Electrophysiological and Behavioral Responses of Oriental Fruit Moth to the Monoterpenoid Citral Alone and in Combination With Sex Pheromone

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ABSTRACT The monoterpenoid citral synergized the electroantennogram (EAG) response of male *Grapholita molesta* (Busck) antennae to its main pheromone compound Z8–12:OAc. The response to a 10-μg pheromone stimulus increased by 32, 45, 54, 71 and 94% with the addition of 0.1, 1, 10, 100 and 1,000 μg of citral, respectively. There was no detectable response to 0.1, 1, or 10 μg of citral; the response to 100 and 1,000 μg of citral was 31 and 79% of the response to 10 μg of Z8–12:OAc. In a flight tunnel, citral affected the mate-seeking behavior of males. There was a 66% reduction in the number of males orientating by flight to a virgin calling female when citral was emitted at 1,000 ng/min $\approx$1 cm downwind from a female. Pheromone and citral induced sensory adaptation in male antennae, but citral did not synergize the effect of pheromone. The exposure of antennae to 1 ng Z8–12:OAc/m^3 air, 1 ng citral/m^3 air, 1 ng Z8–12:OAc + 1 ng citral/m^3 air, or to 1 ng Z8–12:OAc + 100 ng citral/m^3 air for 15 min resulted in a similar reduction in EAG response of 47–63%. The exposure of males to these same treatments for 15 min had no effect on their ability to orientate to a virgin calling female in a flight tunnel. The potential for using citral to control *G. molesta* by mating disruption is discussed.

KEY WORDS *Grapholita molesta*, citral, sex pheromone, sensory adaptation, sexual behavior

Sex pheromone-mediated mating disruption is an effective alternative to the use of insecticide for the control of some moth pests of field crops, orchards, and vineyards (Cardé and Minks 1995). Disruption is achieved by permeating a cropping environment with synthetic pheromone that is dispensed from atomizers, sealed plastic tubes, open-ended hollow fibers, laminated plastic flakes, or microcapsules (Cardé 2007). A reduction in the responsiveness of antennal sensory neurons to pheromone (sensory adaptation), the central nervous system (habituation), or both, and competition between synthetic and natural sources of pheromone (competitive attraction) have been proposed as modes of action of pheromone treatments (Bartell 1982; Cardé 1990, 2007; Cardé and Minks 1995; Sanders 1997; Miller et al. 2006a,b). The effectiveness of combining attractive and antagonistic pheromone compounds in mating disruption systems has been demonstrated (Witzgall et al. 1997, Hapke et al. 2001, Kirchert et al. 2001, Yang et al. 2004, Party et al. 2009). In addition, sex pheromones have been combined with plant volatile compounds in an attempt to augment the disruptive effect of pheromone. For example, the attractiveness of synthetic codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) pheromone was reduced by the addition of the monoterpenoid citral (Hapke et al. 2001) but no additional disruptive effect was detected in an apple (*Malus domestica* Borkh.) orchard when this compound was included in a dispenser with pheromone (Kirchert et al. 2001). The presence of citral also reduced the attractiveness of European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) pheromone in laboratory tests (Meiwald 1995). The use of several terpenes as inhibitors of the response of moths to pheromone has been patented (de Kramer et al. 2002).

The oriental fruit moth, *Grapholita molesta* (Busck) (Lepidoptera: Tortricidae) is a worldwide pest of stone and pome fruits (Rothschild and Vickers 1991) that can be effectively controlled by mating disruption (Trimble et al. 2004). Pheromone treatments for the control of *G. molesta* may cause peripheral nervous system adaptation (Baker et al. 1988; Trimble and Marshall 2007, 2010), central nervous system habituation, or both (Sanders and Lucuik 1996, Rumbo and Vickers 1997), as well as competitive attraction (Sanders and Lucuik 1996, Valeur and Löfstedt 1996, Maini and Accinelli 2001, Stelinski et al. 2004). We report a

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laboratory investigation of the potential for using citral alone or in combination with pheromone to modify the sexual behavior of male *G. molesta.* The electrophysiological and behavioral effects of short- and long-term exposure to citral, and to combinations of citral and pheromone were assessed using an electroantennogram technique and a flight tunnel.

Materials and Methods

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 a photoperiod of 16:8 (L:D) h in Plexiglas cages (33 by 33 by 33 cm; height by width by length) . Adults of both sexes were maintained isolated from each other in separate rooms.

Pheromone and Citral. The main component of *G. molesta* sex pheromone, (Z)-8-dodecen-1-yl acetate (Z8–12: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 (E8–12:OAc). Citral (3,7-dimethyl-2,6-octadienal) was obtained from Sigma-Aldrich Canada, Oakville, ON, Canada. It had a ≥96% chemical purity.

