placed on the floor of the flight tunnel for acclimatization. The female-containing cage or a synthetic pheromone lure was placed on a stand 7.5 cm from the upwind end and 35 cm above the line centre of the floor of the flight tunnel. The lure was removed from storage at 4°C and held for 72 h at 23°C in a fume hood before use in an experiment. A plume of titanium dioxide produced by a small amount of titanium tetrachloride placed at the female/lure position was approximately 35 cm above the floor at the end of the tunnel. It had a cross sectional area of approximately 5 cm. A sprayer nozzle was positioned 1 cm directly downwind from the female containing cage or synthetic pheromone lure.

An 8,000 ng citral/µL ethanol solution was atomized from the nozzle at a rate of 0.125 µL/min for an effective release rate of 1,000 ng citral/min. The average release rate of the Isomate®-OFM Rosso pheromone dispenser used for mating disruption of OFM is approximately 1,000 ng/min (Trimble *et al.*, 2004). The atomization of 0.125 µL ethanol/min was used as a control treatment. A complete pheromone delivery system including a syringe, FEP line and sprayer nozzle were dedicated to the citral and control treatment.

When the female was calling the experiment was begun. A release tube and its contained male moth were placed in the cradle of a stand at the downwind end of the tunnel approximately 130 cm from the calling female or lure. The tube was 35 cm above the floor of the tunnel and located within the space where the plume of titanium dioxide had been observed.

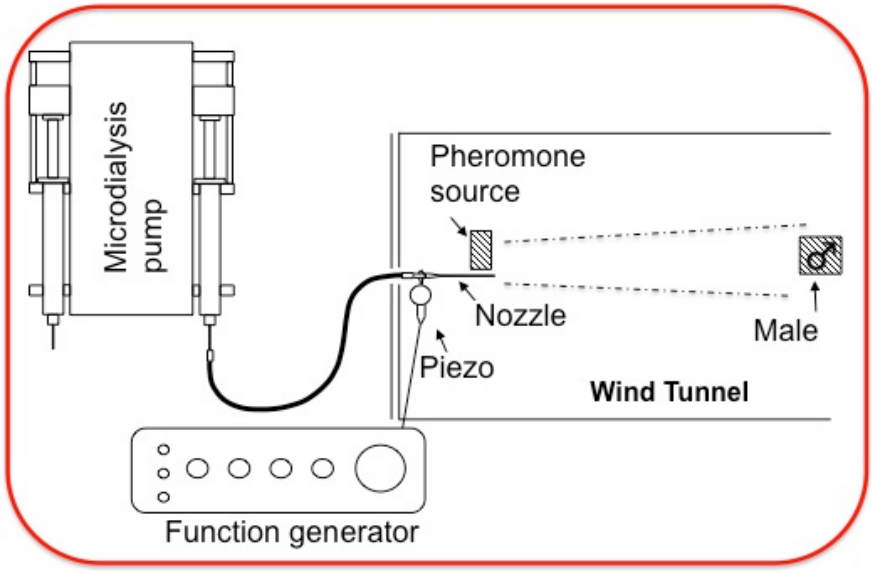
Figure 26: Wind tunnel equipment; pheromone source: virgin calling female (a) and synthetic pheromone lure (b).





An observation was initiated by removing the cotton wool from the openings of the tube. The time for a moth to become activated (wing-fanning and walking in the release tube), and to initiate the take-off (beginning of flight in any direction), lock-on (beginning of upwind flight for at least 10–15 cm), close-in (upwind flight to 10–15 cm from the calling female) and the touchdown (landing on the cage containing the female) phases of upwind flight (El-Sayed *et al.*, 1999) was recorded using The Observer® XT Version 7.0 software (Wageningen, The Netherlands) and a personal computer. The response of 40 males to a virgin calling female or lure was observed for both the citral and control treatments. The observations for each source of pheromone were carried out in four sessions. Ten males were tested with one treatment (i.e. citral or control) and another ten males were tested with the second treatment. The order of use of treatments was rotated between sessions. The pheromone lure was used during two consecutive days. The glass tubing cage for holding the female was washed and rinsed with acetone before being reused, and the glass tubes for the release of males were washed, rinsed in acetone and heated to 300°C for 8 h before being reused.

Figure 27: Wind tunnel equipment to study the effect of citral on sexual response of OFM males to females and synthetic pheromone lure.

