4. *Discussion*

The monoterpenoid citral may have potential for use as an attraction inhibitor in programs that manage *G. molesta* by behavior modification. The emission of citral at a rate similar to that of a commercially available mating-disruption pheromone dispenser 1 cm down wind 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 had no detectable effect on male behavior when a commercially available pheromone lure was used as the source of attraction, demonstrating the importance of using a natural source of pheromone in this type of study. 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 *Z*8-12: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 approximately 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 concentrations of 1 ng of *Z*8-12:OAc plus 1 or 1000 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 *Z*8-12:OAc used in the stimulus delivery system and the EAG response was similar to that previously recorded by Trimble and Marshall (2007), but in the present study the EAG system had greater "sensitivity". A measurable 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. Since the *G. molesta* males originated by the same laboratory colony (Pree et al. 1998) mantained under analogous conditions and were used in similar physiological status (2-3 day-old, virgin), one possible factor contributing to this difference could be the smaller volume (5 mL vs. 36 mL) of air used to deliver the pheromone stimulus in the present study. All of the other EAG

parameters (i.e. airflow of 2 L/min, pulse duration of 0.5 s and 3 mL-capacity Pasteur pipette) and data analysis (Net EAG response calculation) were the same as those used by Trimble and Marshall (2007).
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), stimulating olfactory receptor (ORNs) neurons 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 to activity to pheromone alone (Ochieng *et al.*, 2002). In the palm weevil *Rhynchophorus palmarum* (L.), EAG responses were greater to a racemic blend of sex pheromone (rhynchophorol) and some plant volatiles compounds (i.e. acetoin and ethyl acetate) than to the pheromone alone (Saïd *et al.*, 2005) suggesting that the plant volatile compounds synergized the response to 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 *Cydia pomonella* (L.) 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 and pear were added to codlemone (Light *et al.*, 1993). In field trapping experiments, the response of the southern pine beetle *Dendroctonus frontalis* (Zimmermann) 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 European grapevine moths, *Lobesia botrana* Denis et Schiffemüller 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 present study is the first example of this terpenoid compound reducing male moth response to “natural pheromone”. This is an important observation because natural, female-produced pheromone can be as much as four times as attractive as synthetic pheromone (El-Sayed and Trimble, 2002).

The EAG response of male *G. molesta* antennae to a dose of 10 µg of *Z*8-12:OAc (– 0.462 mV) increased by 32% (– 0.611 mV) with the addition of 0.1 µg of citral. There were successive 10% (– 0.670 mV), 6% (– 0.712 mV), 11% (– 0.792 mV) and 13% (– 0.893 mV) increases in response 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 lack 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 plus 100 µg citral (– 0.792 mV) and to Z8-12:OAc alone (– 0.462) is 2.3x greater than the response to 100 µg of citral, suggesting that the 71% increase in response to the mixture is at least in part due to synergism. By contrast, the 0.431 mV difference in EAG response to a combination of 10 µg Z8-12:OAc plus 1,000 µg citral (– 0.893 mV) and to Z8-12:OAc alone is only 1.2x 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) 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 lepidopterian families, suggesting that the ability to recognize citral may play a role in some important moth behaviors (Jordan *et al.*, 2009).

The antennae of *G. molesta* exhibit sensory adaptation when they are continuously exposed to pheromone (Stelinsky *et al.*, 2005; Trimble and Marshall, 2007). The 47% reduction in antennal sensitivity to *Z*8-12:OAc after 15 min of exposure to 1 ng/m^3 air of this compound observed in the present study is much greater than the reduction of 16% predicted by Trimble and Marshall (2010). The use of the net EAG response in the present 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 present 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 *Z*8-12: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. For example 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 (*Z*8-12:OAc:citral) contrasts with the observation that the addition of increasing relative amounts of citral to *Z*8-12:OAc elicits an increasing EAG response in male antennae. The maximum level of sensory adaptation to *Z*8-12:OAc is 80% in *G. molesta* antennae after long-term 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 adaption 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 *Z*8-12:OAc after 15 min of exposure to 1 ng of *Z*8-12:OAc (47%) or citral/m^3 air (63%), 1 ng *Z*8-12:OAc + 1 ng citral/m^3 air (1:1) (57%) or to 1 ng *Z*8-12:OAc + 100 ng citral/m^3 air (1:100) (59%) 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 *Z*8-12:OAc/m^3 air. A highly efficacious pheromone dispenser for mating disruption of *G. molesta* contains the main pheromone compound *Z*8-12:OAc plus the minor pheromone compounds (*E*)-8-dodecenyl acetate (*E*8-12:OAc) and (*Z*)-8-dodecenol (*Z*8-12:OH) in a 100:6:1 ratio (e.g. Trimble *et al.*, 2004). It is possible that prolonged exposure to this blend of compounds would result in greater levels of adaptation and/or a reduction in the mate seeking ability of males. Rumbo and Vickers (1997) exposed *G. molesta* males to a 100:5:5 mixture of *Z*8-12:OAc, *E*8-12:OAc and *Z*8-12:OH and found that the reduction of sexual flight behavior was dependent on the aerial concentration of pheromone and duration of pre-exposure. The aerial concentration of pheromone required to induce a significant a reduction in mate-seeking ability, however, was sixty-five thousand 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 present study also suggest the treatment of host crops with citral, or combinations of pheromone and citral are unlikely induced sensory adaptation that results in a disruption of mating 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 and/or impairment of the mate locating abilities of males of this species.

It may be possible to use citral in orchards to interrupt the orientation by flight of *G. molesta* males to sexually receptive females. Additional flight tunnel research should be carried out to determine the relationship between the rate of emission of citral downwind from virgin calling females and the ability of males to orientate to them by flight. It is possible that higher rates of emission than the 1,000 ng/min used in the present study would prevent all males from locating and landing at a female. In addition, field studies should be undertaken to determine if dispensers emitting citral have any effect on the ability of males to orientate to virgin females under natural conditions. If these experiments yielded positive results, future research should focus maximizing the rate of disruption by optimizing the density and emission rate of dispensers.

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4R *References*

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