Pheromone Dose–EAG Response. The effect of pheromone stimulus dose on electroantennogram (EAG) response of 10 antennae of 2–3-d-old male *G. molesta* was determined using the Syntech (Hilversum, The Netherlands) EAG system described by Trimble and Marshall (2007). Airflow of 2 liters/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- by 5-cm piece of Whatman No. 1 filter paper (Whatman International Ltd., Kent, United Kingdom) in 50 μl of 99.9% pure ethanol (Commercial Alcohols Inc., Brampton, ON, Canada). 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 first was stimulated with the control (i.e., 50-μl ethanol) and after a 1-min recovery period (Trimble and Marshall 2007) with 10 increasing doses of Z8–12:OAc at 1-min intervals over the range 1.0 by 10^{-11} – 1.0 by 10^{-2} g of pheromone. The stimulus source was renewed after 4 h of use.

Effect of Citral on EAG Response to Pheromone. The EAG response of 10 male *G. molesta* antennae to mixtures of Z8–12:OAc and citral was measured using the same EAG system and methods used to determine the effect of Z8–12:OAc dose on EAG response. An antenna first was stimulated with the control (i.e., 50-μl ethanol). After a 1-min recovery period it was stimulated with 10-μg Z8–12:OAc, and at 1-min intervals thereafter with 10-μg Z8–12:OAc combined with 0.1-μg (1%), 1.0-μg (10%), 10-μg (100%), 100-μg (1,000%), and 1,000-μg (10,000%) citral. The effect of repeated stimulation with pheromone alone on EAG response was measured in 10 antennae. An antenna first was stimulated with the control and after a 1-min recovery period with 10-μg Z8–12:OAc six times at 1-min intervals. The effect of repeated stimulation with the control stimulus on EAG response was measured in 10 antennae by administering the control stimulus seven times at 1-min intervals. The EAG response to citral was measured in 10 antennae. An antenna first was stimulated with the control and then with 0.1, 1.0, 10, 100, and 1,000 μg of citral administered at 1-min intervals.

Effect of Citral on Sexual Response of Males to Females. The effect of citral on the response of a *G. molesta* male to a virgin, calling *G. molesta* female was observed in an acrylic plastic flight tunnel (55.5 by 87 by 160 cm; height by width by length) (El-Sayed et al. 2001) by using an air velocity of 30 cm/s, temperature of 22–24°C, and 50–70% RH. Light intensity was 75 lx on the floor of the tunnel and 150 lx at the release point of the males. The pheromone sprayer 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 were 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 excited a piezo-electric bending motor attached to the nozzle. Experiments were conducted during the 3 hr before the onset of the scotophase when *G. molesta* males and females exhibit the greatest sexual activity (Baker and Cardé 1979). One hour before the test period, a 1–2-d-old virgin female was placed into a glass tubing "cage" (2 by 2 cm, length by diameter) that was closed at each end using 0.8- by 0.8-mm-mesh copper screen. Two to 3-d-old males were placed individually in glass release tubes (15 by 2.5 cm, length by diameter) 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 was placed on a stand 7.5 cm from the upwind end and 35 cm above the centerline of the floor of the flight tunnel. A plume of titanium dioxide produced by a small amount of titanium tetrachloride placed at the female position was ≈35 cm above the floor at the end of the tunnel. It had a cross sectional area of ≈5 cm. A sprayer nozzle was positioned 1 cm directly downwind from the female containing cage. 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 (Vancouver, WA) used for mating disruption of OFM is ≈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 was dedicated to both the citral and control treatments. An experiment was begun by visually confirming that the female was calling. Calling

females spread their wings, elevate their abdomen, and invert their pheromone gland from the tip of their abdomen. A release tube and its contained male moth were placed in the cradle of a stand at the downwind end of the tunnel ≈130 cm from the calling female. 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. 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) were 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 was observed for both the citral and control treatments. Ten males were tested with one treatment (i.e., citral or control) and another 10 males were tested with the second treatment during one session. The order of use of treatments was rotated between sessions. 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.

Effect of Preexposure of Male Antennae to Pheromone, Citral, or Citral Combined With Pheromone on EAG Response to Pheromone. The EAG system 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 *G. molesta* antennae after 15 min of exposure to Z8–12: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. 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 liters/min), control 2 (ethanol: air + ethanol at 6.25 μl/ml air), Z8–12:OAc (air + ethanol + Z8–12:OAc at 1.0 by 10^{-6} ng/ml air), citral (air + ethanol + citral at 1.0 by 10^{-6} ng/ml air), Z8–12:OAc + citral (1:1) (air + ethanol + Z8–12:OAc + citral), and to Z8–12:OAc + citral (1:100) (air + ethanol + Z8–12:OAc + citral at 1.0 by 10^{-4} ng/ml air). The 1.6×10^{-5} mg Z8–12:OAc /ml ethanol solution delivered at 0.125 μl/min into the airflow of 2 liters/min produced a resultant aerial concentration of 1.0 by 10^{-6} ng pheromone/ml air (i.e., 1 ng pheromone/m^3 air) (Trimble and Marshall 2010). Treatments were selected randomly 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 Z8–12: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.