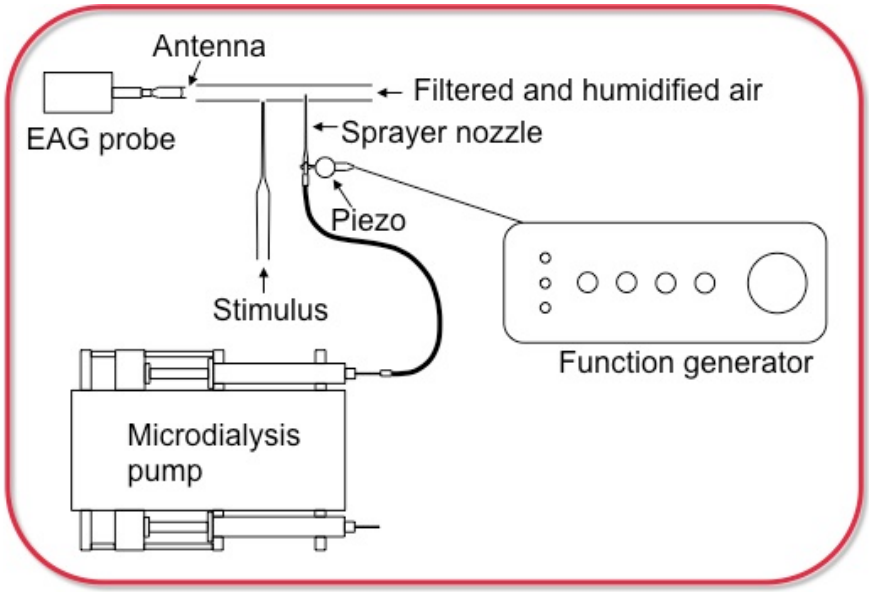


2.6 *Effect of pre-exposure of antennae to citral and citral combined with pheromone on EAG response to pheromone.*

The EAG system (Fig. 28) and methods described by Trimble and Marshall (2010) for inducing and measuring sensory adaptation in moth antennae were used to compare the amount of sensory adaptation in male OFM antennae after 15 min of exposure to (*Z*)-8-12:OAc, citral, and two mixtures of these compounds. The tip of the pheromone sprayer nozzle was positioned in the middle of the EAG air delivery tube through a second 2 mm-diameter hole located 20 cm from the outlet end of the tube (Fig. 29). Ethanol and the solutions of ethanol and test compounds were atomized at 0.125 µL/min. Antennae were exposed to the following treatments: control 1 (air – standard humidified and activated carbon-filtered airflow at 2 L/min), control 2 (air + ethanol at 6.25 µL/mL air), (*Z*)-8-12:OAc (air + ethanol + (*Z*)-8-12:OAc at1.0 x 10^{-6} ng/mL air), citral (air + ethanol + citral at1.0 x 10^{-6} ng/mL air), (*Z*)-8-12:OAc + citral (1:1)

(air + ethanol + (*Z*)-8-12:OAc + citral, i.e. (*Z*)-8-12:OAc + citral), and to (*Z*)-8-12:OAc + citral (1:100) (air + ethanol + (*Z*)-8-12:OAc + citral at 1.0 x 10^{-4} ng/mL air, i.e. (*Z*)-8-12:OAc + citral). The 1.6 x 10^{-5} mg (*Z*)-8-12:OAc/mL ethanol solution delivered at 0.125 µL/min into the airflow of 2 L/min produced a resultant aerial concentration of 1.0 x 10^{-6} ng pheromone/mL air (i.e. 1 ng pheromone/m^3 air) (Trimble and Marshall, 2010).

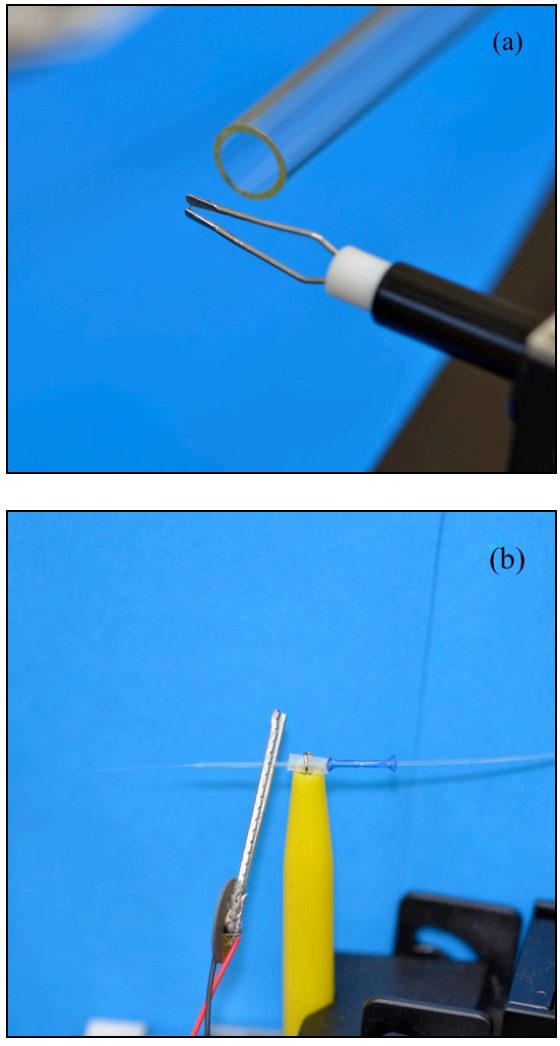
Figure 28: EAG equipment to study sensory adaptation of OFM male antenna.



Treatments were randomly selected and five antennae were tested with each treatment. An experiment was begun by first measuring an EAG response to the control (i.e. 50 µL ethanol) and then 1 min later to 10 µg (*Z*)-8-12:OAc. Exposure to one of the six treatments was begun within 15 s after measuring the EAG response to pheromone by inserting the spray nozzle into the air delivery tube. After 15 min of exposure to the treatment a second EAG response to the control was measured and 1 min later a second EAG response to the pheromone stimulus was measured. A complete treatment delivery system including a 50 µL-capacity syringe,

FEP line, sprayer nozzle and air delivery tube were dedicated to each treatment. The air delivery tube was washed and rinsed with acetone after use.

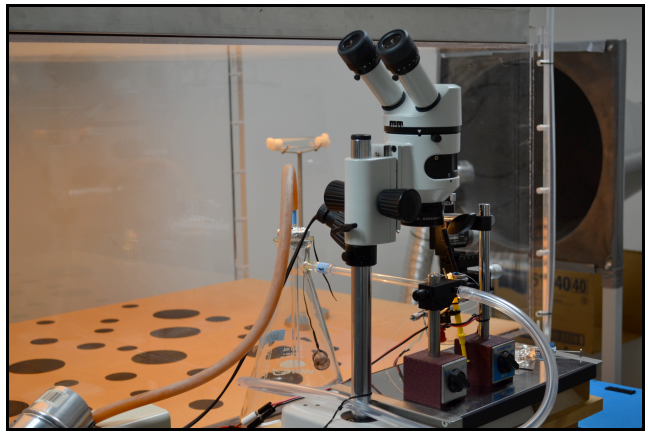
Figure 29: OFM male antenna on probe (a) and nozzle connected to the piezo (b).

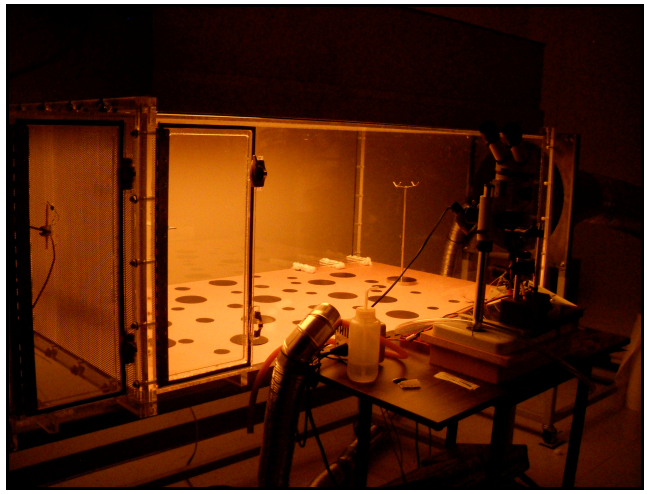


2.7 *Effect of pre-exposure of males to citral and citral combined with pheromone on male response to females.*

The response of a male OFM to virgin calling OFM females after 15 min of exposure to the same treatments used in the sensory adaptation experiment was compared in the previously described flight tunnel and experimental conditions (Fig. 30).