Effect of Preexposure of Males to Pheromone, Citral, or Citral Combined With Pheromone on Male Response to Females. The response of a male *G. molesta* to virgin calling *G. molesta* females after 15 min of exposure of males to the same treatments used in the sensory adaptation experiment was compared in the previously described flight tunnel and experimental conditions. The pheromone sprayer was used to condition air with test chemicals. A method developed by Trimble (2012) was used to preexpose individual males to pheromone, citral, or citral combined with pheromone. One hour before the test period, i.e., 3 hr before the onset of the scotophase, 1–2-d-old females and 2–3-d-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 centerline of the tunnel (center female) and the other two were positioned 20 cm to the right (right female) and left (left female) of the center female. Plumes of titanium dioxide produced by a small amount of titanium tetrachloride placed at the right, center, and left female positions had an estimated cross-sectional diameter of ≈5 cm at the end of the tunnel and did not converge. An experiment was begun when all the three females were observed calling. The effect of preexposure of males to one of the five randomly chosen treatments was tested using the methods of Trimble (2012). The experiment was repeated on 15 d.

Statistical Analyses. Statistical analysis was performed using JMP 7.0 (SAS Institute 2007). EAG responses and the times to initiate a behavioral response were tested for goodness-of-fit of to the normal distribution by using the Shapiro–Wilk W test. The homogeneity of the variances of means was tested using Bartlett's test. Parametric and nonparametric analysis were used as required. The significance of Z8–12: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 Z8–12:OAc:citral expressed as $\log_{10}$(% citral). The EAG response to Z8–12: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 Z8–12:OAc. The significance of the effect of combining increasing amounts of citral with Z8–12:OAc on mean EAG response was tested using multivariate repeated measures ANOVA by modeling the six Z8–12:OAc + 0–1,000-μg 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 Z8–12:OAc stimulus, time of stimulation with the control stimulus, and by dose of a citral stimulus. The significance of the effect of citral or ethanol on the total number of males initiating each of the five behavioral phases of upwind flight to a virgin calling female was tested using Pearson's chi-square test. The Wilcoxon–Mann–Whitney test was used to test the significance of the effect of citral or ethanol on the mean time required to initiate a behavior. The effect of exposing antennae for 15 min to the control, Z8–12:OAc, citral, and Z8–12:OAc + 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 percentage reduction in net EAG response to the pheromone stimulus was computed for each antenna exposed to one of the six treatments as ([net pretreatment EAG response − net posttreatment EAG response]/net pretreatment EAG response)*100 (Trimble and Marshall 2007). The significance of the effect of treatment on differences in mean percentage reduction in response was tested using one-way ANOVA. The effect of treatment on the number of males initiating each of the five behavioral phases of upwind flight response was tested using logistic regression analysis as described by Trimble (2012). The Kruskal–Wallis test was used to test the significance of treatment on the mean time required to initiate a behavior.

Results

Pheromone Dose–EAG Response. EAG response was affected by dose of the Z8–12:OAc stimulus ($F_{10,90}$ = 33.8, $P < 0.001$). A statistically significant response greater than the response to the control was detected using 1×10^{-10} g (i.e., 0.1 ng) of pheromone. There was no change in response with increased dose over the range $1 \times 10^{-10} - 1 \times 10^{-7}$g (0.633 ± 0.173 mV − 0.683 ± 0.044 mV, mean ± SD) of pheromone. Above this range, there was a trend of increase up to 1×10^{-4} g (1.160 ± 0.213 mV), and a sharp decline in response to the two highest doses of pheromone, i.e., 1×10^{-3} (1.068 ± 0.218 mV) and 1×10^{-2} (0.873 ± 0.235 mV) Z8–12:OAc. There was no difference in the response to the 1×10^{-5} (1.035 ± 0.166 mV) and 1×10^{-4} (1.160 ± 0.213 mV) and to the 1×10^{-4} and 1×10^{-3} g (1.068 ± 0.218 mV) dose of Z8–12:OAc. Consequently the 1×10^{-5} g dose (i.e., 10 μg) was selected for use in experiments measuring treatment effect on the responsiveness of antennae. This same dose was used by Trimble and Marshall (2007, 2010) in their studies of sensory adaptation in *G. molesta* antennae.

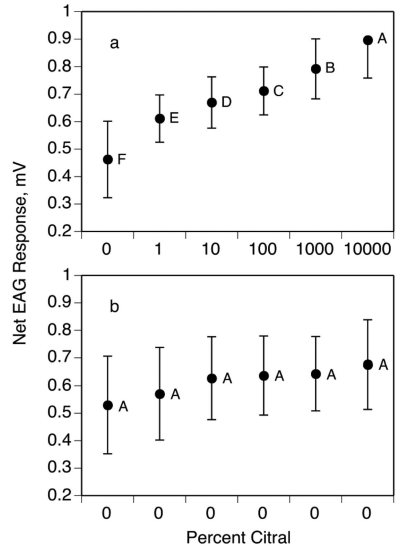

Fig. 1. Mean ± SD net EAG response (mV) of male *G. molesta* antennae after six stimulations at 1-min intervals: (a) with 10-μg Z8–12:OAc alone and with increasing relative amounts (percentages) of citral, (b) with 10-μg Z8–12:OAc. Means followed by the same letter are not significantly different (multivariate repeated measures ANOVA followed by contrasts).