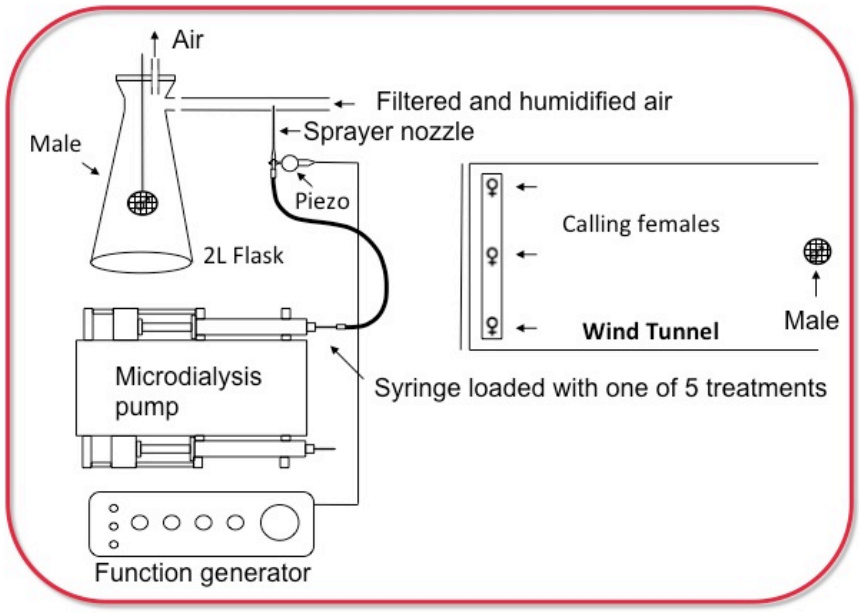
Figure 30: Wind tunnel equipment to study effect of pre-exposure of citral and citral combined with pheromone on OFM male response to females.





The pheromone sprayer was used to condition air with test chemicals (Fig. 31). A 2 L-capacity Büchner (vacuum) flask was fitted with an aluminium foil-covered rubber stopper with a hole through which a 25 cm long glass exhaust tube (8 mm inside diameter, 10 mm outside diameter) was inserted. The glass tube was approximately 10 cm from the bottom of the flask with the stopper firmly in place. Approximately 4 cm of the tube extended from the top of the stopper. A 25 cm-long glass air delivery tube (8 mm inside diameter, 10 mm outside diameter) was attached to the 3 cm-long, vertical vacuum line connecting tube of the flask using a 3.5 cm-long section of Tygon® SE-200 inert tubing (9.5 mm inside diameter) that was lined with fluorinated ethylene propylene (FEP) (Saint-Gobain Performance Plastics Inc., Paris, France). A micromanipulator was used to position the tip of the sprayer nozzle in the centre of the air delivery tube via a 2 mm-diameter hole located 16 cm from the tube's connection with the flask. Activated carbon-filtered and humidified air was pumped to the air delivery tube via Tygon® SE-200 inert tubing at a rate of 2L/min. As in the experiment where antennae were treated with pheromone, citral or pheromone + citral, the 1.6×10^{-5} mg (*Z*)-8-12:OAc/mL ethanol solution delivered at 0.125 µL/min into the airflow of 2 L/min produced a resultant aerial concentration of 1.0×10^{-6} ng pheromone/mL air (i.e. 1 ng pheromone/m^3 air). Air was passively vented from the flask by connecting a surgical rubber tube to the glass exhaust tube. This rubber tube was connected to the down wind air purification filters of the flight tunnel (El-Sayed *et al.*, 2001).

Figure 31: Wind tunnel equipment to study sensory adaptation of OFM males.



One hour before the test period, 1–2 day-old females and 2–3 day-old males were placed individually into the previously described glass tubing cages. Cages holding males were placed in a sealed glass container on the floor of the flight tunnel for acclimatization. Three female-containing cages were placed on a stand 7.5 cm from the upwind end and 35 cm above the floor of the flight tunnel. One cage was positioned above the centre line of the tunnel (centre female) and the other two were positioned 20 cm to the right (right female) and left (left female) of the centre female. Plumes of titanium dioxide produced by a small amount of titanium tetrachloride placed at the right, centre and left female positions had an estimated cross-sectional diameter of approximately 5 cm at the end of the tunnel and did not converge. An experiment was begun when each of the three females were observed to be calling. A male-containing cage was attached to a piece of wire, suspended in the Büchner flask approximately 15 cm from it's bottom and held in place by firmly inserting the aluminium foil-covered rubber stopper into the flask. The male was exposed to a randomly chosen treatment for 15 min. After