Effect of Citral on EAG Response to Pheromone. Net EAG response increased with the addition of increasing relative amounts of citral to the 10-μg Z8–12:OAc stimulus ($F_{5,5}$ = 24.43, P = 0.002) (Fig. 1a). Mean response (0.896 mV) to 10-μg Z8–12:OAc + 1,000 μg of citral (i.e., 10,000%) was 2× greater than the mean response (0.462 mV) to 10 μg of Z8–12:OAc alone. There was a weak relationship between net EAG response and Log_{10} (percentage citral) using both a linear (net EAG response = 0.598 + 0.069 (Log_{10} % citral), $F_{1,48}$ = 45.60; $P < 0.001$; R^2 = 0.487) and a quadratic polynomial (net EAG response = 0.579 + 0.069(Log_{10} % citral) + 0.009(Log_{10} % citral)^2; $F_{2,47}$ = 23.44; $P < 0.001$; R^2 = 0.499) regression model. There was no change in net EAG response when citral was not added to the 10-μg Z8–12:OAc stimulus ($F_{5,5}$ = 2.88, P = 0.14) (Fig. 1b). There was a very weak relationship between net EAG response and the time (time, min) of application of the pheromone stimulus using a linear regression model (net EAG response = 0.517 − 0.028 (time, min); $F_{1,58}$ = 5.78; $P < 0.02$; R^2 = 0.09) and no relationship when using a quadratic polynomial regression model (net EAG response = 0.530 + 0.028 (time, min) − 0.009(time, min − 3.5)^2; $F_{2,57}$ = 3.0; P = 0.06; R^2 = 0.1). The dose of citral alone, without Z8–12:OAc, affected mean EAG response ($F_{5,5}$ = 59.01; df = 5,5; P = 0.001) (Fig. 2). Response to the control (0.501 ± 0.067 mV, mean ± SD) and to the 0.1- (0.498 ± 0.055 mV), 1- (0.480 ± 0.055 mV), and 10- (0.498 ± 0.072 mV) μg doses were statistically similar ($P > 0.05$). The response to a 100-μg dose

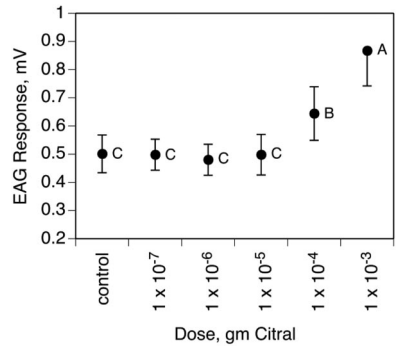

Fig. 2. Mean ± SD EAG response (mV) of male *G. molesta* antennae after stimulation with increasing doses (g) of citral at 1-min intervals. Means followed by the same letter are not significantly different (multivariate repeated measures ANOVA followed by contrasts).

(0.644 ± 0.095 mV) was significantly greater than the response to the 10-μg dose ($P < 0.0001$), and the response to the 1,000-μg dose (0.867 ± 0.125 mV) was greater than the response to the 100-μg dose ($P < 0.001$). The mean ± SD net EAG response was 0.143 ± 0.073 mV using the 100-μg dose and 0.366 ± 0.112 mV using the 1,000 μg dose of citral (Fig. 2). Repeated stimulation with the control (i.e., 50-μl ethanol) seven times at 1-min intervals did not affect EAG response of antennae. ($F_{6,4} = 2.78$; df = 6,4; $P = 0.17$).

Effect of Citral on Sexual Response of Males to Females. The emission of 1,000 ng/min of citral ≈1 cm downwind from a virgin calling female did not affect the number of males initiating the activation and take-off behaviors. By contrast the addition of citral to the virgin calling female effluvia reduced the number of males locking-on to the pheromone plume by 57.9%, reduced the close-in and touchdown phases of upwind flight behavior by 65.8%, and increased the time required for activation by 21.1% but did not affect the time required for the initiation of subsequent phases of upwind flight behavior (Table 1).

Effect of Preexposure of Antennae to Citral and Citral Combined With Pheromone on EAG Response to Pheromone. Mean EAG response of male *G. molesta* antennae to the control stimulus (i.e., 50-μl ethanol) was reduced by 34.7, 38.8, 36.1, 40.8, and 23.7% after 15-min exposure to the air, Z8–12:OAc, citral, Z8–12:OAc + citral (1:1), and to the Z8–12:OAc + citral (1:100) treatments, respectively (Table 2). Net EAG response to the 10-μg Z8–12:OAc stimulus significantly declined after 15 min of exposure to the Z8–12:OAc and citral treatments, but not after the same duration of exposure to the air and air + ethanol treatments (Table 2). The average percentage reduction in net EAG response was similar ($F_{3,16} = 0.5$, $P = 0.7$) after 15 min of exposure to the Z8–12:OAc (46.7 ± 26.4%, mean ± SD), citral (62.5 ± 24.3%), Z8–12:OAc + citral (1:1) (56.8 ± 9.3%), and to the Z8–12:OAc + citral (1:100) (58.8 ± 22.2%) treatments.

Effect of Preexposure of Antennae to Citral and Citral Combined With Pheromone on Male Response to Females. Fifteen minutes of exposure to the air, air + ethanol, Z8–12:OAc, citral, and two Z8–12:OAc + citral treatments had no effect on the number of male *G. molesta* initiating the activation, take-off, lock-on, close-in, and touchdown phases of upwind flight behavior in response to a virgin, calling *G. molesta* female (Table 3). These treatments also had no effect on the number of males initiating these behaviors in response to the first of the three females that were used to test male responsiveness (Table 4). The time required to initiate each of the six upwind flight behaviors was not affected by treatment (Table 5).

Discussion

The monoterpenoid citral may have potential for use as an attraction inhibitor in programs that manage *G. molesta* by behavior modification. In the flight tunnel, the emission of citral at a rate similar to that of a commercially available mating-disruption pheromone dispenser 1 cm downwind from a virgin calling *G. molesta* female increased the time required for male activation by 21% and reduced the number of males landing at a female by 66%. Citral is unlikely to have any potential for affecting the mate-seeking behavior of male *G. molesta* by inducing peripheral nervous system adaptation or central nervous system habituation. The prolonged exposure of male antennae to Z8–12:OAc or to citral at the aerial concentration of 1 ng/m^3 air measured in orchards treated with pheromone for mating disruption of *C. pomonella* (Bächman 1997, Koch et al. 1999, Judd et al. 2005) reduced antennal sensitivity to pheromone by ≈50%; however, there was no detectable effect on a male's mate-seeking behavior when it was subjected to the same treatment. The combination of pheromone with citral at ratios of 1:1 and 1:100, with resultant aerial concen-

Table 1. Number of male *G. molesta* initiating successive phases of upwind flight and mean ± SD time (s) required for initiation in response to a virgin calling *G. molesta* female with and without citral and results of Peason chi-square (df = 1) (number) and Kruskal-Wallis (df = 1) (time) tests

Treatment	Activation		Take-off		Lock-on		Close-in		Touchdown	
	No.	Time	No.	Time	No.	Time	No.	Time	No.	Time
Citral	39	10.4 ± 8.2	38	76.2 ± 85.6	16	5.2 ± 3.0	13	3.4 ± 2.5	13	1.9 ± 1.0
Control	40	7.0 ± 6.1	40	70.0 ± 106.1	38	10.3 ± 8.6	38	4.0 ± 2.2	38	2.3 ± 1.8
χ^2	1.0	5.7	2.1	0.5	27.6	3.4	33.8	1.7	33.8	0.1
P	0.01	0.3	0.2	0.5	<0.001	0.07	<0.001	0.2	<0.001	0.8

The effect of each treatment was tested on the response of 40 males.

Table 2. Mean ± SD electroantennogram response (mV) of male *G. molesta* antennae to a control stimulus and mean ± SD net electroantennogram response to a 10-μg Z8–12:OAc stimulus before and after 15 min of continuous exposure to six treatments and results of paired t-test (df = 4)

Treatment	Stimulus	Preexposure	Postexposure	*t*	*P*
Control 1 (air)	Control	0.147 ± 0.030	0.096 ± 0.019	−6.5	0.002
	10-μg Z8–12:OAc	0.526 ± 0.171	0.566 ± 0.138	0.9	0.8
Control 2 (air + ethanol)	Control	0.131 ± 0.037	0.119 ± 0.053	−1.0	0.2
	10-μg Z8–12:OAc	0.507 ± 0.130	0.539 ± 0.166	0.6	0.7
Z8–12:OAc	Control	0.165 ± 0.035	0.101 ± 0.034	−4.1	0.007
	10-μg Z8–12:OAc	0.486 ± 0.184	0.271 ± 0.174	−4.9	0.004
Citral	Control	0.133 ± 0.034	0.085 ± 0.032	−2.6	0.03
	10-μg Z8–12:OAc	0.514 ± 0.172	0.184 ± 0.110	−4.1	0.008
Z8–12:OAc + citral (1:1)	Control	0.147 ± 0.040	0.087 ± 0.019	−5.1	0.004
	10-μg Z8–12:OAc	0.603 ± 0.222	0.253 ± 0.096	−4.8	0.004
Z8–12:OAc + citral (1:100)	Control	0.131 ± 0.023	0.100 ± 0.010	−2.5	0.04
	10-μg Z8–12:OAc	0.470 ± 0.219	0.182 ± 0.092	−3.7	0.01