treatment the cage was removed from the flask and the male was immediately transferred to a glass release tube (15 x 2.5 cm, L x D) and the ends of the tube were closed using cotton wool. A tube and its contained male moth were then placed in the cradle of a stand at the downwind end of the tunnel approximately 130 cm from the centre female. The tube was 35 cm above the floor of the tunnel and positioned it within the space where the plume of titanium dioxide had been observed. An observation was initiated by removing the cotton wool from the openings of the tube. The time for a moth to initiate each of the five previously described upwind flight behaviors was recorded using The Observer® XT software (Wageningen, The Netherlands) and a personal computer. If the male did not become activated within 1 min, or did not touchdown at a female, it was re-tested using the right female, and if necessary using the left female. Marks on the floor of the tunnel facilitated correct positioning of the stand used to hold the glass release tube. A complete pheromone delivery system including a syringe, FEP line, sprayer nozzle, air delivery tube and flask were dedicated to each treatment. The air delivery tube and flask were washed and rinsed with acetone after the treatment of two males. The glass tubing cages for holding males and females, and glass tubes for release of males were washed, rinsed in acetone and heated to 300°C for 8 hours before being reused. The experiment was repeated fifteen times.

2.8 *Statistical analysis of data.*

Statistical analysis was performed using JMP® 7.0 (SAS Institute, Cary, North Carolina). EAG responses and the times to initiate a behavioral response were tested for goodness-of-fit of to the normal distribution using the *Shapiro-Wilk W* test. The homogeneity of the variances of was tested using *Bartlett's* test. Parametric analysis was used to test the significance of treatment effect when data were normally distributed and variances of means were homogeneous. Nonparametric analysis was used if one or both of these criteria was not fulfilled. The significance of (*Z*)-8-12:OAc dose on EAG response was tested using *Randomized Complete Block Analysis of Variance* (ANOVA). The *Tukey* test was used to identify significantly different mean EAG responses. First- (linear) and

second-order (quadratic) *Polynomial Regression Analysis* was used to determine if there was a relationship between EAG response and the ratio (*Z*)-8-12:OAc:citral expressed as $\log_{10}$ (% citral). The EAG response to (*Z*)-8-12:OAc alone was excluded from this analysis. This same analysis was used to determine if there was a relationship between EAG response and time of administration of six consecutive stimulations with (*Z*)-8-12:OAc alone. The significance of the effect of combining increasing amounts of citral with (*Z*)-8-12:OAc on mean EAG response was tested using multivariate repeated measures ANOVA by modelling the six (*Z*)-8-12:OAc plus 0–10,000% citral treatments as separate factors (Lehman *et al.*, 2005). Contrasts were used to test the significance of the differences in mean EAG response to each of the six treatments. This same analysis was used to determine if mean EAG response was affected by the time of administration of a (*Z*)-8-12:OAc stimulus. The significance of the effect of citral on the total number of males initiating each of the five behavioral phases of upwind flight to a virgin calling female or synthetic pheromone lure was tested using Pearson's chi-square test. The *Wilcoxon-Mann-Whitney* test was used to test the significance of the effect of citral on the mean time required to initiate the behavior. The effect of the control, pheromone, citral, and pheromone + citral treatments on mean EAG response to the control stimulus and net EAG response to the pheromone stimulus (i.e. EAG response to pheromone stimulus – EAG response to control stimulus) was tested using the paired *t*-Test. The % reduction in net EAG response to the pheromone stimulus was computed for each antenna exposed to one of the six treatments as [((net pre-treatment EAG response – net post-treatment EAG response)/net pre-treatment EAG response)*100] (Trimble and Marshall, 2007). The significance of the effect of treatment on differences in mean % reduction in response was tested using *One-way* ANOVA. The effect of the control, pheromone, citral, and pheromone + citral treatments on the number of males initiating each of the five behavioral phases of upwind flight response was tested using *Logistic Regression Analysis* and likelihood ratios were used to identify significantly different treatments (Freund *et al.*, 2003). In cases where all 15 of the tested moths initiated a behavior the analysis contingency table contained a structural zero (Ten Have, 2010). As recommended by Hosmer and Lemeshow (2000) these

treatments were eliminated from the analysis. *Logistic Regression Analysis* was also used to test the significance of treatment on the number of males initiating the behavior on the first attempt, i.e. exhibiting the behavior in response to the centre female. The *Kruskal-Wallis* test was used to test the significance of treatment on the mean time required to initiate the behavior.

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