Five antennae were exposed to each treatment. Antennae were exposed to 6.25-μl ethanol/ml air in the control 2, pheromone, and pheromone + citral treatments. Antennae were also exposed 1.0 by 10^{-6} ng Z8–12:OAc/ml air in the Z8–12:OAc treatment, 1.0 by 10^{-6} ng citral/ml air in the citral treatment, 1.0 by 10^{-6} ng Z8–12:OAc + 1.0 by 10^{-6} ng citral/ml air in the Z8–12:OAc + citral (1:1) treatment, and to 1.0 by 10^{-6} ng Z8–12:OAc/ml air + 1.0 by 10^{-3} ng citral/ml air in the Z8–12:OAc + citral (1:100) treatment.

trations of 1 ng of Z8–12:OAc + 1 or 1,000 ng of citral/m^3 air did not increase the level of sensory adaptation or result in a detectable effect on the mate-seeking behavior of males.

The relationship observed between the amount of Z8–12:OAc used in the stimulus delivery system and the EAG response was similar to that recorded previously by Trimble and Marshall (2007), but in the current study the EAG system had greater "sensitivity." A measureable response to pheromone was obtained using a dose of 0.1 ng, whereas in the earlier study a dose of 100 ng was required to elicit a detectable EAG response. In addition, the maximum average EAG response was −1.160 mV in the current study, but only −0.655 mV in the previous study. One possible factor contributing to this difference could be the smaller volume (5 versus 36 ml) of air used to deliver the pheromone stimulus in the current study.

The EAG response of male *G. molesta* antennae to a dose of 10 μg of Z8–12:OAc increased when the amount of citral was increased to 1, 10, 100, and 1,000 μg, respectively. There was no measureable EAG response to doses of 0.1, 1, or 10 μg of citral, suggesting that the combination of these amounts of citral with 10 μg of Z8–12:OAc synergizes the response to pheromone. In contrast to the absence of a measurable EAG response to doses of citral smaller than 10 μg, doses of 100 and 1,000 μg of this compound elicited responses of −0.143 and −0.366 mV, respectively. The 0.330-mV difference in EAG response to a combination of 10-μg Z8–12:OAc + 100-μg citral (−0.792 mV) and to Z8–12:OAc alone (−0.462) was 2.3× greater than the response to 100 μg of citral, suggesting that the 71% increase in response to the mixture is at least in part because of synergism. By contrast, the 0.431-mV difference in EAG response to a combination of 10-μg Z8–12:OAc + 1,000-μg citral (−0.893 mV) and to Z8–12:OAc alone was only 1.2× greater than the response to 1,000 μg of citral, suggesting that citral acts less synergistically and more additively at this concentration. The antennae of male and female light brown apple moths, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), respond to citral (Suckling et al. 1996), and this species has genes that encode citral sensitive olfactory receptors (ORs) (Jordan et al. 2009). Interestingly, orthologues of citral encoding genes occur in six different lepidopteran families, suggesting that the ability to recognize citral may play a role in some important moth behaviors (Jordan et al. 2009).

The synergistic effect of plant volatile compounds on the electrophysiological response to pheromone has been observed in other Lepidoptera. For example, in the corn earworm *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), stimulating olfactory receptor neurons (ORNs) with binary mixtures of the main pheromone compound (Z)-11-hexadecenal and increasing doses of either linalool or (Z)-3-hexenol significantly increased the ORNs activity compared with activity to pheromone alone (Ochieng et al. 2002). The results of the current study demonstrate for the first time a synergistic effect on the electrophysiolog-

Table 3. Number of male *G. molesta* initiating successive phases of upwind flight to a conspecific virgin calling female in a flight tunnel after 15 min of exposure to one of six treatments and results of logistic regression analyses

Treatment	Activation	Take-off	Lock-on	Close-in	Touchdown
Control 1 (air)	15	15	15[a]	15[a]	15[a]
Control 2 (air + ethanol)	15	15	15[a]	15[a]	15[a]
Z8–12:OAc	15	15	14	14	14
Citral	15	15	15[a]	14	14
Z8–12:OAc + citral (1:1)	15	15	11	11	11
Z8–12:OAc + citral (1:100)	15	15	14	14	14
χ^2	-	-	3.3	3.8	3.8
df	-	-	2	3	3
P	-	-	0.2	0.3	0.3

[a] Excluded from analysis. Fifteen males were tested within each treatment. Antennae were exposed to 6.25-μl ethanol/ml air in the control 2, pheromone, and pheromone + citral treatments. Antennae also were exposed 1.0 by 10^{-6} ng Z8–12:OAc/ml air in the Z8–12:OAc treatment, 1.0 by 10^{-6} ng citral/ml air in the citral treatment, 1.0 by 10^{-6} ng Z8–12:OAc + 1.0 by 10^{-6} ng citral/ml air in the Z8–12:OAc + citral (1:1) treatment, and to 1.0 by 10^{-6} ng Z8–12:OAc/ml air + 1.0 by 10^{-3} ng citral/ml air in the Z8–12:OAc + citral (1:100) treatment.

Table 4. Number of male *G. molesta* initiating successive phases of upwind flight to a conspecific virgin calling female in a flight tunnel on the first of three attempts after 15 min of exposure to one of six treatments and results of logistic regression analyses

Treatment	Activation	Take-off	Lock-on	Close-in	Touchdown
Control 1 (air)	15[a]	14	11	11	11
Control 2 (air + ethanol)	15[a]	15[a]	11	11	11
Z8–12:OAc	13	13	9	9	9
Citral	15[a]	14	11	10	10
Z8–12:OAc + citral (1:1)	15[a]	14	10	10	10
Z8–12:OAc + citral (1:100)	14	13	12	12	12
χ^2	0.4	0.9	1.7	1.8	1.8
df	1	4	5	5	5
P	0.5	0.9	0.9	0.9	0.9

[a] Excluded from analysis. Fifteen males were tested with each treatment. Antennae were exposed to 6.25-μl ethanol/ml air in the control 2, pheromone, and pheromone + citral treatments. Antennae also were exposed 1.0 by 10^{-6} ng Z8–12:OAc/ml air in the Z8–12:OAc treatment, 1.0 by 10^{-6} ng citral/ml air in the citral treatment, 1.0 by 10^{-6} ng Z8–12:OAc + 1.0 by 10^{-6} ng citral/ml air in the Z8–12:OAc + citral (1:1) treatment, and to 1.0 by 10^{-6} ng Z8–12:OAc/ml air + 1.0 by 10^{-3} ng citral/ml air in the Z8–12:OAc + citral (1:100) treatment.

ical response of a moth antenna when citral is combined with pheromone.

Plant volatile compounds can both enhance and inhibit the behavioral response of insects to pheromone (Reddy and Guerrero 2004). For example, in a flight tunnel, the host plant volatile compounds linalool, (E)-β-farnesene, and (Z)-3-hexen-1-ol increased the attraction of codling moth males *C. pomonella* to (E,E)-8,10-dodecadien-1-ol (codlemone) (Yang et al. 2004), and in orchards the trap capture of *C. pomonella* males increased when a blend of green-leaf volatile compounds from walnut (*Juglans regia* L.) and pear (*Pyrus communis* L.) were added to codlemone (Light et al. 1993). In field trapping experiments, the response of the southern pine beetle *Dendroctonus frontalis* (Zimmermann) (Coleoptera: Curculionidae) to its sex pheromone was reduced by the addition of 4-allyl anisole, a common compound produced by loblolly pine, *Pinus taeda* L., and other conifer species (Hayes et al. 1994). Citral is the only terpenoid plant volatile compound that has been reported to have inhibitory activity when added to the pheromone of a moth (de Kramer et al. 2002). In a laboratory flight tunnel experiment, the recapture rate of male *L. botrana* was reduced by 90% when citral was combined in a dispenser with synthetic sex pheromone (E,Z)-7-9-dodecadienyl acetate at a ratio of 1:1,000 pheromone:citral (Meiwald 1995). In a similar flight tunnel experiment, the recapture rate of *C. pomonella* was reduced by 67% when citral was combined in a dispenser with codlemone at a ratio of 1:1,000 pheromone:citral (Hapke et al. 2001). The inhibitory effect of citral on the response of male *G. molesta* to virgin calling females reported in the current study is the first example of this terpenoid compound reducing male moth response to "natural pheromone."

The antennae of *G. molesta* exhibit sensory adaptation when they are exposed continuously to pheromone (Stelinski et al. 2005, Trimble and Marshall 2007). The 47% reduction in antennal sensitivity to Z8–12:OAc after 15 min of exposure to 1 ng/m^3 air of this compound observed in the current study is much greater than the reduction of 16% predicted by Trimble and Marshall (2010). The use of the net EAG response in the current study versus the use of a response that was not adjusted for response to the control stimulus in the study of Trimble and Marshall (2010) may be one reason for this discrepancy. Another possible reason for the difference may be the greater sensitivity of EAG system in the current study. The induction of adaptation in the antennae of *G. molesta* after long-term exposure to citral represents the first reported example of a reduction in sensitivity to pheromone in a moth antenna after prolonged exposure to a volatile plant compound. The exposure of antennae to 1 ng citral/m^3 air for 15 min induced a similar level of sensory adaptation to that observed in antennae exposed to same aerial concentration of Z8–12:OAc, suggesting that the electrophysiological response to both Z8–12:OAc and citral could be mediated through a common sensory channel involving the same OR on the antenna. The antennae of male *C. pomonella* contain ORNs that respond to both pheromone and pear ester, ethyl (E,Z)-2–4-decadienoate (De Cristofaro et al. 2004, Ansebo et al. 2005, Witzgall et al. 2008). The absence of a detectable increase in sensory adaptation with combinations of pheromone and citral at ratios of 1:1 and 1:100 (Z8–12:OAc:citral)

Table 5. Mean ± SD time (s) required for male *G. molesta* to initiate successive phases of upwind flight to a virgin calling *G. molesta* female in a flight tunnel after 15 min of exposure to one of six treatments and results of Kruskal–Wallis test (df = 5)

Treatment	Activation	Take-off	Lock-on	Close-in	Touchdown
Control 1 (air)	5.9 ± 8.2 (15)	41.2 ± 36.5 (15)	3.7 ± 2.5 (15)	4.0 ± 2.5 (15)	1.4 ± 1.3 (15)
Control 2 (air + ethanol)	5.6 ± 3.3 (15)	54.1 ± 51.6 (15)	4.3 ± 3.0 (15)	3.3 ± 1.5 (15)	2.2 ± 1.9 (15)
Z8–12:OAc	6.7 ± 7.7 (15)	105.3 ± 115.4 (15)	5.1 ± 2.6 (14)	3.3 ± 1.7 (14)	2.4 ± 3.1 (14)
Citral	7.7 ± 13.9 (15)	54.0 ± 56.7 (15)	4.7 ± 6.4 (14)	3.3 ± 0.9 (14)	1.4 ± 1.0 (14)
Z8–12:OAc + citral (1:1)	6.0 ± 3.8 (15)	66.5 ± 55.2 (15)	3.7 ± 1.7 (11)	3.6 ± 1.6 (11)	1.8 ± 1.5 (11)
Z8–12:OAc + citral (1:100)	13.7 ± 17.9 (15)	142.9 ± 129.7 (15)	3.5 ± 1.6 (14)	4.1 ± 1.9 (14)	1.7 ± 1.8 (14)
χ^2	8.5	8.9	5.0	2.3	3.2
P	0.1	0.1	0.4	0.8	0.7

Number of males in parentheses. Antennae were exposed to 6.25-μl ethanol/ml air in the control 2, pheromone, and pheromone + citral treatments. Antennae also were exposed 1.0 by 10^{-6} ng Z8–12:OAc/ml air in the Z8–12:OAc treatment, 1.0 by 10^{-6} ng citral/ml air in the citral treatment, 1.0 by 10^{-6} ng Z8–12:OAc + 1.0 by 10^{-6} ng citral/ml air in the Z8–12:OAc + citral (1:1) treatment, and to 1.0 by 10^{-6} ng Z8–12:OAc/ml air + 1.0 by 10^{-3} ng citral/ml air in the Z8–12:OAc + citral (1:100) treatment.

contrasts with the observation that the addition of increasing relative amounts of citral to Z8–12:OAc elicits an increasing EAG response in male antennae. The maximum level of sensory adaptation to Z8–12:OAc is 80% in *G. molesta* antennae after prolonged exposure to this compound (Trimble and Marshall 2007). If citral acts at the level of the OR to facilitate more rapid movement of pheromone to the binding site, or binds to the OR, an increase in the level of adaptation would be expected with an increase in the aerial concentration of citral. The similar levels of adaptation of 58% induced with 1:1 and 1:100 ratios of pheromone:citral suggests that the synergistic effect of the plant volatile compound is not operable under conditions of continuous exposure to pheromone.

There was no measureable behavioral manifestation of sensory adaptation in the flight tunnel. The reduction in EAG response to Z8–12:OAc after 15 min of exposure to different treatments did not measurably affect the ability of males to orientate to a virgin, calling female. The results suggest that sensory adaptation induced by prolonged exposure to the main pheromone compound of *G. molesta* is unlikely to be a mechanism of mating disruption in this species. In a similar flight tunnel study, the number of males touching down at virgin calling females was reduced by only 9% after 30 min of exposure to 1 ng Z8–12:OAc/m^3 air (Trimble 2012). A highly efficacious pheromone dispenser for mating disruption of *G. molesta* contains the main pheromone compound Z8–12:OAc + the minor pheromone compounds (E)-8-dodecenyl acetate (E8–12:OAc) and (Z)-8-dodecenol (Z8–12:OH) in a 100:6:1 ratio (Trimble et al. 2004). It is possible that prolonged exposure to this blend of compounds would result in greater levels of adaptation, a reduction in the mate seeking ability of males, or both. Rumbo and Vickers (1997) exposed *G. molesta* males to a 100:5:5 mixture of Z8–12:OAc, E8–12:OAc, and Z8–12:OH and found that the reduction of sexual flight behavior was dependent on the aerial concentration of pheromone and duration of preexposure. The aerial concentration of pheromone required to induce a significant reduction in mate-seeking ability, however, was 65,000 times greater than the 1–2 ng pheromone/m^3 air that has been measured in orchards treated with pheromone for mating disruption. The results of the current study also suggest the treatment of host crops with citral, or combinations of pheromone and citral, are unlikely to induce sensory adaptation that results in a mating disruption of *G. molesta.* Additional studies should be conducted to determine if longer periods of exposure to these treatments results in greater levels of sensory adaptation, impairment of the mate locating abilities of males of this species, or both.

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